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1 SOLICITUD DE

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☐ Capítulo I ☒ Capítulo II

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- [ ] Comprobante de pago por reivindicación de prioridad
- [ ] Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica
- [ ] Poderes, si fuere el caso
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- [ ] Declaraciones
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(11) **FIGURA CARACTERISTICA.**

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(54) Title AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

(57) Abstract An antibody of the invention interacts with human DR5 to produce agonistic or antagonistic effects downstream of the receptor including inhibition of cell proliferation and apoptosis Nucleic acid sequences and amino acid sequences of anti DR5 antibodies have been elucidated and vectors and cells containing and expressing these sequences have been generated. Methods and uses for the antibodies are detailed including treatment of apoptosis-related disease and treatment of dysregulated cell growth

AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

Field of the Invention

The present invention relates to an antibody capable of specifically binding a single type of tumor necrosis factor (hereinafter referred to as "TNF")-related apoptosis-inducing ligand (hereinafter referred to as "TRAIL") receptor, more particularly, to a monoclonal antibody that induces apoptosis in *in vivo* and *vitro* cells expressing the single type receptor and therapies based thereon

Background of the Invention

TRAIL is a member of the TNF family of proteins, which also includes TNF-α and Fas ligand (1) These proteins are potent inducers of apoptosis To date, five receptors for TRAIL have been identified, two of which, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (2-7), are capable of transducing the apoptosis signal while the other three DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG) do not transduce the apoptosis signal (8-12) All five receptors for TRAIL share significant homology in their extracellular ligand binding domains Similar to Fas and TNF receptor I (hereinafter referred to as "TNFRI"), the intracellular segments of both DR4 and DR5 contain a death domain, and transduce an apoptosis signal through a pathway that involves the Fas-associated death domain protein (hereinafter referred to as "FADD") and caspase 8 (6,7) In addition to transducing the apoptosis signal, the DR4 and DR5 receptors can also activate a pathway involving NFκb (6,7)

The biological functions of TRAIL that have been demonstrated include the capability of TRAIL to selectively induce apoptosis of transformed tumor cells, with normal cells being relatively resistant to TRAIL-mediated apoptosis (13-15) This selectivity suggests that, in contrast to Fas ligand, the administration of TRAIL is associated with very low levels of toxicity as demonstrated by systemic administration of TRAIL in an animal model without inducing significant toxicity (13) Thus, TRAIL has been proposed as a potent apoptosis inducing agent that would be a suitable therapeutic agent for the

treatment of cancer and other diseases associated with abnormal cell proliferation. TRAIL also has been proposed to be a potent apoptosis inducing agent that would be suitable for the treatment of autoimmune and inflammatory diseases It has been demonstrated that TRAIL-mediated apoptosis is involved in activation-induced cell death of T cells, thereby serving as an alternative mechanism to Fas ligand (16,17) TRAIL-mediated apoptosis may also function in the induction of apoptosis of T cells and other inflammatory cells (18), and plays a role in the killing activity of NK cells (19-21), and in the immunomodulatory function of dendritic cells (22,23) Thus, TRAIL-mediated apoptosis may also function in immunoprivilege and immunosurveillance

The TRAIL receptor system is complex, and includes at least two death receptors, DR4 and DR5, and at least two non-apoptotic receptors, DcR1 and DcR2 All of these receptors not only share a high amino acid sequence homology, but also exhibit a similar binding affinity to TRAIL (2-12) The ability of the DcR1 and DcR2 receptors to compete for binding of TRAIL without inducing apoptosis suggests that they may act as decoy receptors that block or modulate the activity of the TRAIL ligand Moreover, it has been reported that untransformed cells express higher levels of decoy receptors than do transformed cells Thus, it has been proposed that the differential modulation of the expression of the death and decoy receptors may represent a key regulatory mechanism that determines the susceptibility of cells to TRAIL-mediated apoptosis, but due to the lack of receptor-specific antibodies (2) Although the expression and function of DR4 and DR5 have been studied extensively, progress has been impeded by the lack of receptor-specific monoclonal antibodies The cell surface expression of DR5 has not been documented. It has been reported that a panel of anti-TRAIL receptor antibodies have been generated that are capable of inducing apoptosis of melanoma cells *in vitro* but only upon immobilization of the antibodies, to promote cross-linking, and, in some cases, the cells require culturing with actinomycin D (24) Several anti-DR5 antibodies have been generated (24) However, these previously generated anti-DR5 monoclonal antibodies have low apoptosis-inducing activity

in vitro, even under the conditions of crosslinking No *in vivo* activity has been reported. These antibodies have not been used for examining cell surface expression of TRAIL receptors (24) Thus, there exists a need for a monoclonal antibody selective for each specific TRAIL receptor that is not only able to bind to cell surface receptor but also to strongly induce apoptosis of various types of abnormal cells, including tumor cells, both *in vivo* and *vitro* without the requirement for crosslinking or immobilization Such an antibody would not only provide potential therapeutic agent but also a diagnostic tool for functional analysis of TRAIL receptor There exists a particular need for an antibody specific against each of the death inducing receptors DR4 and DR5

In the development, or progression, of many diseases it is often the case that cells are not deleted In many autoimmune diseases and inflammatory conditions, the surviving activated cells attack normal tissues or cells Further, progression of tumorigenesis and the proliferative panus formation of rheumatoid arthritis are characterized by the unchecked proliferation of cells Thus, insufficient apoptosis leads to the development of disease, and the uses of apoptosis-inducing ligand or agonistic monoclonal antibody to enhance apoptosis are considered as a potential therapeutic strategy for eliminating those unwanted cells

For example, rheumatoid arthritis (hereinafter referred to as "RA") is a common human autoimmune disease The current understanding of the pathophysiology of RA is that autoimmune T cells and B cells initiate an inflammatory response in the joints, which drives hyperproliferation of the synoviocytes As a consequence of the hyperproliferation of synovial cells, metalloproteinases (hereinafter referred to as "MMPs") are over-produced, which further leads to the erosive destruction of the cartilage and bone that is characteristic of RA (25) Thus, the control of hyperproliferation of inflammatory synovial cells is a key step in the treatment of RA The molecular mechanisms leading to the hyperproliferation of synovial cells are still unknown Although the hyperproliferative synovial cells are non-malignant and non-transformed, many studies have suggested that they share some common

features with transformed cells (46) These cells, the so-called, "transformed-appearing synoviocytes", are characterized by a dense rough endoplasmic reticulum, numerous irregular nuclei, and changes in the normally spindle-shaped cell skeleton. It has been proposed that the incorporation of the oncogenes and virus-derived genes might be the primary triggers for the transformed appearance of RA synovial cells (46)

At least two aspects of RA suggest that dysregulated apoptosis may contribute to the disease process and that therapeutic elicitation of apoptosis may be an effective treatment the failure of the deletion of the activated T cells suggests that there is defective activation-induced cell death of these T cells, which is a process that involves Fas-mediated apoptosis and TRAIL-mediated apoptosis, and the hyperproliferative nature of the RA synovial cells is a contributing factor in the later stages of RA pathophysiology Indeed, it has been shown that the administration of anti-Fas antibody into the inflammatory joint inhibits the development of chronic arthritis in *tax* transgenic mice, which are an animal model for human RA (26) Moreover, localized transduction with the *fas ligand* gene by an adenoviral vector is effective in prevention of collagen-induced arthritis (27) Inhibition of the proliferation of inflammatory synovial cells by enhancement of Fas-mediated apoptosis is observed in both cases Although Fas ligand is a strong apoptosis inducer in RA synovial cells, the application of Fas ligand-mediated apoptosis as a therapy for humans has been limited by lethal liver toxicity Thus, TRAIL receptor induced apoptosis represents a safer and more effective therapeutic for the treatment of RA than Fas-ligand induced apoptosis

TRAIL receptor induced apoptosis also represents a safer and more effective therapeutic for the treatment of cancer than Fas-ligand induced apoptosis TRAIL-mediated apoptosis is known to specifically induce apoptosis of transformed tumor cells without affecting normal cells It has been shown that the systemic administration of the trimerized soluble TRAIL did not cause toxicity in experimental animals yet was able to induce regression of implanted tumors (13,28) Its potential as an adjunctive therapy for traditional treatments

was underscored by the recent finding that the expression of DR5 and susceptibility to TRAIL-induced apoptosis of breast cancer cells is enhanced by the radiation, suggesting that combined with radiation, the efficiency of TRAIL would be increased in cancer therapy (29)

In addition, the gene encoding the TRAIL receptor DR5 has been mapped to chromosome 8p21-22, a loci with a high frequency of mutation in some cancer cells (30) It has been reported that at least two kinds of tumor cells, small lung cancer (31) and head and neck cancer (32) exhibit mutations in the death domain of the DR5 gene Thus, there exists a need for an anti-DR5 antibody in cancer research to determine the effect of receptor epitope variation on the development and progression of cancers Further, the functionality of TRAIL receptor mutations would prove a useful clinical diagnostic tool when used in conjunction with other biomarkers in the early detection of cancers and as a predictor of the tumor aggressiveness

Summary of the Invention

An antibody is disclosed which recognizes a TRAIL receptor DR5 and which induces apoptosis in a DR5-expressing cell in vivo Further disclosed is an antibody that recognizes DR5 but not DR4, DcR1, nor DcR2 Specifically detailed is a monoclonal antibody produced by a hybridoma.

A method provided by the invention allows inhibition of cell proliferation by exposing a cell to a therapeutic quantity of an antibody capable of binding to DR5 Also disclosed is a pharmacological composition that includes a therapeutic amount of monoclonal antibody active against a DR5, a pharmaceutically acceptable carrier and a container enclosing the antibody and the carrier Further provided by the invention is the use of an antibody recognizing DR5 for preparing a therapeutic for selective apoptosis of abnormal or dysregulated cells

An antibody of the present invention interacts with a tumor necrosis factor ligand receptor such as DR4, DR5, DcR1, DcR2 and OPG, inducing apoptosis in a cell expressing such a receptor Disclosed is an antibody of the

invention capable of selectively binding an agonistic or antagonistic tumor necrosis factor ligand receptor epitope

The present invention provides a treatment for an apoptosis related disease by a method that includes exposing a target tissue having an apoptosis related disease to a therapeutic quantity of an antibody of the invention.

Further described is a fusion protein that includes an antigenic TRAIL receptor amino acid sequence having at least ten bases, coupled to an immunoglobulin protein or fragment thereof capable of eliciting an immune response within a subject

The present invention provides a method of gene therapy in which a target cell is transfected with a TRAIL receptor nucleic acid sequence in an expression vector so that the TRAIL receptor is expressed on the target cell The target cell is then exposed to an antibody that selectively binds the TRAIL receptor

Provided are nucleic acid sequences and amino acid sequences encoding the heavy and light chain immunoglobulins of an antibody selective for DR5 Also detailed are vectors that include a nucleic acid sequence of the invention and host cells transformed with a vector of the invention

The present invention provides a host cell producing a humanized TRA-8

A process for producing a humanized DR5 antibody is described in which a host is transformed with nucleic acid sequences encoding a humanized immunoglobulin light chain and a humanized immunoglobulin heavy chain after which the transformed host is incubated for a predetermined period of time

Also described is a process for inhibiting cell proliferation that includes contacting a target cell with a pharmaceutically effective amount of a humanized DR5 antibody

A commercial kit is provided for inducing cell death that includes a humanized TRA-8 antibody selective for DR5, packaged in a suitable container together with instructions for use

Brief Description of the Drawings

Figure 1 Characterization of TRA-8 (a) Binding specificity of TRA-8 Western blot analysis (upper panel) Recombinant fusion proteins of the TNFR family probed with TRA-8 or anti-human IgG Lane 1 DR5/hIgG1 fusion protein (immunogen), Lane 2 DR4/hIgG1 (TRAIL-R1), Lane 3 DR5/hIgG1, Lane 4 TRAIL-R3 (DcR-1)/hIgG1, Lane 5 TRAIL-R4 (DcR-2)/hIgG1, Lane 6, CD95/hIgG1, Lane 7 soluble TNFRI ELISA analysis (lower panel) The well numbers match those of the Western blot except well 8 which is a murine DR5/hIgG1 fusion protein (b) Binding activity of soluble TRAIL and TRA-8 to DR5 and DR4 ELISA plates were coated with DR5/hIgG1 (left panel) or DR4/hIgG1 (middle panel) and then incubated with TRAIL or TRA-8 (c) Flow cytometry analysis of the surface expression of DR5 Cos-7 cells transfected with pcDNA3 expression vector containing the full-length DR5 cDNA (solid histogram), DR4 cDNA (open histogram, solid line) or empty vector (open histogram, dashed line) Forty-eight hours after transfection, cells were stained with TRA-8 followed by PE-conjugated anti-mouse IgG1 (d) in situ immunohistochemistry reactivity for DR5 Cytospin slides of Cos-7 cells transfected with DR5 expression or control vector were stained with TRA-8 at 48 hours after transfection, (e) Killing activity of TRA-8 Jurkat cells were incubated with the indicated concentrations of TRA-8 Cell viability was determined by ATPLite, MTT, and PI exclusion assays after overnight culture The results of ATPLite and MTT assays are presented as percent of medium control, and PI assay are presented as percent of PI negative cells (f) Western blot analysis of caspase activation Jurkat cells were incubated with 500 ng/ml TRA-8 for indicated time Cell lysates were separated by 15% SDS-PAGE, blotted, and probed with anti-caspase antibodies The arrows indicate the cleaved subunits of each caspase g Caspase inhibition assay Jurkat cells were incubated with 50 ng/ml TRA-8 overnight in the presence of various concentrations of indicated caspase inhibitors Cell viability was determined by the ATPLite assay

Figure 2 Cell surface expression of DR5 and susceptibility to DR5-mediated apoptosis Normal T and B cells, freshly isolated from peripheral blood, T cell (a and a'), glioma (b and b'), prostate cancer cell (c) and B cell (d) cell lines were incubated with TRA-8 or murine IgG1 isotype control antibody followed by PE-conjugated goat anti-mouse IgG1 The open histograms represent the isotype antibody control while the solid histograms represent TRA-8 staining Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles) as shown in a, b' and d.

Figure 3a' T cell line U937 was incubated with TRA-8 or murine IgG1 isotype control antibody Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles)

Figure 3 Glioma (b) and prostate cancer (c) cell lines were incubated with TRA-8 or murine IgG1 isotype control antibody Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles)

Figure 4 is a series of graphs showing cell viability for human Jurkat cells after exposure to indicated concentrations of (A) antibody strains TRA-1, -8 and -10 and (B) TRAIL in the presence of a fixed concentration of the inventive antibody strains depicted in Figure 4A,

Figure 5 Expression of DR5 in normal and cancer tissues Normal and cancer tissue homogenates were probed with TRA-8 and developed by chemiluminescence (a) Western blot analysis of DR5 protein in normal tissues lane 1 liver, lane 2 brain, lane 3 lung, lane 4 kidney, lane 5 spleen, lane 6 testes Lane 7 ovary, lane 8 heart, lane 9 pancreas b Western blot analysis of DR5 protein in cancer tissues The cancer tissue blot containing cancers from the ovary (lane 1), lung (lane 2), liver (lane 3), rectum (lane 4), cervix (lane 5), skin (lane 6), testes (lane 7), thyroid (lane 8), uterus (lane 10), stomach (lane 11), larvngopharynx (lane 12), and pancreas (lane 13) was probed *In situ*

immunohistochemistry of normal human tissues(c) and of cancer tissues (d) Frozen sections were immunostained with TRA-8

Figure 6 Tumoricidal activity of TRA-8 SCID mice were inoculated subcutaneously with 1321N1 cells Mice were injected intravenously with a single dose of 100 μg TRA-8 on the second day after tumor inoculation (a), or with three doses of 100 μg TRA-8 beginning 7 days after tumor inoculation (b) Tumor growth was determined by the weight and examined histologically with H&E staining The photographs show viable tumor growth in control mice but not in TRA-8 treated mice (c , upper panel), and H&E staining of tumor (c , lower panel) SCID mice were injected intravenously with 10^6 Jurkat cells and treated with a single dose of TRA-8 on the second day after injection. Seven days later, spleen cells were harvested, stained with anti-human CD3 antibody and analyzed by flow cytometry (d), or by immunohistochemistry (e)

Figure 7 shows expression of cell surface DR5 in RA (A) and OA (B) synovial cells 1 X 10^6 primary cultured synovial cells were stained with affinity-purified TRA-8 and followed by PE-conjugated goat anti-mouse IgG1 antibody 10,000 viable cells analyzed by FACSvantage

Figure 8 is a series of graphs showing cell viability as a function of TRAIL and TRA-8 concentration induced apoptosis of representative strains of RA (A) and OA (B) synovial cells with various concentrations of the recombinant soluble TRAIL (the open circles) or affinity-purified TRA-8 (the closed circles) Cell viability is the percentage of the cpm of treated cells versus the cpm of untreated cells

Figure 9 is a series of graphs showing the caspase dependence of DR5-mediated apoptosis of RA synovial cells RA synovial cells (RA512) are incubated with 50 ng/ml of soluble Fas ligand (open squares), anti-Fas antibody (CH-11) (closed squares), soluble TRAIL (open circles), or anti-DR5 antibody (TRA-8) (closed circles) in the presence of variable concentrations of caspase inhibitors After overnight culture, cell viability is determined by ATPLite

Figure 10A is an electrophoretic gel-shift assay indicating NFkb activation RA1016 cells are incubated with 20 ng/ml TNF-a, 50 ng/ml soluble

TRAIL or 50 ng/ml TRA-8 for indicated time points before being subjected to electrophoresis Figures 10B and C are graphs showing the production of MMP-1 and MMP-3 1 X 10^6/ml of indicated RA synovial cells are incubated with the indicated concentrations of TNF-a (the open circles), TRAIL (the open triangles) or TRA-8 (the closed circle) After overnight culture, the culture supernatants are collected. The levels of MMPs in culture supernatants are determined by ELISA.

Figure 11 TRA-8 does not induce hepatocellular toxicity (a) Normal liver tissues do not express DR5 The paraffin sections of two normal liver tissues, one hepatocellular carcinoma tissue, and the cytospin preparation of HepG2 cells were prepared for H&E staining, and corresponding frozen sections were stained with TRA-8 (b) Flow cytometry analysis of cell surface expression of DR5 Hepatocytes, isolated from two normal liver tissues and from a case of hepatocellular carcinoma tissue, and HepG2 cells were stained with TRA-8, anti-Fas antibody (DX2) or an isotype control antibody The solid histograms indicate TRA-8 or DX2 staining, and the open histograms are the corresponding isotype controls

Figure 12 TRAIL but not TRA-8 induces hepatocellular toxicity Fresh normal human hepatocytes were maintained in Hepatocyte Culture Medium (a.) Apoptosis of hepatocytes was induced with 1 µg/ml soluble TRAIL plus crosslinker or TRA-8 for the indicated time points Cell viability was determined by ATPLite The results are presented as percent viable cells compared to the medium control The shaded bars indicate TRAIL and the black bars indicate TRA-8 (b) The condensed nuclei of hepatocytes were stained with Hoechst 33352 and analyzed by flow cytometry (c) Effect of cycloheximide on hepatocytes apoptosis Hepatocytes were cultured in control medium or with 1 µg/ml TRAIL or TRA-8 in the presence (closed bars) or absence (open bars) of 1 ug/ml cycloheximide for 8 hours Cell viability was determined by ATPLite The results are presented as mean±SEM of triplicate cultures of two experiments d A comparison of the susceptibility of normal hepatocytes to DR5 and Fas-mediated apoptosis Freshly isolated hepatocytes were incubated

with indicated concentrations of soluble TRAIL, TRA-8, soluble FasL or the anti-Fas mAb CH11 for 6 hours Cell viability was determined by ATPLite assay The results are presented as the percentage of viable cells compared to medium control For normal hepatocytes, mean±SEM of four normal individuals are presented. The results of hepatocellular carcinoma cells from one patient and HepG2 cells are presented as the average of triplicate cultures

Figure 13 TRAIL induces hepatitis B6 mice were intravenously inoculated with 10^9 pfu of adenoviral vector encoding the full length of human TRAIL under the control of the "Tet-on" transcriptional element TRAIL expression was induced by the indicated dose of tetracycline (a.) Northern blot analysis of human TRAIL expression in the liver 24 hours after inoculation of vector and induction with tetracycline, total RNA was isolated from the livers and probed with human TRAIL cDNA or β-actin. (b) Serum levels of AST 24 hours after transduction of TRAIL, serum levels of AST were determined (c) TRAIL-mediated cell death of adenoviral vector infected hepatocytes B6 mice were intravenously inoculated with tetracycline-inducible adenoviral vector 48 hours after inoculation, hepatocytes from inoculated and non-inoculated control mice were isolated and incubated with indicated concentrations of TRAIL for 8 hours (left panel) Cell viability of hepatocytes was determined by the ATPLite assay Mice, inoculated with adenoviral vector as above, were intravenously injected with 10 μg of soluble human TRAIL 48 hours later Serum levels of AST were measured at 24 hours after TRAIL injection (right panel) (d. and e) Histology analysis of liver damage induced by TRAIL The livers were collected at 24 hours (d.) or 7 days (e) after transduction with TRAIL The paraffin sections were H&E stained, and photographed at 100X (top panel) and 400X (lower panel)

Figure 14 is a series of graphs showing that activated T cells and B cells purified from human PBMC express increased levels of DR5 as determined by flow cytometry for resting (unfilled) and activated (shaded) cells

Figure 15 is viability graphs as a function of TRA-8 concentration for the purified T cells and B cells depicted in Figure 14 that have been stimulated for

48 hours with anti-CD3 or anti-μ, with activated and blast cells collected by different density of Ficoll-Paque Viability is determined by ATPLite assay

Figure 16 is a histogram and flow cytometry plots showing CD3 expression in a gated lymphocyte population for NK cell depleted NOD/SCID mice injected with human PBMC and TRA-8 or IgG (control)

Figure 17 shows CD3 and TUNEL stained cellular micrographs for mouse spleen tissue as detailed in Example 13

Figure 18 shows cycotoxicity plots for chronic lympholytic leukemia (CCL) and normal B cell humans in the presence of TRA-8, BISVIII, and the combination thereof

Detailed Description of the Invention

The failure to delete cells is due to defects in the apoptosis inducing system which are associated with defects illustratively including expression or function of the ligand, the receptor, or the intracellular regulatory and effector molecules The present invention affords a method to correct a deficient apoptosis inducing system as well as elucidate the specific defects inherent in a given defective apoptosis inducing system

The present invention relates to a new class of monoclonal antibodies that have selective *in vivo* and *in vitro* apoptosis inducing activity against specific TRAIL receptors, including DR5, DR4, DcR1 and DcR2 The present invention has utility as a reagent for apoptosis signaling research, as well as a therapeutic effective against cells expressing TRAIL receptors illustratively including broad classes of cancer cells, disregulation of the apoptosis system and abnormally proliferating synovial cells of autoimmune diseases Antibodies according to the present invention are specific in binding particular types of TRAIL receptors in spite of the homology therebetween. The inventive antibodies afford targeted apoptosis of only those cells expressing a target TRAIL receptor or alternatively, blocking TRAIL apoptosis of cells expressing a target receptor

An anti-DR5 monoclonal antibody of the present invention serves as a potent inducer of apoptosis in cells expressing DR5 *in vitro* and as a potent

inducer of apoptosis *in vivo* Humanized fragmentary CDR sequences engrafted on humanized antibody backbones and fusion protein anti-DR5 antibodies of the present invention exhibit similar apoptotic properties

To date, no monoclonal antibody is available which binds to cell surface DR5 and induces apoptosis of cells expressing DR5 both *in vitro* and *in vivo* in the absence of a crosslinker The present invention includes an anti-DR5 antibody operative as a therapeutic agent in animal models of disease, such as xenografted animals, or *in vivo* Although soluble TRAIL has been shown to be effective in induction of apoptosis of tumor cells *in vivo*, the killing activity appeared to be very low with the large and repeated doses often being required (13) TRA-8, one of a series of anti-DR5 antibodies according to the present invention, is pharmaceutically effective in animals carrying a human DR5 transgene and also has utility in establishing a model for the investigation of the role of DR5 and TRAIL

An antibody according to the present invention raised against a TRAIL receptor is harvested according to the present invention from an experimental animal By humanizing the antibody according to the present invention to maintain receptor binding activity while eliciting a diminished and therapeutically tolerable immune response within a human subject, a humanized anti-TRAIL receptor antibody according to the present invention is used as therapeutic agonist or antagonist for a given TRAIL receptor The present invention being operative as an *in vivo* therapeutic since secondary crosslinking of the anti-TRAIL receptor antibody is not required.

The present invention extends beyond a single anti-TRAIL receptor antibody having agonist or antagonistic apoptotic effects Rather, two or more anti-TRAIL receptor antibodies are brought into contact with a cell culture *in vitro* or a subject body tissue *in vivo* to create a synergistic treatment For example, glioma cell line U87 and hematopoietic cell lines U937 and Molt-4 are responsive to exposure to a synergistic exposure to agonistic anti-DR4 and anti-DR5 antibodies whereas exposure to agonistic anti-DR5 antibody alone shows only limited success in inducing apoptosis

Additionally, antagonistic anti-TRAIL receptor antibodies have particular utility in the present invention when an antibody is specific to binding one of the decoy receptors DcR1, DcR2 or OPG Selective blocking of a decoy receptor with an antibody according to the present invention has the effect in cell types expressing decoy receptors of shifting the TRAIL binding equilibrium towards those TRAIL receptors capable of transducing the apoptosis signal Thus, in another combined therapy according to the present invention, a decoy receptor binding antibody sensitizes an expressing cell towards agonistic apoptosis signal transducing TRAIL receptor binding

In another embodiment, the present invention affords a method of elucidating agonistic and antagonistic epitopes of a given TRAIL receptor Further, polymorphisms between individuals associated with a given TRAIL receptor are elucidated according to the present invention through the use of a panel of monoclonal antibodies each having a differing variable or CDR region. A characterized panel of monoclonal antibodies provides the ability to define agonistic and antagonistic epitopes and polymorphisms Thus, a panel of monoclonal antibodies according to the present invention has utility in drug discovery and/or subject screening for disease proclivity

Still another embodiment of the present invention involves fusion proteins including an antigenic fragment of a TRAIL receptor coupled to an immunoglobulin protein, polypeptide or fragment thereof A TRAIL receptor fragment being defined as containing a sufficient number of bases to elicit an immunogenic response to a native TRAIL receptor expressed on a subject cell surface A TRAIL receptor fusion fragment including at least ten amino acids An immunoglobulin fusion protein or fragment thereof is defined herein to include a native or synthetic protein or polypeptide segment having a sufficient number of amino acid bases to activate an immunogenic cascade response within a subject An immunogen of the present invention including a fusion of a TRAIL receptor fragment coupled to an immunoglobulin fragment has utility as an *in vivo* therapeutic to elicit an anti-TRAIL receptor antibody in situ within a subject

In still a further embodiment, the present invention is operative as a gene therapy In a gene therapy aspect of the present invention, targeted cells are transfected with a vector carrying an expressible sequence corresponding to a TRAIL receptor The vector being conventional and chosen on the basis of the targeted cell susceptibility to the vector Gene therapy vectors illustratively include adenovirus, pAdCMV5 Upon the targeted cells or tissue expressing the transfected TRAIL receptor, the cells or tissue are exposed to an antibody according to the present invention specific for binding the transfected TRAIL receptor It is appreciated that the anti-TRAIL receptor antibody is either agonistic or antagonistic thereto consistent with the desired therapeutic result

The antibodies of the present invention are also operative in conjunction with a sensitizer A sensitizer as used herein is defined to include any stimulus that induces apoptosis including ultraviolet light, organic molecules specifically including the class of bisindolmaleimides, heavy metals and free radical species

In the context of a malignancy therapy, TRA-8, is able to induce apoptosis of most TRAIL-sensitive tumor cells in a caspase-dependent fashion in the absence of the secondary crosslinking TRA-8 exhibits a strong tumoricidal activity *in vivo* The ability of TRA-8 to induce apoptosis of most TRAIL-sensitive cells confirms that DR5 alone is sufficient to trigger apoptosis The majority of tumor cells detailed herein express cell surface DR5 and their susceptibility to TRA-8 induced cell death paralleled their susceptibility to TRAIL, indicating that DR5 is a primary death receptor for TRAIL-mediated apoptosis in most tumor cells Thus, differential expression of DR5 by normal and cancer cells is operative in the selectivity of TRAIL-mediated apoptosis TRA-8 bypasses the decoy receptors to induce TRAIL-mediated apoptosis Only a minority of TRAIL resistant tumor cells are sensitive to TRA-8, however, indicating that the decoy receptors do not appear to play a major role in the resistance of tumor cells to TRAIL-mediated apoptosis

Although previous studies have indicated that systemic administration of the soluble form of TRAIL in animals does induce tumor regression without causing toxicity[3,4,22], the membrane-bound form of human TRAIL induces liver

damage in mice as shown herein. However, the hepatic toxicity of TRAIL is much less potent than that of Fas ligand as demonstrated by the lesser susceptibility of normal hepatocytes to TRAIL-induced injury compared to Fas ligand and by the lack of lethality of TRAIL *in vivo* Thus, titration of TRAIL has utility in cancer therapy

As detailed herein, the absence of significant levels of DR5 protein expression by normal hepatocytes is shown and is associated with hepatocyte resistance to TRA-8 induced apoptosis Crosslinking of DR5 with monoclonal antibody is insufficient to organize the homopolymeric forms of the death receptor able to trigger apoptosis Experiments in marmoset indicate no evidence of hepatic toxicity of TRA-8 administration. Thus, an agonistic monoclonal anti-DR5 antibody is likely to be more selective and safer than soluble TRAIL as a therapeutic agent

As a screening assay, the present invention is well suited for detecting small clusters of malignant cells which may still exhibit normal cell morphology *In situ* cell section staining of human cancer cells including lung, prostate and liver cancers with labeled antibodies according to the present invention readily identifies cancerous cells These cancer cells are observed to express very high levels of DR5 as compared to normal cells of the same type Thus, the present invention has utility as a sensitive screening method for early stage malignancies within tissue including at least lung, prostate and liver A therapeutic process is detailed herein for the inhibition of abnormal cell proliferation associated with diseases illustratively malignant cancers and lymphatic leukemias

The present invention is detailed herein with particularity to an anti-human DR5 monoclonal antibody designated as TRA-8, having ATCC Accession Number PTA-1428 It is appreciated that the techniques and results detailed with regard to the agonistic anti-human DR5 monoclonal antibody TRA-8 are wholly extendable and applicable to antagonistic DR5 antibodies, as well as antibodies raised against DR4, DcR1 and DcR2 acting in both agonistic and antagonistic manners

The levels of expression of an apoptosis receptor, such as Fas, do not necessarily correlate with the susceptibility of the cells to apoptosis For TRAIL-mediated apoptosis, it has been suggested that the expression of the decoy receptors for TRAIL influences the susceptibility of the cells Moreover, it has been suggested that DR5 must be associated with DR4 for effective transduction of the apoptosis signal through FADD and the caspase 8 pathway The availability of agonistic monoclonal anti-DR5 antibody allowed evaluation of the regulation of DR5 signaling and its relative role in TRAIL-mediated apoptosis Comparison of the susceptibility of the cells to TRA-8-mediated apoptosis with their susceptibility to TRAIL-mediated apoptosis offers insight into the role of DR5 in TRAIL-mediated apoptosis and the mechanisms that may affect susceptibility

This advantage generally extends to humanized anti-DR5 antibodies of the present invention A molecular clone of an antibody to DR-5 is prepared by known techniques as detailed with respect to the following Examples Recombinant DNA methodology (33) is operative herein to construct nucleic acid sequences which encode a monoclonal antibody molecule or antigen binding region thereof

The present invention allows the construction of humanized anti-TRAIL receptor antibodies that are unlikely to induce a human anti-mouse antibody (hereinafter referred to as "HAMA") response (34), while still having an effective antibody effector function. As used herein, the terms "human" and "humanized", in relation to antibodies, relate to any antibody which is expected to elicit a therapeutically tolerable weak immunogenic response in a human subject

The present invention provides for an anti-DR5 antibody, a humanized anti-DR5 antibody, TRA-8 heavy and light chain immunoglobulins and humanized heavy and light chain immunoglobulins Certain truncations of these proteins or genes perform the regulatory or enzymatic functions of the full sequence protein or gene For example, the nucleic acid sequences coding therefor can be altered by substitutions, additions, deletions or multimeric

expression that provide for functionally equivalent proteins or genes Due to the degeneracy of nucleic acid coding sequences, other sequences which encode substantially the same amino acid sequences as those of the naturally occurring proteins may be used in the practice of the present invention. These include, but are not limited to, nucleic acid sequences including all or portions of the nucleic acid sequences encoding the above polypeptides, which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change It is appreciated that the nucleotide sequence of an immunoglobulin according to the present invention tolerates sequence homology variations of up to 25% as calculated by standard methods ("Current Methods in Sequence Comparison and Analysis," *Macromolecule* Sequencing and Synthesis, Selected Methods and Applications, pp 127-149, 1998, Alan R. Liss, Inc) so long as such a variant forms an operative antibody which recognizes a TRAIL receptor DR5 For example, one or more amino acid residues within a polypeptide sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine The positively charged (basic) amino acids include arginine, lysine and histidine The negatively charged (acidic) amino acids include aspartic acid and glutamic acid Also included within the scope of the present invention are proteins or fragments or derivatives thereof which are differentially modified during or after translation, e g , by glycosolation, protolytic cleavage, linkage to an antibody molecule or other cellular ligands, etc In addition, the recombinant vector encoding nucleic acid sequences of the anti-DR5 antibodies of the present invention may be engineered so as to modify processing or expression of a vector

Additionally, an inhibitor encoding nucleic acid sequence can be mutated *in vitro* or *in vivo* to create and/or destroy translation, initiation, and/or termination sequences or to create variations in coding regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification Any technique for mutagenesis known in the art can be used, including but not limited to *in vitro* site directed mutagenesis, *J Biol. Chem* 253 6551, use of Tab linkers (Pharmacia), and the like

X-ray crystallography data indicates that the antibody immunoglobulin fold generally forms a long cylindrical structure comprising two layers of antiparallel b-sheets, each consisting of three or four b-chains In a variable region, three loops from each of the V domains of H and L chains cluster together to form an antigen-binding site Each of these loops is termed a complementarity determining region (CDR) The CDRs have the highest variability in amino acid sequence with the antibody The portions of the variable region that are not part of a CDR are called "framework regions" ("FR" regions) and generally play a role in maintaining CDR structure Preferably, all the CDRs from a given antibody are grafted into an acceptor antibody, in order to preserve the binding region for the TRAIL receptor epitope region. It is appreciated that grafting a portion of the total amount of CDRs into a donor is operative herein It is understood that grafting generally entails the replacement, residue for residue, of one amino acid or region, for another However, occasionally, especially with the transfer of a region, one or more residues may be added or omitted or substituted therefor, as desired, and that such deletions and insertions, as well as appropriate replacements and inversions, are within the skill of those in the art

An antibody of the present invention is obtained by, for example, grafting each CDR of L chain and H chain subunit of an anti-TRAIL receptor monoclonal antibody into a corresponding CDR region of a human antibody, thereby humanizing a mouse monoclonal antibody effective against a TRAIL-receptor

Antibody fragments which contain the idiotype of the molecule are also generated and operative herein using known techniques For example, such fragments illustratively include the anti-TRAIL receptor (AB')2 fragment which can be produced by pepsin digestion of the antibody molecule, the TRAIL receptor antibody AB' fragments generated through reduction of the disulfide bridges of the TRAIL receptor (AB')2 fragment, and the antibody fragment which are generated by treating the antibody molecule with papain and a reducing agent

In particular, the anti-DR5 monoclonal antibody TRA-8 may be obtained by culturing a hybridoma which, in turn, may be obtained by immunizing a mouse with human DR5 and subsequently fusing the spleen cells or lymph node cells from the mouse with mouse myeloma cells

Preparation of a monoclonal antibody illustratively involves the following steps

a) purification of a biomacromolecule for use as an antigen,

b) preparation of antibody producing cells, after first immunizing an animal using injections of the antigen, bleeding the animal and assaying the antibody titer, in order to determine when to remove the spleen,

c) preparation of myeloma cells,

d) fusing the antibody producing cells and myeloma cells,

e) selecting a hybridoma producing a desired antibody,

f) preparing a single cell clone (cloning),

g) optionally, culturing the hybridoma cells, or growing animals into which the hybridoma cells have been transplanted, for large scale preparation of the monoclonal antibody, and

h) testing the biological activities and the specificity, or assaying marker agent properties, of the monoclonal antibody thus prepared

The procedure for the preparation of an anti-DR5 monoclonal antibody is detailed below with reference to the above described steps This method for

preparing an antibody of the present invention is intended only to be illustrative of the methods of preparation and is not limited thereto Other known procedures may be followed, or the following method modified, for instance by using antibody producing cells other than spleen cells and myeloma.

(a) Preparation of antigen

A recombinant protein (hereinafter referred to as "recombinant human DR5"), effective as the antigen, is obtained by transfecting QBI-293A cells with the expression vector pAdDR5-IgG for a fusion protein comprising the extracellular domain of human DR5 and the Fc region of human IgG1 antibody (hereinafter referred to as "IgG"), (cf PTA-1428) to express it by using the ADENO-Quest kit (Quantum Biotechnologies Inc , Canada), and collecting and partially purifying the expression product. The plasmid pAdDR5-IgG is constructed by inserting DNA encoding a human DR5 and human IgG fusion protein into pAdCMV5, which is an expression vector for animal cells Other materials, such as the DNA encoding DR5, the vector, and the host, are operative herein.

The human DR5 and IgG fusion protein produced in the culture supernatant of the QBI-293A cells transfected with the vector pAdDR5-IgG may be partially purified by ProteinA-Sepharose affinity chromatography or ProteinG-Sepharose affinity chromatography, or ion-exchange chromatography using a Resource Q column (trade name, Pharmacia)

Alternatively, purified DR5 obtained from the cell membranes of human cell lines is used as the antigen Further, since the primary structure of DR5 is known (cf PTA-1428), a peptide comprising the amino acid sequence of SEQ ID No 1, may be chemically synthesized by a known method such as the Sanger method, and used as the antigen

(b) Preparation of antibody producing cells

A mouse is immunized with the immunogen produced in step (a), mixed with an adjuvant, such as Freund's complete or incomplete adjuvant or alum Other suitable experimental animals illustratively include rats, guinea pigs, rabbits, dogs, chickens, horses, pigs, cows and sheep

Suitable administration routes to immunize an experimental animal include the subcutaneous, intraperitoneal, intravenous, intradermal, and intramuscular injection routes, with subcutaneous and intraperitoneal injections being preferred.

Immunizations are optionally performed by a single dose or, by several repeated doses at appropriate intervals (preferably 1 to 5 weeks) Immunized animals are monitored for antibody titer in their sera, and an animal with a sufficiently high antibody titer is selected as the source of antibody producing cells Selecting an animal with a high titer makes the subsequent process more efficient. Cells for the subsequent fusion are generally harvested from the animal 3 to 5 days after the final immunization

Methods for assaying antibody titer include various well known techniques such as radioimmunoassay (hereinafter, referred to as "RIA"), solid-phase enzyme immunoassay (hereinafter, referred to as "ELISA"), fluorescent antibody assay and passive hemagglutination assay, with RIA and ELISA preferred for reasons of detection sensitivity, rapidity, accuracy and potential for automation.

Determination of antibody titer may be performed, for example, by ELISA, as follows First, purified or partially purified DR5 is adsorbed onto the surface of a solid phase, such as a 96-well ELISA plate, followed by blocking any remaining surface, to which DR5 has not been bound, with a protein unrelated to the antigen, such as bovine serum albumin (BSA) After washing, the well surfaces are contacted with serially diluted samples of mouse sera to enable binding of the anti-DR5 antibody in the samples to the antigen An enzyme-labeled, anti-mouse antibody, as the secondary antibody, is added to be bound to the mouse antibody After washing, the enzyme substrate is added, and antibody titer is estimated by determining absorbance change due to color development caused by the alteration of the substrate or the like

(c) Preparation of myeloma cells

Cells from established mouse cell lines serve as the source of myeloma cells, including for example 8-azaguanine resistant mouse, derived from

BALB/c myeloma strains P3X63Ag8U 1 (P3-U1) (35), P3/NSI/1-Ag4-1(NS-1) (36) Sp2/0-Ag14 (SP-2) (37), P3X63Ag8 653 (653) (38) and P3X63Ag8 (X63) (39) The cell line selected is serially transferred into an appropriate medium, such as 8-azaguanine medium. 8-azaguanine medium includes Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM") or Dulbecco's, Modified Eagle Medium (hereinafter referred to as "DMEM") RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, fetal calf serum (hereinafter referred to as "FCS"), and 8-azaguanine The cells are then transferred to a normal medium, such as ASF104 medium (Ajinomoto, K. K.) containing 10% FCS, 3 to 4 days prior to fusion, in order to ensure that at least 2 x 10^7 cells are available on the day of fusion.

(d) Cell fusion

Lymphocytes and plasma cells obtained from any suitable part of the animal are precursor cells to produce the antibody Lymphocyte or plasma cell sources illustratively include spleen, lymph nodes, peripheral blood, or any appropriate combination thereof, with spleen cells being the most common source

After the last booster injection, tissue in which antibody producing cells are present is removed from a mouse having the predetermined antibody titer The currently favored technique for fusion of spleen cells with myeloma cells prepared in step c), employs polyethylene glycol

The fusion technique includes washing spleen and myeloma cells with serum-free medium (such as RPMI 1640) or phosphate buffered saline (hereinafter referred to as "PBS") so that the number ratio of spleen cells to myeloma cells is approximately between 5 1 and 10 1, and then centrifuged. After the supernatant has been discarded and the pelleted cells sufficiently loosened, 1 ml of serum-free medium containing 50%(w/v) polyethylene glycol (m w 1,000 to 4,000) is added dropwise with mixing Subsequently, 10 ml of serum-free medium is slowly added and then centrifuged The supernatant is discarded again, and the pelleted cells are suspended in an appropriate amount of HAT medium containing a solution of hypoxanthine, aminopterin and thymidine

(hereinafter referred to as "HAT") and mouse interleukin-2 (hereinafter referred to as "IL-2") The suspension is then dispensed into the wells of culture plates (also referred herein simply as "plates") and incubated in the presence of 5% v/v CO_2 at 37°C for about 2 weeks, with the supplementary addition of HAT medium as appropriate

e) Selection of hybridomas

When the myeloma strain used is resistant to 8-azaguanine, i e , it is deficient in the hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme, any unfused myeloma cells and any myeloma-myeloma fusions are unable to survive in HAT medium On the other hand, fusions of antibody producing cells with each other, as well as hybridomas of antibody producing cells with myeloma cells can survive, the former only having a limited life Accordingly, continued incubation in HAT medium results in selection of only the desired hybridomas

The resulting hybridomas grow into colonies that are then transferred into HAT medium lacking aminopterin (HT medium) Thereafter, aliquots of the culture supernatant are removed to determine anti-Fas antibody titer by, for example, ELISA. When the above-mentioned fusion protein is used as the ELISA antigen, it is also necessary to eliminate clones producing an antibody which is specifically bound to the Fc region of human IgG1 The presence or absence of such a clone may be verified, for example, by ELISA using Fas-IgG1or IgG1, as the antigen

(f) Cloning

Hybridomas which have been shown to produce specific antibodies, using a method similar to that described in step b) to determine antibody titer, are then transferred to another plate for cloning Suitable cloning methods include the limiting dilution method, in which hybridomas are diluted to contain one cell per well of a plate and then cultured, the soft agar method in which colonies are recovered after culturing in soft agar medium, a method of using a micromanipulator to separate a single cell for culture, and "sort-a-clone", in which single cells are separated by a cell sorter

The cloning procedure according to, for example, the limiting dilution method is repeated 2 to 4 times for each well demonstrating an antibody titer, and clones having stable antibody titers are selected as anti-DR5 monoclonal antibody producing hybridomas Hybridomas producing an anti mouse DR5 antibody are selected by a similar method to obtain an anti-DR5 monoclonal antibody producing cell line

The mouse-mouse hybridoma TRA-8 which is a basis for antibodies of the present invention was deposited with American Type Culture Collection on March 1, 2000, and has the accession number PTA-1428 Accordingly, when preparing an antibody using the mouse-mouse hybridoma TRA-8 or any other established hybridoma, the preparation may be performed by following a procedure starting from the step (g) below, with the steps (a) to (f) omitted

(g) Culture of hybridoma to prepare monoclonal antibody

The hybridoma obtained by the cloning is then cultured in normal medium, not in HT medium. Large-scale culture is performed by roller bottle culture, using large culture bottles, or by spinner culture The supernatant from the large-scale culture is then harvested and purified by a suitable method, such as gel filtration, which is well known to those skilled in the art, to obtain an anti-DR5 monoclonal antibody which is a basis for antibodies of the present invention The hybridoma may also be grown intraperitoneally in a syngeneic mouse, such as a BALB/c mouse or a nu/nu mouse, to obtain ascites containing an anti-DR5 monoclonal antibody in large quantities Commercially available monoclonal antibody purification kits (for example, MAbTrap GII Kit, Pharmacia) are conveniently used to purify the harvested antibodies

Monoclonal antibodies prepared as above have a high specificity for human DR5

(h) Assay of monoclonal antibody

Suitable identification methods of the isotype and the subclass of the monoclonal antibody include the Ouchterlony method, ELISA and RIA. Preferably, a commercial kit is used for identification, such as a Mouse Typer Kit (trade name, BioRad)

Quantification of protein may be performed by the Folin-Lowry method, or by calculation based on the absorbance at 280 nm (1 4 (OD280) = Immunoglobulin 1 mg/ml)

Identification of the epitope that the monoclonal antibody recognizes are performed as follows First, various partial structures of the molecule that the monoclonal antibody recognizes are prepared The partial structures are prepared by the method wherein various partial peptides of the molecule are synthetically prepared by known oligopeptide synthesis technique, or the method wherein DNA encoding the desired partial polypeptide is incorporated in a suitable expression plasmid, and is expressed in a suitable host, such as *E coli*, to produce the peptides Generally, both methods are frequently used in combination for the above object For example, a series of polypeptides having appropriately reduced lengths, working from the C- or N-terminus of the antigen protein, can be prepared by established genetic engineering techniques By establishing which fragments react with the antibodv, an approximate idea of the epitope site is obtained

The epitope is more closely identified by synthesizing a variety of smaller oligopeptides corresponding thereto or mutants of the peptide using established oligopeptide synthesis techniques to determine a binding property of the peptides to the anti-DR5 monoclonal antibody which is a basis for preparation of the antibody of the present invention and a competitive inhibition of binding of the peptide to an antigen with the monoclonal antibody Commercially available kits, such as the SPOTs Kit (Genosys Biotechnologies, Inc) and a series of multipin peptide synthesis kits based on the multipin synthesis method (Chiron Corp) may be conveniently used to obtain a large variety of oligopeptides

An antibody of the present invention has the various functional properties a) to f) described below, each of which is verified by, for example, a method described hereinbelow

a) Specific binding of TRA-8 to cells expressing human DR5

A unique feature of the present invention is the ability to bind cell surface DR5 This is demonstrated by flow cytometry analysis of cells expressing DR5 First, specific cell surface binding of DR5 is confirmed by the COS-7 cells transfected with the full-length cDNA encoding human DR5 Specifically, TRA-8 only recognizes COS-7 cells transfected with DR5 but not empty control vector or vector encoding DR4 Second, three different origins hematopoietic, glioma, and prostate cancer of human malignant tumor cells are tested The majority of these transformed tumor cells expressed significant levels of cell surface DR5, although expression levels varied largely Third, two panels of human primary synovial fibroblast cells from RA and OA patients are examined All RA synovial cells expressed significantly higher levels of DR5 compared to OA cells

b) Induction of apoptosis of human malignant tumor cells *in vitro* in the absence of crosslinking

The ability of an antibody raised according to the present invention to recognize TRAIL receptor and to directly induce apoptosis of malignant human tumor cells is determined by cell viability assay (ATPLite) during *in vitro* culture of cells with various concentrations of an antibody, specifically TRA-8 The majority of tumor cells are susceptible to TRA-8 induced apoptosis For some cells, TRA-8 exhibited a strong apoptosis-inducing activity, for example, TRA-8 is able to induce apoptosis of human Jurkat cells within the pg/ml levels Importantly, TRA-8 induced apoptosis did not require crosslinking, and in most cells, TRA-8 exhibited a stronger apoptosis-inducing activity than the recombinant soluble TRAIL in the presence of the enhancer

c) Tumoricidal activity of TRA-8 *in vivo*

Tumoricidal activity of TRA-8 is evaluated in two SCID/human tumor cell models First, SCID mice are intravenously inoculated with human leukemia Jurkat cells, and treated with a single dose (100 μg) of TRA-8 The results show that the majority of implanted Jurkat cells are eliminated from the peripheral blood and spleen by the treatment with TRA-8, as determined by flow cytometry analysis and in situ immunohistochemical staining of Jurkat cells

Second, human astrocytoma cells, 1321N1, are subcutaneously inoculated in SCID mice, and the tumor-bearing mice are treated with a single dose of TRA-8 The growth of implanted 1321N1 cells is significantly inhibited in TRA-8 treated mice as determined by the sizes of tumor and histological analysis

d) Identification of RA synovial cells by TRA-8

The primary synovial cells isolated from 8 RA and 4 OA patients are tested for cell surface expression of DR5 TRA-8 is able to positively strain all RA cells but negatively stain all OA cells Thus, RA is differentiated from OA by the surface expression of DR5 as detected by TRA-8

e) Induction of apoptosis in RA synovial fibroblast cells by TRA-8

The ability of TRA-8 to induce apoptosis of RA synovial cells is determined by cell viability assay during *in vitro* culture in the presence of various concentrations of TRA-8 All RA cells exhibited high to intermediate levels of susceptibility to 100 ng/ml of TRA-8 In contrast, all OA cells are essentially resistant to TRA-8 induced apoptosis Importantly, TRA-8 exhibited a better apoptosis-inducing activity to RA synovial cells than soluble TRAIL with the enhancer Moreover, compared to anti-Fas antibody (CH-11), TRA-8 exhibited a better selectivity to RA synovial cells

f) TRA-8 does not induce production of MMPs in RA synovial cells

Since TRA-8 is able to induce NF-kb activation in RA synovial cells as TNF-a, the effect of TRA-8 on the production of MMP1 and MMP3 of synovial cells is determined. While TNF-a induced a dose-dependent increase of MMPs, TRA-8 is unable to induce any production of MMPs, and in some concentrations, TRA-8 slightly decreased the production of MMPs in RA synovial cells

g) TRA-8 induces multiple caspase activation.

Since caspases play a crucial role in induction of apoptosis The ability of TRA-8 to induce caspase activation is determined in human Jurkat cells When Jurkat cells are incubated with a low dose (50 ng/ml) of TRA-8, the activation of caspase 8, caspase 9, and caspase 3 is observed as early as 15 minutes after incubation as demonstrated by Western blot analysis and caspase cleavage analysis In term of timing, number and strength of caspase activation,

antibodies of the present invention including the demonstrative antibody TRA-8 exhibited a much better activity than any other known apoptosis-inducing antibodies, such as anti-human Fas antibody (CH-11)

Thus, an antibody of the present invention is a substance having a property to selectively induce apoptosis in pathogenic cells as shown in effect (a) and (g) Accordingly, it is useful as a prophylactic and therapeutic agent for diseases associated with inappropriate survival of cells or inappropriate proliferation of cells, such as those attributable to dysregulation of apoptosis systems including the Fas/Fas ligand system

The ability of an antibody of the present invention to induce apoptosis is confirmed by culturing cells such as the human leukemia cell line Jurkat (American Type Culture No TIB-152) and astrocytoma cell line 1321N1 in medium in which the test sample has been added, and determining the survival rate by, for example, an ATPLite assay

Antibody of the present invention, especially anti-DR5 antibodies having almost the same immunogenicity to human as that of human antibodies, is used as an agent for prophylaxis or treatment of diseases associated with inappropriate survival or proliferation of cells, including those attributable to dysregulation of the apoptosis systems in autoimmune diseases illustratively including systemic lupus erythematosus, Hashimoto's disease, rheumatoid arthritis, graft-versus-host disease, Sjögren's syndrome, pernicious anemia, Addison disease, scleroderma, Goodpasture's syndrome, Crohn's disease, autoimmune hemolytic anemia, sterility, myasthenia gravis, multiple sclerosis, Basedow's disease, thrombopenia purpura, insulin-dependent diabetes mellitus, allergy; atopic disease, arteriosclerosis, myocarditis, cardiomyopathy, glomerular nephritis, hypoplastic anemia, rejection after organ transplantation and numerous malignancies of lung, prostate, liver, ovary, lymphatic and breast tissues

Such a prophylactic or therapeutic agent may be administered in various forms Suitable modes of administration include oral administration, such as by

tablets, capsules, granules, powders and syrups, or parenteral administration, such as by injection, dropping injection and suppositories

The antibody or therapeutic agent may be administered orally, rectally, intracisternally, intraventricular, intracranial, intrathecal, intravaginally, parenterally (intravenously, intramuscularly, or subcutaneously), locally (powders, ointments, or drops), by intraperitoneal injection, transdermally, by inhalation or as a buccal or nasal spray The exact amount of the antibody or therapeutic agent required will vary from subject to subject, depending on the age, weight and general condition of the subject, the severity of the disease that is being treated, the location and size of the tumor, the particular compounds used, the mode of administration, and the like An appropriate amount may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. Typical single dosages of antibody range from 0 1-10,000 micrograms, preferably between 1 and 100 micrograms Typical antibody concentrations in a carrier range from 0 2 to 2000 nanograms per delivered milliliter

Depending on the intended mode of administration, the antibody or therapeutic agent can be in pharmaceutical compositions in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules powders, liquids, or suspensions, preferably in unit dosage form suitable for single administration of a precise dosage The compositions will include an effective amount of the selected substrate in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, or diluents By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, which can be administered to an individual along with the selected substrate without causing significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions,

suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like It may also be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates, and sodium carbonate, (e) solution retarders, as for example, paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol, and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, or mixtures thereof

In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like

Solid dosage forms such as tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner Examples of embedding compositions which can be used are polymeric substances and waxes The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore, melt in the rectum or vaginal cavity and release the active component

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays, and inhalants The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers, or propellants as may be required. Ophthalmic formulations, eye ointments, powders, and solution are also contemplated as being within the scope of this invention

The term "pharmaceutically acceptable salts, esters, amides, and prodrugs" as used herein refers to those carboxylate salts, amino acid addition salts, esters, amides, and prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, nitrate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate mesylate, glucoheptonate, lactobionate, methane sulphonate and laurylsulphonate salts, and the like These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary

ammonium and amine cations including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. (See, for example, S.M Berge et al, "Pharmaceutical Salts," *J Pharm. Sci.*, 1977, 66 1-19 which is incorporated herein by reference.)

The term "prodrug" refers to compounds that are rapidly transformed *in vivo* to yield the parent compounds of the above formula, for example, by hydrolysis in blood A thorough discussion is provided in T Higuchi and V Stella, "Pro-drugs as Novel Delivery Systems," Vol 14 of the A C S Symposium Series, and in *Bioreversible Carriers in Drug Design*, ed. Edward B Roche, American Pharmaceutical Association and Pergamon Press, 1987

A target cell is a cell of an animal illustratively including human, non-human primate, rat, mouse, guinea pig, rabbit, goat, sheep, cow, horse, chicken, pig, marmoset and ferret

In addition, the antibody or therapeutic agent of the present invention can exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like In general, the solvated forms are considered equivalent to the unsolvated forms for the purposes of the present invention

Antibody molecules are purified by known techniques illustratively including amino absorption or amino affinity chromatography, chromatographic techniques such as high pressure liquid chromatography, or a combination thereof

Another aspect of the present invention includes a pharmaceutical product for use in delivering biologically active anti-TRAIL receptor antibody or humanized anti-TRAIL receptor antibody to a vertebrate The pharmaceutical product includes a pharmaceutically effective quantity of anti-TRAIL receptor antibody or fragment thereof, a pharmaceutically acceptable carrier, and a container enclosing the carrier and the antibody in a sterile fashion

In a preferred embodiment of the invention, a pharmaceutically effective amount of an anti-DR5 antibody inhibits cell proliferation by contact with a

target cell A pharmaceutically effective amount of an antibody recognizing DR5 or a humanized antibody recognizing DR5 is an amount administered to an individual sufficient to cause a desired effect. Desired effects of administration of a pharmaceutically effective amount of DR5 recognizing antibodies include death of a target cell, growth inhibition of a target cell, stimulation of DR5, binding to DR5 and increased NFkB levels or activity in a target cell A target cell is a cell that expresses DR5 and illustratively includes abnormally growing cells and tumors such as papillomas and warts, breast cancer, colon cancer, hepatomas, leukemias, lung cancer, melanoma, myelomas, osteosarcomas, ovarian cancer, pancreatic cancer, prostate cancer, cancer of the head and neck, thyroid cancer, uterine cancer and tumors of the brain such as astrocytomas In vivo, the target cell is a cell of an individual with a pathological condition, including those where cell proliferation is abnormal or dysregulated such as malignant or benign cancer and rheumatoid arthritis

In another preferred embodiment, the target cell is also contacted by a therapeutic agent.

A therapeutic agent is a compound or composition effective in ameliorating a pathological condition. An illustrative example of a therapeutic agent includes an anti-cancer compound.

An anti-cancer compound is a compound or composition effective in inhibiting or arresting the growth of an abnormally growing cell. A pharmaceutically effective amount of an anti-cancer compound is an amount administered to an individual sufficient to cause inhibition or arrest of the growth of an abnormally growing cell Illustrative examples of anti-cancer compounds include bleomycin, carboplatin, chlorambucil, cisplatin, colchicine, cyclophosphamide, daunorubicin, dactinomycin, diethylstilbestrol doxorubicin, etoposide, 5-fluorouracil, floxuridine, melphalan, methotrexate, mitomycin, 6-mercaptopurine, teniposide, 6-thioguanine, vincristine and vinblastine Further examples of anti-cancer compounds and therapeutic agents are found in The Merck Manual of Diagnosis and Therapy, 15th Ed , Berkow et al., eds ,

1987, Rahway, N J and Sladek et al Metabolism and Action of Anti-Cancer Drugs, 1987, Powis et al eds , Taylor and Francis, New York, N Y

Antibody of the present invention can be further combined with other therapies, such as chemotherapy and radiotherapy in the treatment of malignance, and therapeutic efficacy can be enhanced by apoptosis-inducing compounds such as bisindolylmaleimide VIII

Compared to previously published anti-DR5 antibody (24), the apoptosis-inducing activity of the demonstrative TRA-8 antibody of the present invention is very strong, and is able to induce apoptosis of Jurkat cells with the pg/ml levels *in vitro* and demonstrates superior *in vivo* tumoricidal activity as compared to previously reported soluble TRAIL The intravenous administration of a single dose of TRA-8 is sufficient to inhibit the growth of both solid tumor and hematopoietic tumor cells, whereas induction of *in vivo* tumor regression with the soluble TRAIL requires much high dose (500 ug every day for 10 days) The anti-TRAIL receptor antibodies of the present invention appear to be as safe as soluble TRAIL since exemplary antibody TRA-8 does not induce apoptosis of non-transformed fibroblast cells

Vectors of the present invention include a nucleic acid sequence encoding a heavy or light chain immunoglobulin of an anti-DR5 antibody operably linked to a regulatory element such as a promoter or enhancer "Operably linked" refers to an arrangement of nucleotide sequences configured so as to perform their usual function Thus, a regulatory element operably linked to a nucleotide sequence encoding a polypeptide is capable of directing the transcription, replication and/or translation of the polypeptide It will be recognized by those skilled in the art that a single vector optionally includes coding sequences for both a heavy and a light chain immunoglobulin of an anti-DR5 antibody

The following examples are set forth below to illustrate the methods and results according to the present invention These examples are not intended to be inclusive of all aspects of the present invention, but rather to illustrate representative methods and results These examples are not intended to exclude

equivalents and variations of the present invention which are apparent to one skilled in the art

Example 1 Preparation of DR5 antigen

1 1 Cloning of DR5 cDNA

DNA encoding the human DR5 protein is cloned by the following RT-PCR method using

a) Template

The total RNA of HeLa cells is extracted by using TRIzol Reagent (GIBCO BRL) The template for the PCR reaction used cDNA that is obtained by using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the instruction manual provided with the kit

b) PCR Primers

The following oligonucleotide primers are synthesized for the PCR.

5'-gacgatgcccgatctactttaaggg-3' (DR5p1 SEQ ID No 1),

5'- ccactgggtgatgttggatggg-3' (DR5p2 SEQ ID No 2),

Unless otherwise specified, all oligonucleotides in these Examples are synthesized by Lifetechnologies All oligonucleotides are stored at –20°C after being dissolved in distilled water

c) PCR reaction

Composition of the PCR reaction solution

template cDNA, 5 µl of total 33 µl reaction

primer DR5p1, 10 pmol,

primer DR5p2, 10 pmol,

10 x concentrated PCR buffer (provided with the kit), 10 µl,

dNTPs (each 2 5 mM), 4 µl, and

Taq polymerase (Promega), 5 units

Sterile distilled water is added to the solution to a total volume of 100 µl Unless otherwise specified, dNTPs are an equimolar mixture of dATP, dCTP, dGTP and dTTP (2 5 mM each)

The PCR reaction is conducted as follows The solution is first heated at 94°C for 2 minutes, after which a cycle of heating to 94°C for 30 sec, 52°C for 1

minute and 72°C for 3 minutes, is repeated 40 times After completion of this procedure, the reaction solution is heated at 72°C for 10 minutes

The amplified DNA fragments, thus obtained, are separated on a 1% agarose gel containing 0 25 ug/ml ethidium bromide The bands determined to contain the desired DNA fragments are cut out using a razor blade and the DNA is recovered therefrom using the Gene Clean kit (BIO101) The DNA fragment is cloned using the TA Cloning Kit (Invitrogen, CA) This is performed as follows

The DNA fragment recovered from the PCR reaction solution, together with 50 ng of pCR2 1 vector which is provided with the TA Cloning kit, is mixed with 1 µl of 10 X ligase reaction buffer (6 mM Tris-HCl (pH 7 5), 6 mM magnesium chloride, 5 mM sodium chloride, 7 mM β-mercaptoethanol, 0 1 mM ATP, 2 mM DTT, 1 mM spermidine, and 0 1 mg/ml bovine serum albumin), to which 4 units of T4 DNA ligase (1 µl) has been added. The total volume of the mixture is adjusted to 10 µl with sterile deionized water, and the resulting ligase solution is incubated at 14°C for 15 hours After this time, 2 µl of the ligase reaction solution is added to 50 µl of competent *E coli* strain TOP10F', which is provided with the TA cloning kit and brought to competence in accordance with the instruction manual, to which 2 µl of 0 5 M β-mercaptoethanol has been added, and the resulting mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 5 minutes Next, 500 µl of medium containing 2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM potassium chloride, 1 mM magnesium chloride, and 20 mM glucose (hereinafter referred to as "SOC" medium) is added to the culture, and the mixture is incubated for 1 hour at 37°C with shaking After this time, the culture is spread on an L-broth agar plate (1% v/v tryptone, 0 5% w/v yeast extract, 0 5% w/v sodium chloride, 0 1% w/v glucose, and 0 6% w/v bacto-agar (Difco)), containing 100 µg/ml Ampicillin resistant colonies appearing on the plate are selected and scraped off with a platinum transfer loop, and cultured in L-broth medium containing 100 µg/ml ampicillin at 37°C, overnight, with shaking at 200

r p m. After incubation, the cells are harvested by centrifugation, from which plasmid DNA is prepared by the alkali method EcoRI-EcoRI DR5cDNA fragment from the thus obtained plasmid is subcloned into pcDNA3 plasmid (Invitrogen, CA) The full length of the DR5 gene in pcDNA3 are sequenced and matched the published sequence The thus obtained plasmid is designated as plasmid pcDNA3-DR5

1 2 Construction of DR5-IgG expression vector

In order to obtain a soluble form of human DR5 lacking the transmembrane domain, an expression plasmid vector is constructed. This vector is designed to encode a fusion protein comprising the extracellular domain of human DR5 fused to the human IgG1 Fc DNA (41) DNA encoding the human DR5 lacking the transmembrane domain is obtained by the following PCR reaction.

a) Template

The template for the PCR reaction used pcDNA3-DR5

b) PCR Primers

The following oligonucleotide primers are synthesized for the PCR

5'-gacgatgcccgatctactttaaggg-3' (DR5p1 SEQ ID No 1),

5'-ggatccgtggacacattcgatgtc-3' (DR5p3 SEQ ID No 3),

Unless otherwise specified, all oligonucleotides in these Examples are synthesized by Lifetechnologies All oligonucleotides are stored at -20°C after being dissolved in distilled water

c) PCR reaction

The PCR reaction is conducted and amplified DNA isolated as per Example 1 1(c)

The thus obtained plasmid is designated as plasmid pCR-ΔDR5 The BamHI-EcoRI fragment encoded human Fc fragment which is recovered from pmFas-hIgG1Fc is subcloned into BamHI and EcoRI multi-cloning sites of pcDNA3 The plasmid thus obtained is designated pcDNAFc Furthermore, the BamHI-BamHI fragment encoding the human soluble DR5 region which is recovered from pCR-ΔDR5 is subcloned into the BamHI site of pcDNAFc

plasmid The thus obtained plasmid is designated as plasmid pcDNAΔDR5-Fc The EcoRI fragment encoding the human soluble DR5-human IgG Fc region which is recovered from the pcDNAΔDR5-Fc plasmid is blunt ended by using the DNA polymerase Klenow fragment (GIBCO BRL) and then subcloned into the shuttle vector pAdCMV5 (Quantum Biotechnologies Inc , Canada) which is blunt ended after cutting by BamHI The plasmid thus obtained is designated pAdΔDR5-Fc

1 3 Expression and purification of the human DR5-IgG1 fusion protein

QBI-293A cells (provided with the ADENO-Quest Kit) are co-transfected with pAdΔDR5-Fc and QBI-viral DNA (provided with the ADENO-Quest Kit) using the ADENO-Quest kit (Quantum Biotechnologies Inc , Canada) according to the instruction manual The recombinant virus plaques are cultured and screened for expression of DR5-IgG fusion protein by ELISA analysis of the supernatant. The positive plaques are amplified in QBI-293A cells and stored at –80°C as virus stock Fifty dishes (150 mm) of QBI-293A cells are transfected with pAdΔDR5-Fc recombinant virus at 10 m o i (Multiplicity of Infection) The culture media are harvested after transfection for 48 hours

The transfected cells having the DR5-IgG gene are grown to a cell density of 1 x 10^6 cells/ml by incubation in 500 ml of DMEM (GIBCO) medium, containing 10% v/v FCS, at 37°C in an atmosphere of 5% v/v CO_2 for 2 days The culture is then centrifuged (1,000 r p m , 5 minutes) and the supernatant collected The purification of DR5-IgG from the supernatant is achieved using ProteinA-Sepharose CL-4B affinity chromatography (Pharmacia) under the following conditions

column ProteinA-Sepharose CL-4B column (column size 2 ml, Pharmacia),

elution buffer 0 1 M glycine (pH 2 4), 0 15 M NaCl,

neutralization buffer 1M Tris-HCl (pH 8 5)

After all of the supernatant is applied to the column, it is washed three times with 20 ml of PBS and then 1 ml of elution buffer is added 10 times The optical density of each eluted fraction (1 ml) is measured. The second fraction through the fifth fraction (with $OD_{280} \geq 0.1$) are collected and after addition of 100 µl of neutralization buffer, the eluates are placed separately in dialysis tubing, and the eluates dialyzed against 1 liter of PBS (pH 7.5) at 4°C The dialysis buffer being changed twice

The eluates are then assayed for expression of the DR5-IgG gene product by ELISA. First, 100 µl of each fraction are placed separately into wells of a 96-well microplate (Costar) and incubated at 37°C for 1 hour After this time, the solution in the wells is removed, and the plate is washed 3 times with 100 µl/well of PBS containing 0.1% v/v Tween 20 (hereinafter referred to as "PBS-Tween") After washing, PBS containing 2% w/v bovine serum albumin (hereinafter referred to as "BSA") is added in quantities of 100 µl/well, and the plate is then incubated at 37°C for 1 hour After this time, the wells are washed a further 3 times with 100 µl/well of PBS-Tween, after which 100 µl/well of a solution of anti-human IgG1 monoclonal antibody diluted 1000-fold with PBS-Tween is added to each well, and the plate is once again incubated at 37°C for 1 hour The wells are then washed 3 times with 100 µl/well of PBS-Tween. 3,3',5,5'-Tetramethyl-benzidine (hereinafter referred to as "TMB") liquid substrate system (Sigma) is then added in an amount of 100 µl/well and the plate is allowed to stand at room temperature for 5 minutes and then the reaction stopped by adding 100 µl/well of 0.2N H_2SO_4 The absorbance of each well is read at 450 nm to estimate the concentration of the bound antibody, using the absorbance at 650 nm as the control reading The absorbance is measured using a microplate reader (Molecular Devices) The production of DR5-IgG1 is confirmed using this ELISA method The molecular weight of the expressed DR5-IgG1 fusion protein is determined using western blotting analysis in which anti-human IgG1 mAb (Sigma) is used to detect the antibody on the gel The molecular weight of the expressed DR5-IgG1 fusion protein has an approximate

molecular weight of 50 kDa. The purity achieved being greater than 90% as evaluated by analysis on SDS-PAGE and detection of the protein by Coomassie blue staining

Example 2 Generation of monoclonal antibodies against human DR5

2 1 Immunization

Female, Balb/c mice (Jackson Laboratory, Bar Harbor, ME) of 6-8 weeks of age, are immunized with the affinity-purified human DR5/hIgG1 fusion protein. For the initial foot-pad immunization, the fusion protein (50 μg) is emulsified in Freund's complete adjuvant (Difco, Detroit, MI) The mice are then boosted with four injections of 50 μg of fusion protein administered without adjuvant every other day Three days after the last injection, lymphocytes from the local lymph nodes are fused with NS-1 myeloma cells, and the hybridomas are cultured in F104 media supplemented with 10% fetal calf serum Positive hybridomas are selected by ELISA in which the plates are coated either with 1 μg/ml DR5/hIgG1 or the same amount of Fas/hIgG1 as a control The isotype of the hybridomas is determined by ELISA using a panel of mouse Ig isotype-specific goat antibodies (Southern Biotechnology, Birmingham, AL) Monoclonal antibodies are purified by affinity chromatography using immobilized anti-mouse IgG1 or protein G (Sigma)

2 2 Cell fusion

On the third day after the booster injection, the local lymph nodes are removed from the mouse and placed into 10 ml of serum-free RPMI 1640 medium (GIBCO BRL) containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 300 μg/ml L-glutamic acid, and disrupted by passing the organ through a mesh (Cell Strainer, Falcon) using a spatula The resulting cell suspension is centrifuged to pellet the local lymph nodes cells which are then washed twice with serum-free RPMI medium The washed cells are then resuspended in serum-free RPMI medium and counted

In the meantime, myeloma NS1 cells (American Type Culture Collection TIB-18) had been grown to a cell density not exceeding 1 x 10^8 cells/ml in

ASF104 medium (Ajinomoto, K. K.) containing 10% v/v FCS (Gibco BRL) ("ASF medium with serum") at 37°C under 5% v/v CO_2, and these are likewise disrupted, washed, resuspended and counted.

An amount of the NS1 cell suspension calculated to contain 3 x 10^7 cells is mixed with an amount of the spleen cell suspension calculated to contain 3 x 10^8 cells. The resulting mix is centrifuged and the supernatant discarded. The following steps of the cell fusion are performed whilst at all times keeping the plastic tube containing the pellet at 37°C in a beaker of warm water.

One ml of 50%(w/v) polyethylene glycol 1500 (Boehringer Manheim) is then slowly added to the tube, all the while stirring the pellet using the tip of a pipette. Subsequently, 1 ml of serum-free RPMI medium, prewarmed to 37°C, is slowly added in 2 portions, followed by the addition of a further 7 ml of serum-free RPMI medium. The resulting mix is then centrifuged, the supernatant discarded and 10 ml of HAT medium containing 10% v/v FCS are added while stirring gently with the tip of a pipette. A further 20 ml of HAT medium containing 10% v/v FCS is added, and the suspension is dispensed into 96-well cell culture microplates at 100 µl/well and incubated at 37°C in an atmosphere of 5% v/v CO_2. After 7 or 8 days, 100 µl/well of fresh HAT medium are used to replace medium in any wells exhibiting a yellowish hue. The fusion cells from these wells are cloned by limiting dilution as described below.

2.3 Cloning by limiting dilution

Thymuses from 4 to 10 week-old female BALB/c mice (from Japan SLC, Inc.) are removed, disrupted on a mesh (Cell Strainer; Falcon) as described above, and the disrupted cells are washed twice with HT medium containing 10% v/v FCS. An amount of thymus cells corresponding to those from one mouse is suspended in 30 ml of HT medium containing 10% v/v FCS to produce a feeder cell suspension. The fusion cell preparation obtained above in Example 2.2 is diluted with this feeder cell suspension 10- to 100-fold, and further diluted serially with the feeder cell suspension to make suspensions having fusion cell densities of 5, 1 and 0.5 cells/ml. The thus prepared samples are dispensed into

wells of 96-well cell culture microplates at 100 µl/well and incubated for 5 days at 37°C under 5% v/v CO_2

2.4 Screening

The culture supernatants from the growing hybridomas are screened by ELISA using plates coated either with 1 µg/ml DR5/hIgG1 or the same amount of Fas/hIgG1 (41) as a control The bound antibodies are detected using horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulins (Southern Biotechnology Birmingham, AL) with TMB (Sigma, St Louis, MI) as the substrate Purified DR5-IgG1 at a concentration of 1 µg/ml or the same amount of Fas-hIgG1 are introduced into a well of a 96-well ELISA/RIA STRIP PLATE (Costar, NY) The plate is kept standing at 4°C overnight to allow adsorption of the protein onto the well surface After this time, the solution in the wells is discarded and each well is washed 3 times with PBS-Tween Then, 100 µl of PBS containing 1% (w/v) bovine serum albumin (A3803, Sigma Chemicals Co) is added to each well and the plate is incubated at 37°C for 1 hour The wells are then washed a further 3 times with PBS-Tween, and then 50 µl of each culture supernatants from the growing hybridomas is added to each well The plate is then incubated at 37°C for 1 hour, and the wells are again washed 4 times with PBS-Tween After washing, 50 µl of horseradish peroxidase labeled goat anti-mouse immunoglobulin antibody (Southern Biotechnology Birmingham, AL), diluted 1000-fold with PBS, is added per well, and the plate is again incubated at 37°C for 1 hour, after which the wells are washed 4 times with PBS-Tween. 3,3',5,5'-Tetramethyl-benzidine (TMB) liquid substrate system (Sigma) is then added in an amount of 100 µl/well and the plate is allowed to stand at room temperature for 5 minutes and then the reaction stopped by addition of 100 µl/well of 0 2N H_2SO_4. The absorbance of each well at 450 nm (control 650 nm) is measured using a microplate reader (Molecular Devices) and fusion cells are selected from the sample which had the absorbance (450 nm-650 nm, OD values, > 0 5) clearly higher than those to which no fusion cells supernatant had been added (OD values, ≈ 0 03)

Furthermore, the culture supernatants from the growing hybridomas are also functionally screened by measuring the apoptosis-inducing activity using Jurkat cell Fifty µl of RPMI medium containing Jurkat cells (1000 cells per well) and 5 uM Bisindolylmaleimide VIII (BisVIII, Alexis, San Diego, CA) are added in 96-well plates in the presence of 50 ul of the culture supernatants from the growing hybridomas The cells are cultured in a humidified incubator at 37°C overnight. Apoptosis is determined by cell viability using the ATPLite kit as instructed by the manufacturer (Packard Instruments), and the samples are counted using the TopCounter (Packard Instruments)

2 5 ELISA binding of TRAIL and TRA-8 to the receptors

ELISA plates are coated with 2 µg/ml of DR4-Ig or DR5-Ig fusion protein overnight. After blocking with 3% BSA, the soluble TRAIL-FLAG or TRA-8 is added at indicated concentrations and incubated at 37°C for one hour The binding of TRAIL or TRA-8 is detected by HRP-conjugated anti-Flag antibody (Alexis) or HRP-conjugated anti-murine IgG1 (Southern Biotechnology), respectively The reactions are developed by TMB substrate buffer and measured by the Benchmark Microplate Reader (BioRad) The Kd values are estimated by the one-site binding model of non-linear regression using GraphPad Prism software (GraphPad Software, San Diego, CA) For competitive ELISA, 100 ng/ml TRAIL-FLAG is added and incubated in the presence of various concentrations of TRA-8 The binding of TRAIL is determined as above

2 6 Cloning

The steps described in Examples 2 3 and 2 4 above are repeated 5 times for the cells selected in 2 4, thereby enabling the selection of several hybridoma clones each of which produced a single antibody that bound DR5-IgG but did not bind Fas-IgG As a result of this selection procedure, a mouse-mouse hybridoma, designated TRA-8 and producing an antibody binding to DR5-IgG, but not Fas-IgG, is obtained This hybridoma, TRA-8, was deposited with the American Type Culture Collection on March 1, 2000, and has been assigned accession No PTA-1428

The subclass of the antibody produced by the mouse-mouse hybridoma TRA-8 (hereinafter referred to simply as "TRA-8") is demonstrated to be IgG1, κ, after testing with a monoclonal antibody isotyping kit (Pierce)

Using our human DR5-IgG1 fusion protein as immunogen, seven hybridoma clones are obtained by initial ELISA screening, all of which are strongly positive for DR5-IgG but not the Fas-IgG fusion protein, indicating that the obtained hybridomas produce antibodies that recognize the extracellular portion of DR5 but not the Fc portion of IgG1 (data not shown)

2.7 Western blot analysis

Filters for Western blot analysis of normal human and cancer tissue homogenates are purchased from Geno Technology (St Louis, MO) Each lane is loaded with an equal amount of protein as determined by an anti-β-actin antibody The blots are probed with 1 μg/ml TRA-8 overnight, and followed by HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology) at room temperature for one hour, and developed by chemiluminescence

2.8 *In situ* immunohistochemistry

Human tissues are obtained from the Tissue Procurement Center of UAB Frozen sections are fixed in 70% ethanol, blocked with 10% horse serum in PBS, and then incubated with 10 μg/ml of affinity-purified TRA-8 at room temperature for 60 minutes The anti-mouse IgG ABC kit with diaminobenzidine (Vector, Burlingame, CA) as the colorimetric substrate is used to visualize the reactivity

2.9 Analysis of caspase activation

Jurkat cells ($1x10^6$/ml) are incubated with 500 ng/ml TRA-8 Aliquots (30 μg of protein) of the cell lysate are separated on 15% SDS-PAGE, blotted onto a nylon membrane, and the blots are probed with anti-caspase 8, 9, and 3 antibodies (BD Pharmingen, San Diego, CA) followed by HRP-conjugated secondary antibody and chemiluminescence visualization of cleaved products The caspase inhibitor set is purchased from R&D Systems (Minneapolis, MN) Each caspase inhibitor is added into culture at indicated concentrations

Example 3 Purification of TRA-8 Monoclonal Antibody

The mouse-mouse hybridoma, TRA-8, is grown to a cell density of 1 x 10^6 cells/ml by incubation in 500 ml of ASF medium, containing 10% v/v FCS, at 37°C under 5% v/v CO_2 for 5 days The culture is then centrifuged (1,000 r p.m , 5 minutes) and the supernatant collected. The purification of TRA-8 from the supernatant is achieved using ProteinG-Sepharose CL-4B affinity chromatography (Pharmacia) under the following conditions

column ProteinG-Sepharose CL-4B column (column size 2 ml, Pharmacia),

elution buffer 0 1 M Glycine (pH 2 4), 0 15 M NaCl,

neutralization buffer 1M Tris-HCl (pH 8 5)

After all of the supernatant is applied to column, 20 ml of PBS is washed three times and then elution buffer is added in 1 ml volumes for 10 times The optical density of each eluted fraction (1 ml) is measured The fractions from No 2 to No 5 (> OD_{280} = 0 1) are collected separately

After adding 100 ul of neutralization buffer, the eluates are placed in dialysis tubing separately, and the eluates dialyzed against 1 liter of PBS (pH 7 5) at 4°C The dialysis buffer being changed twice This sample is assayed for anti-DR5 antibody activity by ELISA using the human DR5-IgG fusion protein prepared above using the technique described above

Example 4 Preparation of DR4 antigen, DR4-IgG expression vector and anti-DR4 monoclonal antibody

The procedures of Examples 1-3 are repeated with DR4 template cDNA and primers in place of those detailed in Example 1 to obtain a DR4 antigen which is utilized as per Examples 1 2-3 to obtain a monoclonal antibody specific against DR4

Example 5 Monoclonal antibodies against DcR1 and DcR2

Monoclonal antibodies are raised against decoy receptors DcR1 and DcR2 by substituting the corresponding cDNA and primers to create the respective antigens as per Example 1 Expression vectors for DcR1 or

DcR2-fusions with immune globulin G and resulting purified monoclonal antibodies are created as per Examples 2 and 3

Example 6 The specificity of a monoclonal antibody

As all of the receptors for TRAIL and other proteins of the TNFR family share significant homology, the specificity of exemplary antibody TRA-8 for DR5 is determined by western blot analysis using two different human DR5-IgG fusion proteins and soluble, recombinant forms of other related proteins A first DR5-Ig fusion protein is constructed by fusing cDNA from residues 1-180 of the extracellular portion of DR5 and cDNA encoding the constant region of human IgG1 The fused cDNA is cloned into a recombinant adenoviral vector (Quantum Biotechnogies, Inc , Montreal, Canada) The expressed DR5/hIgG1 fusion protein, which had a relative molecular weight of 50 kDa, is purified using an anti-human IgG affinity column (Sigma, St Louis, MO) For western blot analysis of specificity, a second recombinant human DR5/IgG1 fusion protein (aa. 52-212), as well as TRAIL-R1, R3 and R4 fusion proteins, are purchased from Alexis The soluble forms of human Fas and TNFR1 are kindly provided by Dr Carl Edwards of Amgen, Inc , Thousands Oaks, CA, USA. The soluble recombinant human DR4, DcR1, DcR2, TNFR1, R4, and Fas molecules used are human IgG1 fusion proteins 0 5 µg of each protein is separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane The blots are blocked with 5% dry milk in PBS at room temperature for one hour, and probed with 1 µg/ml of purified monoclonal anti-DR5 antibody (clone TRA-8) or 0 1 µg/ml of HRP-conjugated goat anti-human IgG at 4°C overnight. Horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG is used as secondary antibody to detect bound TRA-8 The blots are developed by chemiluminescence

Cos-7 cells transfected with the pcDNA3 vector (Clontech, Palo Alto, CA) containing the full-length DR5 or DR4 or empty vector are used for flow cytometry analysis The full-length cDNA encoding human TRAIL or murine Fas ligand is cloned into the pTRE vector down-stream of the tetracycline-controllable promoter (Clontech) The XhoI-HindIII fragments of

pTRE-hTRAIL or pTRE-mFasL are further cloned into the adenoviral shuttle vector pAdBN (Quantum Biotechnologies, Inc) The 293 host cells are co-transfected with the linearized pAd-TRE-hTRAIL or pAd-TRE-mFasL and the large fragment DNA of adenovirus The expression of functional human TRAIL or murine Fas ligand from the recombinant virus plaques is screened using a ^{51}Cr-release assay with Jurkat as the targets

TRA-8 reacted strongly with the DR5-IgG fusion protein (~50 kDa), which is used for immunization as shown in Figure 1a, DR5, #1, and weakly with the second DR5-IgG fusion protein (~60 kD) as shown in Figure 1a, DR5, #2 There is no significant binding of TRA-8 to DR4, DcR1, DcR2, Fas (CD95) or TNFRI These results indicate that TRA-8 recognizes the epitopes that are specific for DR5 but not shared by the other members of the family

TRA-8 does not react with other members of the TNF receptor superfamily, such as Fas (CD95) and TNF receptor I, nor does TRA-8 cross-react with the murine homologue of DR5 as shown by optical absorbance ratios for 450 nm and 650 nm, wherein lower panel numbers 1-7 (Fig 1a, the column 8 of lower panel) Soluble TRAIL and TRA-8 bound comparably to immobilized DR5 (Fig 1b, left panel) In contrast, TRAIL bound to DR4, but TRA-8 did not exhibit any binding activity to DR4 (Fig 1b, middle panel) The Kd values for the binding of TRAIL and TRA-8 to DR5 are estimated at 59 nM and 3 nM, respectively Importantly, TRA-8 efficiently competing with TRAIL for binding to DR5 but not for binding to DR4, as shown in competitive ELISA (Fig 1b, right panel) These results establish the specificity of TRA-8 for human DR5

TRA-8 is able to detect cell surface expression of DR5, with flow cytometric analysis indicating specific binding to the cell surface of Cos-7 cells transfected with full-length human DR5, but not of Cos-7 cells transfected with DR4 or empty vector (Fig 1c) Similarly, *in situ* immunohistochemistry with TRA-8 demonstrated reactivity with Cos-7 cells transfected with full-length DR5 DNA but not with those transfected with control vector (Fig 1d) TRA-8 does not induce apoptosis of untransfected Cos-7 cells, and RT-PCR of RNA

from Cos-7 cells using paired primers encoding human DR5 showed that no specific PCR products Further functional analysis using human Jurkat cells as targets showed that, in the absence of crosslinking, TRA-8 strongly induces cell death, demonstrated by three different assays for cell viability including ATPLite, MTT and PI exclusion (Fig 1e) Greater than 50% of Jurkat cells are killed by nanogram levels of TRA-8 as shown by ATPLite assay The killing activity of TRA-8 is specific for DR5 as it could be blocked by DR5-Ig but not DR4-Ig fusion protein (data not shown) Cleavage of caspases 8, 9, and 3 could be detected by western blot analysis as early as 30 minutes after TRA-8 treatment of Jurkat cells (Fig 1f), and cell death of Jurkat cells is completely inhibited by the general caspase inhibitor (Z-VAD) (Fig 1g) Individual caspase inhibitors for caspase 8, 3, 9, and 10 partially inhibited cell death, further indicating that TRA-8-mediated cell death is primarily through a caspase-dependent apoptotic mechanism

Example 7 Flow cytometric analysis of the expression of cell surface DR5 A major death receptor on many tumor cells but not on normal cells

The ability of TRA-8 to bind DR5 expressed on the cell surface and the specificity of this reaction is then assessed using COS-7 (American Type Culture Collection No CRL-1651) cells transfected with the expression vector containing the full-length human DR5 or DR4 cDNA or empty vector as control Phycoerythrin (PE)-conjugated anti-mouse IgG1 (Pharmingen) is used as the second antibody to detect the bound TRA-8 The fluorescence of 1 X 10^4 cells is measured, using a flow cytometer (FACSVantage) under the following conditions

Excitation wave length 488 nm,

Detection wave length, 600 nm

Flow cytometry analysis showed that TRA-8 stained approximately 30% of COS-7 cells transfected with the DR5 vector as shown in the solid histogram of Figure 1c This percentage parallels the transfection efficiency as determined by analysis of transfection using green fluorescent protein (GFP) (data not

shown) TRA-8 did not significantly stain cells transfected with either DR4 (the open histogram) or control vector (the dotted histogram), indicating that TRA-8 is specific for cell surface DR5

Although DR5 expression in tumor cells has been studied extensively at the mRNA level (Rieger J et al 1 FEBS Lett 1998 May 1, 427(1) 124-8), the surface expression of DR5 is not well understood Gibson S B et al 1 Mol Cell Biol 2000 Jan, 20(1) 205-12, Kim K. et al. 1 Clin Cancer Res 2000 Feb, 6(2) 335-46 Thus, the availability of monoclonal anti-DR5 antibody allows us to examine the surface levels of DR5, and to correlate the expression with the susceptibility of the cells to TRAIL-mediated apoptosis The following panel of cells (1×10^6) is incubated with 10 μg/ml of affinity purified TRA-8 at room temperature for 30 min, and then stained with PE-conjugated anti-mouse IgG1 (Pharmingen) for another 30 min 10,000 viable cells are analyzed using the FACS vantage flow cytometer under the following conditions

Excitation wave length 488 nm,

Detection wave length 600 nm

The five hematopoietic cell lines tested are Jurkat, CEM-6, Molt-4, H-9 and U937 cells DR5 expression is detectable on the surface of Jurkat, CEM-6, H-9, and U937 cells but is almost undetectable on Molt-4 cells as shown in Figure 2a and 2a' Although high levels of DR5 RNA expression has been described previously (43), the FACs analysis indicated that these cells do not express high levels of the surface DR5 These results indicate that cell surface expression of DR5 does not correlate with the transcriptional expression of DR5, which is not unexpected for such a receptor The level of cell surface expression of DR5 may be cell lineage-specific since most of the cells of hematopoietic origin expressed low levels whereas most glioma and prostate cells expressed high levels of DR5

TRA-8 monoclonal antibody is used determine the role of DR5 in induction of TRAIL-mediated apoptosis by examining its cell surface expression among a panel of different types of human tumor cells as well as the susceptibility of these cells to both TRAIL and TRA-8-mediated apoptosis

Primary peripheral blood T cells did not express significant levels of cell surface DR5 and are resistant to both TRAIL and TRA-8-mediated apoptosis (Figs 2*a*, 2*a'* and 3*a'*) Although all five of the human T-leukemia cell lines tested expressed detectable albeit relatively low levels of cell surface DR5, two of them (Jurkat and CEM-6) are highly susceptible to both TRAIL-mediated and TRA-8-mediated apoptosis, indicating that DR5 alone is sufficient to induce apoptosis of these cells Molt-4 and U937 cells are partially susceptible to TRAIL-mediated apoptosis but are relatively resistant to TRA-8-mediated apoptosis, suggesting that other TRAIL receptors might be involved in transduction of an apoptosis signal H-9 cells are resistant to both TRAIL and TRA-8-mediated apoptosis, implicating a block mediated by an intracellular anti-apoptosis pathway

The panel of cells included the human malignant glioma cell lines, Hs683, U251MG, D37MG, D54MG, U373MG, CH235MG, U87 and normal human astrocytes, which were provided by Dr Yancey Gillespie of the Neurosurgery Department of the University of Alabama at Birmingham The human prostate cancer cell lines, Du154, PC3 and LnCap, were provided by Dr William Grizzle of the Pathology Department of the University of Alabama at Birmingham who had obtained the cell lines from the American Type Culture Collection The human leukemia T cell lines, B-cell lymphoma, HepG2 Jurkat (American Type Culture Collection TIB-152) and CCRF-CEM CEM-6 (American Type Culture Collection CCL-119), monocyte cell lines, U937 (American Type Culture Collection CRL-2367), were purchased from the American Type Culture Collection All above cell lines are cultured in RPMI 1640 supplemented with 10% FCS The human astrocytoma cell line, 1321N1, was kindly provided by Dr Richard Jope of Psychiatry Department of the University of Alabama at Birmingham, and cultured in DMEM supplemented with 5% FCS

Soluble recombinant human TRAIL, purchased from Alexis Corporation (San Diego, CA), is a fusion protein comprised of the extracellular domain of human TRAIL (aa residues 95 to 281) fused at N-terminus to a FLAG-tag and an

8 amino acid linker peptide Unlike previously reported His-tagged TRAIL, this preparation of TRAIL alone does not induce a strong apoptotic response in Jurkat cells and requires an anti-FLAG antibody as a crosslinker to enhance apoptosis The anti-FLAG antibody was also purchased from Alexis

All of the 10 human malignant glioma cells tested expressed detectable levels of DR5 at the cell surface Most expressed intermediate to high levels of DR5 as shown in Figure 2b Three lines, D-54MG, U373MG and CH-235MG expressed high levels of DR5 while six lines, Hs-683, U251-MG, D37-MG, U87, SMK1 and 1321N1, expressed intermediate levels of DR5 Only one cell line, H-465 expressed low levels of DR5 All three prostate cancer cell lines expressed high levels of DR5 as shown in Figure 2c

Like the normal primary T cells, primary B cells did not express significant levels of DR5 and did not undergo apoptosis after treatment with either TRAIL or TRA-8 (Fig 2*d*) Three (SKW6 4, EB-3, and Raji) out of the four B lymphoma cell lines tested expressed relatively high levels of DR5 and are very susceptible to both TRAIL and TRA-8-mediated apoptosis The fourth cell line, Daudi, expressed very low levels of DR5 and is much less susceptible to either TRAIL or TRA-8-mediated apoptosis Although primary astrocytes did not express detectable levels of cell surface DR5 (Fig 2*b'*), all four glioma cell lines tested expressed high levels of DR5 The higher level of expression of DR5 on glioma cells than on T and B cells is not accompanied by a significantly greater susceptibility to TRAIL and DR5-mediated apoptosis, suggesting that the level of cell surface expression of DR5 is not necessarily correlated with the level of apoptosis of tumor cells RT-PCR, performed to determine message levels of DR4, DR5 and DcR2, detected message in all cells tested (Table 1) However, in general, primary normal cells expressed relatively low levels of DR5 compared to transformed tumor cells

Table 1 RT-PCR analysis of TRAIL receptor expression*

Cells	DR5	DR4	DcR2
Primary T cells	<0 001	<0 001	0 015
Jurkat	0 10	<0 001	0 21
CEM-6	0 50	0 59	0 25
Molt-4	0 10	<0 001	0 05
H-9	0 73	0 61	0 07
Primary B cells	<0 001	<0 001	0 024
SKW6 4	0 95	0 66	0 45
EB3	0 40	<0 001	0 35
Raji	0 55	0 11	0 45
Daudi	0 73	0 36	0 63
Normal Astrocytes	0 05	<0 001	0 12
SH683	0 56	0 96	0 14
U87	0 44	0 56	0.21
D54	1 15	0 46	0 12
1321N1	0 25	0 35	0 05

* Total RNA was isolated from cells and RT-PCR was performed as described in Methods The PCR products were separated in 3% agarose gel and analyzed by the Fluor-S MAX MultiImager System (BioRad) The values are presented as a ratio relative to β-actin

Example 8 Induction of apoptosis in vitro in malignant cells

To determine whether TRA-8 induces apoptosis in transformed cells *in vitro*, all DR5-positive tumor cells are examined for their susceptibility to apoptosis induced either by TRA-8 or TRAIL

Target cells (1 x 10^3 per well) are cultured in 96-well plates in the presence of the indicated concentrations of soluble TRAIL plus crosslinker (Alexis) or TRA-8 at 37°C overnight Cell viability is determined using (1) the ATPLite kit according to the manufacturer's instructions (Packard Instruments, Meriden, CT), (2) the MTT Cell proliferation/viability kit (Sigma), or (3) PI staining of dead cells and analyzed by flow cytometry At end of culture, cells are stained with 10 µg/ml PI and PI negative cells are gated as viable cells For analysis of condensed nuclei of hepatocytes, cells are stained with 10 ng/ml Hoechst 33352 (Molecular Probes) and analyzed by flow cytometry

The TRA-8 antibody is capable of inducing apoptosis in the majority of the malignant human glioma cell lines (9/10), in 2 of the 3 prostate cancer cell lines, and in 2 of the 4 DR5-positive hematopoietic cell lines It did not induce apoptosis in the Molt-4 cell line, which expressed almost undetectable cell surface levels of DR5 The levels of susceptibility of the cells to TRA-8-mediated apoptosis varied considerably among the cell lines, however

The variability of the susceptibility of the cells to TRA-8 antibody induced apoptosis suggests that although a minimal level of cell surface expression of DR5 is required, the level of cell surface expression of DR5 is not necessarily the primary determinant of susceptibility and other factors influence this process Although all of the glioma cells generally expressed significantly higher levels of the surface DR5 than did the hematopoietic cells, glioma cell susceptibility to apoptosis induced by TRA-8 is not proportionally increased compared to the hematopoietic cells The susceptibility of five of the glioma cell lines, D-37MG, D54-MG, U373-MG, CH235-MG and 1321N1 to TRA-8-induced apoptosis is high and is equivalent to their susceptibility to TRAIL-mediated apoptosis as shown in Figure 3b Two of the glioma cell lines, H-456 and SMK1, are much less susceptible to apoptosis induced by TRA-8 In the case of the H-456 cells, the surface expression of DR5 is low, however, the surface expression of DR5 on SMK1 is similar to the more susceptible cell lines, suggesting that other mechanisms might play a role in the determining the susceptibility to TRAIL-mediated apoptosis Although all three prostate cancer cell lines expressed high levels of DR5, the Du145 cells are most sensitive to TRA-8-induced apoptosis, the PC3 cells are partially sensitive while LnCAP cells are completely resistant as shown in Figure 3c Among the hematopoietic cells, it is found that Jurkat and CEM-6 are very susceptible to TRA-8- apoptosis as shown in Figure 2a although both these cell lines had been found to express low levels of DR5 Although DR5 is detectable on U937 cells, these cells are resistant to TRA-8-induced apoptosis Similarly, although the H-9 cells expressed detectable levels of DR5, H-9 cells are resistant to apoptosis induced

by TRA-8 These results implicated the existence of regulatory mechanisms that influence DR5-mediated apoptosis

Additional surface binding anti-DR5 antibodies are produced as per the procedures of Examples 1-3 Two additional anti-DR5 antibodies designated TRA-1 and TRA-10 are studied along with TRA-8 to determine comparative ability induce apoptosis and thereby act as an agonist or conversely block TRAIL-mediated apoptosis, thereby acting as an antagonist. Human Jurkat cells are used as a target to determine the agonist and/or antagonist activity of the three anti-DR5 antibodies denoted TRA-1, TRA-8 and TRA-10 As shown in Figure 4, cell viability is about 90%, 70% and 20% for TRA-10, TRA-1 and TRA-8, respectively upon overnight incubation with 2 5 μg per mL TRA-8 induced a strong apoptotic response in a dose dependent fashion while TRA-1 induced only a moderate apoptotic response and TRA-10 only induced a weak response TRA-8 is therefore classified as an agonist anti-DR5 antibody In Figure 4, the viability of human Jurkat cells is shown as a dose dependent function of TRAIL-induced apoptosis TRA-10 blocked apoptosis of human Jurkat cells to a significant extent in a low dose TRAIL-induced apoptosis study Thus, TRA-10 is classified as an antagonist anti-DR5 antibody TRA-1 is on deposit with American Type Culture Collection under Accession Number PTA-1741 TRA-10 likewise is on deposit with American Type Culture Collection under Accession Number PTA-1742

The susceptibility of five of the glioma cell lines, D-37MG, D54-MG, U373-MG, CH235-MG and 1321N1 to TRA-8-induced apoptosis is equivalent to their susceptibility to TRAIL-mediated apoptosis as shown in Figure 3b, indicating that TRAIL-induced apoptosis in these cells is mediated primarily through DR5 Moreover, two of the glioma cell lines, Hs683 and U251-MG, are resistant to TRAIL-induced apoptosis but partially sensitive to TRA-8-induced apoptosis, indicating that the decoy receptors function in these cells and that use of the TRA-8 antibody bypassed this regulatory mechanism In the prostate cancer cell lines, despite the varying sensitivity to apoptosis induced by TRA-8, this paralleled the sensitivity of the cells to apoptosis induced by TRAIL, again

suggesting that DR5 plays a major role of TRAIL-mediated apoptosis in the prostate cancer cells Among the hematopoietic cells, it is found that Jurkat and CEM-6 are very susceptible to both TRA-8 and TRAIL-mediated apoptosis The level of apoptosis induced by TRA-8 is comparable to that induced by TRAIL as shown in Figures 2a and 3a' Only one of the glioma cell lines, U87, and two hematopoietic cell lines, U937 and Molt-4, exhibited sensitivity to TRAIL-induced apoptosis but are less sensitive or resistant to TRA-8-induced apoptosis One cell line, the H-9 cell line, expressed detectable levels of DR5 but are resistant to apoptosis induced by either TRA-8 or TRAIL While minimal levels of expression of DR5 are required for TRA-8-induced apoptosis, the level of expression of DR5 does not necessarily predict the susceptibility of the cells to TRA-8 mediated apoptosis, decoy receptors play a role in modulating TRAIL-mediated apoptosis in some cells, but does not appear to play a major role in most of the cells tested to date, as anticipated the TRA-8 antibody bypasses the effects of the decoy receptors, functional mutations of the DR5 receptor may occur in transformed cells, and, finally, intracellular regulatory mechanisms may be as important, or more important than the decoy receptors in defining the susceptibility of the cells to TRAIL and DR5-mediated apoptosis

Previous studies have shown that the mRNA for DR5 is distributed widely in normal tissues[7] To evaluate the expression of DR5 at the protein level, a panel of normal human tissue homogenates (Geno Technology, St Louis, MO) is probed with the TRA-8 antibody in western blot analysis Among nine normal human tissues, brain tissue is weakly positive (Fig *5a*, lane 2) DR5 protein is not detectable by TRA-8 reactivity in liver (lane 1), lung (lane 3), kidney (lane 4), spleen (lane 5), testes (lane 6), ovary (lane 7), heart (lane 8), or pancreas (lane 9) In contrast, all thirteen human cancer tissues stained positively with TRA-8 (Fig *5b*), including cancers of the ovary (lane 1), lung (lane 2), liver (lane 3), rectum (lane 4), cervix (lane 5), skin (lane 6), testes (lane 7), thyroid (lane 8), uterus (lane 10), stomach (lane 11), laryngopharynx (lane 12), and pancreas (lane 13) Moreover, *in situ* immunohistochemistry of normal and cancer tissues with TRA-8 confirmed that aside from a few scattered

positive cells in spleen, DR5 expression in normal breast, lung and spleen tissues is not detectable (Fig 5*c*) The corresponding cancer tissues including breast infiltrating ductal carcinoma, small cell lung cancer, and lymphoma reacted positively with TRA-8 (Fig 5*d*) Among a total of 22 cancer tissues examined, 5 of 6 breast cancers, 2 of 2 cancers of the cervix, 4 of 5 liver cancers, 5 of 8 lymphomas, 2 of 2 lung cancers, and 2 of 2 prostate cancers reacted positively with TRA-8 These results are consistent with those of the flow cytometry analysis and indicate that cancerous tissues express higher levels of DR5 protein than do normal tissues

Example 9 Tumoricidal activity of TRA-8 *in vivo*

For various reasons, many agents that show promise in *in vitro* studies do not show efficacy *in vivo* It is therefore important to test the efficacy of TRA-8 in an *in vivo* animal model To accomplish this the TRA-8 anti-human DR5 antibody is administered to mice bearing human xenografts that express the human DR5 molecule The mice used are 6 to 8 week-old NOD/SCID mice (Jackson Laboratory), which are inoculated subcutaneously with human astrocytoma 1321N1 cells ($1x10^7$), or inoculated intravenously with human leukemia Jurkat cells ($1x10^6$) At day 2 after tumor inoculation, mice are inoculated intravenously with TRA-8 (100 μg) Five days after the treatment with TRA-8, 1321N1 tumor growth is determined by the size and weight of the tumor mass The growth of Jurkat cells is determined by the weight of the spleen and the percentage of human CD3-positive Jurkat cells in the spleen of inoculated animals Biopsies of tumor tissues are taken and examined histologically

Early treatment with a single intravenous dose of 100 μg of TRA-8 at one day after tumor inoculation completely inhibited the 1321N1 cells from forming a solid tumor of (Fig 6*a*) Late treatment with three doses of 100 μg TRA-8 at one week after tumor inoculation reduced tumor weight 4-fold or more (Fig 6*b*) Tumor formation is not visible in animals treated with TRA-8 at an early time point (Fig 6*c*, upper panel) Histologic analysis revealed dramatically degenerated tumor tissue in animals treated with TRA-8 (Fig 6*c*, lower panel)

Similarly, TRA-8 treatment inhibited population of the spleen by Jurkat cells as demonstrated by the scarcity of CD3-positive Jurkat cells in the spleen (Fig 6*d*, 6*e*) Histological analysis of the implanted tumor showed a few tumor cells scattered in the soft tissue in TRA-8-treated animals while controls showed the formation of a solid tumor as shown in Figure 6c In the Jurkat cell model, the number of Jurkat cells in the spleens of TRA-8 treated animals is less than 2% compared to nearly 10% in the spleen of control animals as demonstrated by flow cytometry analysis as shown in Figure 6a and in situ CD3 staining of Figure 6c

These results confirm the recent demonstration that systemic administration of cross-linked recombinant TRAIL inhibits growth of tumor *in vivo* (13) These results indicate that a single dose of TRA-8 is highly effective in the elimination of tumor cells *in vivo*

As an anti-human antibody is used in a murine model, the toxicity of the TRA-8 treatment could not be assessed However, the study of administration of TRAIL *in vivo* indicated that no significant toxicity is associated with this treatment (13)

Example 10 RA synovial cells are susceptible to TRAIL and TRA-8-induced apoptosis

Most of the prior art studies of TRAIL-mediated apoptosis have focused on malignant cells TRAIL-mediated apoptosis according to the present invention is also therapeutic in autoimmune and inflammatory conditions, such as RA

10 1 Flow cytometric analysis of the expression of cell surface DR5 in RA synovial cells

The expression of DR5 on a panel of eight primary cultured synovial cells from patients with RA is compared with that on eight primary cultured synovial cells from patients with osteoarthritis (hereinafter referred to as "OA") The eight human primary RA synovial cell cultures RA-1014, RA-1016, RA-1021, RA-512, RA-707, RA-811, RA-716, and RA-929 are kindly provided by Dr M Ohtsuki (Sankyo Co Ltd., Tokyo, Japan) and cultured in DMEM

supplemented with 10% FCS, penicillin, streptomycin, and glutamine The seven OA synovial cell primary cell cultures are isolated from the synovial tissues of OA patients by a standard collagenase method and cultured under the same conditions The passage number of all primary cells is under 10 The expression of DR5 is determined by FACs analysis as described in Example 5

All of the primary cultures of RA cells expressed high levels of surface DR5, and there is little variation in the expression levels among these synovial cells isolated from different patients as shown in Figure 7a. In contrast, the expression of surface DR5 on the surface of synovial cells isolated from the OA patients is very low or undetectable as per Figure 7b SV40-transformed synovial cell are found to express high levels of DR5 comparable with those exhibited by the RA cells In contrast, non-transformed fibroblast cells expressed low levels of DR5 comparable to those exhibited by the OA cells in Figure 7b

10 2 Susceptibility of RA synovial cells to apoptosis mediated by TRA-8 or TRAIL

In general, all synovial cells isolated from the RA patients are susceptible to both TRAIL and anti-DR5 antibody induced apoptosis, and all OA cells are resistant to TRAIL and anti-DR5 antibody induced apoptosis as per Figure 8a, b These studies indicate that the TRA-8 antibody targets altered cells in preference to normal cells Moreover, the pattern of the susceptibility or resistance to apoptosis induced by TRAIL is correlated with that induced by anti-DR5 antibody, indicating that the synovial cells primarily utilize DR5 to trigger TRAIL apoptosis

As described for the malignant cells, the susceptibility to apoptosis induced by TRAIL or anti-DR5 antibody varied among the RA synovial cells although expressing similar levels of DR5 RA-512 and RA-707 are the most susceptible as over 80% cells are killed by concentrations of TRAIL or TRA-8 below 20 ng/ml RA-1014, RA-811, RA-716, and RA929 are among those with the intermediate susceptibility to TRAIL or TRA-8, with nearly 100% cell death occurring in the presence of high concentrations (>50 ng/ml) of TRAIL or

TRA-8 In RA-1016 and RA1021 cells, although the majority (over 60%) of cells are killed by a low dose of TRAIL or TRA-8, a portion of cells survived in the presence of high concentrations of TRAIL or TRA-8, indicating that a sub-population of cells are resistant to TRAIL-mediated apoptosis In contrast, all OA cells are much less susceptible to TRAIL and TRA-8 induced apoptosis No greater than 60% cells are killed in the OA52F and OA69F even in the presence of high concentration of TRAIL or TRA-8 OA72M cells are completely resistant to TRAIL or TRA-8 induced apoptosis The SV40 transformed synovial cells are also susceptible to TRAIL and TRA-8 induced apoptosis (data not shown) In contrast, the non-transformed fibroblast cells appeared to be resistant to TRAIL and TRA-8

It has been shown previously that DR5 utilizes a FADD/caspase 8 dependent pathway to trigger apoptosis (44) To determine the caspase-dependence of DR5-mediated apoptosis of RA synovial cells, RA cells are cultured with TRAIL or anti-DR5 antibody in the presence of specific caspase inhibitors Among eight caspase inhibitors tested, caspase 6, 8 and 10 inhibitors are able to inhibit apoptosis of RA synovial cells induced by both TRAIL and DR5 as shown in Figure 9, indicating that these three caspases are involved in DR5-mediated apoptosis

10 3 TRA-8 or TRAIL induce NF-κb activation in RA synovial cells without increased release of MMPs

There is considerable evidence to support the concept that there are close links between the signaling of apoptosis and the signaling of proliferation (45) It has been established that DR5 is able to activate a NF-kb pathway in addition to apoptosis signaling transduction, and that NF-κb activation may be able to transduce an anti-apoptosis signal Therefore a gel-shift assay is carried out Cells are stimulated with 50 ng/ml of the recombinant soluble TRAIL, Fas ligand in the presence of the 1 mg/ml enhancer, or 50 ng/ml of TRA-8 for the indicated time The nuclear extracts are prepared and incubated with the double-stained [^{32}P]-labeled oligo-DNA probe The results are analyzed using the cyclone phospha-imager (TopCount NXT, Packard Instrument Company,

CT) After RA synovial cells are incubated with TNF-α or TRAIL, NF-κb is activated in a time-dependent fashion The TRA-8 antibody is able to strongly activate NF-κb In contrast, Fas ligand is unable to induce NF-κb activation as per Figure 10a.

Thus, although TRAIL and TRA-8 antibody induce a strong apoptosis response in RA synovial cells, they also activate NF-κb, and NF-κb activation has been believed to contribute to the proinflammatory role of TNF-α in RA. Thus, it is possible that TRAIL, like TNF-α, may serve as a pro-inflammatory cytokine To determine whether there is a similar biological consequence of NF-kb activation induced by TRAIL and TNF-α, the production of MMPs is determined by ELISA Synovial cells are cultured in medium alone or with 50 ng/ml interleukin 1b, 10 ng/ml TNF-a, 50 ng/ml TRAIL, or 50 ng/ml TRA-8 overnight The levels of the MMP-1 and MMP-3 in the culture supernatants are determined by the ELISA kits

When RA synovial cells are incubated with a proinflammatory cytokine, TNF-α or IL-1b, the production of MMP-1, 3, and 13 is increased compared to the medium control as shown in Figure 10 b,c In contrast, treatment with TRAIL or anti-DR5 antibody is not associated with increased release of these MMPs

Example 11A. Failure to induce hepatocellular toxicity

For 24-hour cell viability assays, fresh normal human hepatocytes in 96-well plates were purchased from In Vitro Technology (Baltimore, MD) The hepatocytes are cultured in the Hepatocyte Culture Medium containing 1 μg/ml of either soluble TRAIL or TRA-8 For 6-hour viability assays, normal hepatocytes or hepatocellular cancer cells are isolated from fresh surgical specimens collected from UAB Tissue Procurement Center All reagents for isolation of human hepatocytes including hepatocytes perfusion buffer, digest medium, washing medium, and attachment medium were purchased from Gibco The tissue slides are digested in the Hepatocyte Digest Medium at 37°C with shaking (50 rpm) for one hour The isolated hepatocytes are harvested by low speed centrifugation (50 g, 3 min), and washed with the Hepatocyte

Washing Medium six times Single cell suspension of hepatocytes are cultured in the Attachment Medium containing 10% FCS in 96-well Matrigel plates (BD) for six hours Non-attached hepatocytes are removed by twice washing with pre-warmed attachment medium. Attached hepatocytes are further incubated with various concentrations of soluble TRAIL or FasL in the presence of crosslinker, or TRA-8 or CH11 for 6 hours

TRAIL has at least two receptors (DR4 and DR5) that are capable of inducing apoptosis TRA-8 is used to determine whether crosslinking of DR5 alone is sufficient to induce apoptosis of normal hepatocytes DR5 expression at the protein level is examined initially in five normal human liver tissues and five liver cancer tissues by *in situ* immunohistochemistry using TRA-8 Sections from the normal liver tissues showed normal architecture and cell morphology on H&E staining (Fig 11*a*, left upper panels) in the absence of positive reactivity with TRA-8 for DR5 (Fig 11*a*, left lower panels) In contrast, the human hepatocellular carcinoma tissue reacted positively with TRA-8 in a pattern consistent with both cell membrane and cytoplasmic presence of DR5 on the cancerous cells The human hepatocellular carcinoma cell line HepG2 is also positive for DR5 These results are consistent among the five normal liver tissues, and only one (liver adenoma) out of five liver cancer tissues is DR5-negative These results are consistent with the Western blot data, shown in Figure 5a, that, as with other normal tissues, normal human liver tissue does not express significant levels of DR5 protein Furthermore, Western blot analysis of isolated, normal human hepatocytes probed with TRA-8 does not reveal detectable levels of DR5

Cell surface expression of DR5 on human hepatocytes by flow cytometry analysis demonstrated that freshly prepared normal hepatocytes did not express detectable levels of cell surface DR5 (Fig 11*b*, top left panels) Neither is it detected on normal human hepatocytes that had been cryopreserved or placed in short-term culture In contrast, freshly isolated hepatocellular carcinoma cells as well as HepG2 cells express cell surface DR5 Using Fas as a comparison, the normal hepatocytes, hepatocellular carcinoma cells, and HepG2 cells all

expressed equivalent levels of Fas (Fig 11*b*, lower panels) These results are consistent with those obtained using *in situ* immunohistochemistry and Western blot and indicate that cell surface DR5 is highly expressed in cancerous liver cells but not normal hepatocytes The presence of mRNA levels for DR4, DR5, DcR1 and DcR2 in human hepatocytes, demonstrated by RT-PCR[23], suggests that human hepatocytes might express very low levels of DR5 protein that are below the threshold for detection by TRA-8

To determine whether TRA-8 induces hepatocellular toxicity, the susceptibility of normal human hepatocytes to apoptosis induced by TRA-8 and by soluble TRAIL plus crosslinker is examined When normal hepatocytes are cultured in the presence of a high concentration of TRAIL, a time-dependent decrease in cell viability is observed by ATPLite (Fig 12a) and MTT assays TRAIL-mediated cell death of normal hepatocyes could be seen as early as four hours after addition of TRAIL At end of a 24-hour culture, more than 80% of the hepatocytes are killed by TRAIL In contrast, during the same culture period, TRA-8 did not induce significant cell death in normal hepatocytes The condensed nuclei stained with Hoechst, a characteristic of apoptosis, are increased in TRAIL-treated but not TRA-8-treated hepatocytes (Fig 12b) The number of apoptotic hepatocytes is well correlated with decreased cell viability as determined by ATPLite assay, suggesting that TRAIL-induced cell death of hepatocytes is mediated by apoptosis This is confirmed by the ability of Z-VAD to inhibit TRAIL-mediated toxicity of hepatocytes As cycloheximide is a potent apoptosis enhancer, the effect of this compound on TRAIL and TRA-8-treated hepatocytes is investigated During a four-hour culture, cycloheximide significantly enhanced the cell death of hepatocytes induced by TRAIL, with greater than 70% hepatocytes being killed by TRAIL in the presence of cycloheximide (Fig 12c) However, cycloheximide treatment is unable to enhance TRA-8-mediated cell death in hepatocytes To compare the characteristics of apoptosis induced by TRA-8 with that induced by TRAIL in hepatocytes, normal hepatocytes as well as cancer cells are incubated with variable concentrations of soluble TRAIL with crosslinker or TRA-8 During a

6-hour culture period, TRAIL induced a moderate apoptotic response in normal hepatocytes Over 20% of hepatocytes are killed in the presence of 500 ng/ml TRAIL (Fig 12*d*, upper left) TRA-8-treatment of normal hepatocytes did not elicit any significant cell death over the same time period In contrast to normal hepatocytes, primary hepatocellular carcinoma cells (Fig 12*d*, upper middle) and HepG2 cells (Fig 12*d*, upper right) are highly susceptible to apoptosis mediated by either TRAIL or TRA-8 Over 80% of hepatocellular carcinoma cells and nearly 100% of HepG2 cells are killed during the 8-hour culture period These results indicate that normal hepatocytes are completely resistant to TRA-8-mediated apoptosis, and are much less susceptible to TRAIL-mediated apoptosis than are liver cancer cells Using Fas ligand and anti-Fas antibody (CH-11), there is no significant difference in the susceptibility to Fas-mediated apoptosis among normal hepatocytes, hepatocellular carcinoma cells, and HepG2 cells (Fig 12*d*, lower panels)

Comparative Example 11B Human membrane-bound TRAIL induction of hepatitis *in vivo*

8-10 week-old female B6 mice are intravenously injected with 10^9 pfu of Ad/hTRAIL with the equal number of Ad/Tet-on Mice are fed with different concentrations of tetracycline in their drinking water immediately after inoculation of adenoviral vectors Liver injury is determined by serum levels of AST using an AST diagnostic kit (Sigma) Expression of TRAIL is determined by Northern blot analysis

To determine whether the membrane bound form of TRAIL induces liver damage *in vivo*, a recombinant adenoviral vector encoding the full length human TRAIL (Ad/hTRAIL) is constructed, the expression of which is under the control of the tetracycline-inducible promoter Twenty-four hours after intravenous inoculation of B6 mice with Ad/hTRAIL, tetracycline-induced expression of human TRAIL is observed in the liver in a dose-dependent fashion as demonstrated by Northern blot analysis (Fig 13*a*) The expression levels of TRAIL correlated well with liver damage as shown by a tetracycline-dependent increase in serum levels of transaminases, again in a dose-dependent fashion

(Fig 13*b*) As the inoculation with adenoviral vector *per se* might increase the susceptibility of hepatocytes to TRAIL-mediated apoptosis, the hepatocytes from mice inoculated with Ad/TRAIL are isolated and tested for TRAIL-mediated cell death. There is no significantly increased cell death of Ad/TRAIL infected hepatocytes compared to those from control mice (Fig 13c, left panel) Moreover, Ad/TRAIL inoculated mice did not exhibit increased liver injury after intravenous injection of soluble human TRAIL Thus, it follows that hepatitis induced by Ad/TRAIL is mediated by high levels of TRAIL expression in its membrane form Histologic analysis of liver sections revealed that damage to the hepatocytes is apparent as early as 24 hours after vector inoculation (Fig 13d), and persisted for at least 7 days (Fig 13*e*) These pathologic alterations in the liver also are tetracycline-dependent and occurred in a dose-dependent manner The early phase, within 24 hours of treatment, of TRAIL-induced liver damage is characterized by foci of necrosis Infiltration of inflammatory cells is not observed at this stage, but hemorrhage had occurred. By day 7 after inoculation, diffuse liver damage is apparent with marked lobular disarray, severe degeneration of hepatocytes with irregularly clumped cytoplasm and large clear spaces, and prominent apoptosis and necrosis An extensive infiltrate of mononuclear cells is a characteristic feature at this stage These results indicate that human TRAIL in its membrane-bound form is able to induce liver damage *in vivo* Despite the propensity of human TRAIL to cause severe hepatitis in mice, it did not induce a lethal response In contrast, mice inoculated with similar tetracycline-controlled vectors encoding Fas ligand developed fulminant hepatitis with massive apoptosis and necrosis of hepatocytes accompanied by severe hemorrhage and by mortality occurring in a tetracycline dose-dependent within 72 hours of inoculation. The mortality rate reached 100% within 48 hours in those subgroups receiving 3 mg/ml or more of tetracycline In contrast, all of the mice that received Ad/hTRAIL, regardless of the dose of tetracycline, are still alive four weeks after inoculation. Thus, it follows that, *in vivo*, the membrane-bound form of TRAIL is a less potent inducer of hepatocellular damage than Fas ligand They further suggest that

TRAIL might induce liver damage through a mechanism that differs from the mechanism underlying the toxicity of Fas ligand

Example 12 Activated human T and B cells express increased levels of DR5

To determine whether DR5 plays a role in TRAIL-mediated apoptosis of activated T cells and B cells, surface expression of DR5 on resting and activated T and B cells using TRA-8 is examined. The unstimulated human T cells in PBMC did not express significant levels of DR5 (Fig 14) At 48 hours after either anti-CD3 or Con-A stimulation, cell surface DR5 expression is significantly increased. Similarly, the unstimulated B cells expressed very low levels of DR5 Stimulation with anti-μ but not LPS resulted in increased cell surface expression of DR5 These results indicate that both activated T and B cells express higher levels of cell surface DR5 Cells are stained with 20 μg/ml TRA-8 and PE anti-mouse IgG1

Example 13 Activated T and B cells become susceptible to TRA-8 mediated apoptosis

To determine whether activated T and B cells are susceptible to TRA-8-mediated apoptosis, the T cells and B cells of human PBMC are stimulated with anti-CD3 or anti-μ in vitro for 48 hours, respectively The viable cells and proliferating blast cells are collected by gradient centrifugation, and incubated with various concentrations of TRA-8 Unstimulated T cells and B cells are not susceptible to TRA-8-mediated apoptosis (Fig 15) Total stimulated T cells and B cells showed a moderately increased susceptibility to TRA-8-mediated apoptosis, with 20% cells being killed by TRA-8 after overnight culture The highly proliferating blast T cells are even more susceptible to TRA-8 mediated apoptosis More than 70% of the blast T cells could be killed by TRA-8 The blast B cells are also more susceptible to TRA-8 mediated apoptosis compared to others These results indicate that activated T and B cells are susceptible to DR5-mediated apoptosis

Example 14 TRA-8 depletes activated T cells in human/SCID mice

To determine the *in vivo* anti-T cell efficacy of TRA-8, NOD/SCID mice are intravenously injected with $1x10^8$ human PBMC Normally, the human T cells in SCID mice are quickly activated in response to xenogeneic stimulation. The human PBMC/SCID mice are intraperitonally injected with 100 µg TRA-8 or control IgG1 from the day of transfer, repeated daily for three days Five days after transfer, the mononuclear cells are isolated from the spleen and stained with anti-human CD3 antibody, and the lymphocyte population is gated by flow cytometry analysis, and CD3 positive human T cells are analyzed Approximately 30% of splenic lymphocytes are human T cells as determined by anti-human CD3 staining in control treated mice However, only a few human T cells (less than 3%) are observed among the splenic lymphocytes in TRA-8 treated mice (Fig 16) *In situ* histological study revealed that in the spleen of control mice, the human T cells are repopulated in the spleen with only a few apoptotic cells observed as demonstrated by TUNEL staining In contrast, repopulation with viable human T cells is not observed in the spleen of TRA-8 treated mice, rather many apoptotic cells are observed (Fig 17) These results demonstrate that TRA-8 has anti-T cell activity *in vivo*, and indicate the utility of the inventive antibodies for the treatment of GVH disease

Example 15 Anti-cancer therapeutic activity of TRA-8

15 1 DR5 expression and function in human cancer tissues and cell lines

i) DR5 expression in human cancer tissues by in situ staining with TRA-8 To determine whether cancer cells and tissues differentially express higher levels of DR5, a panel of human cancer tissues including over 20 breast cancers, 6 ovarian cancers, 5 colon cancers and 5 prostate cancers are stained with TRA-8 for immunohistochemistry The majority of these cancer tissues expressed detectable DR5 The expression levels of DR5 in these cancer tissues varied In general, cancer tissues expressed higher levels of DR5 than uninvolved tissues In addition, DR5 expression is apparently not correlated with the mutation of p53

ii) DR5 expression and function in human cancer cell lines (Table 2) Nine human breast cancer cell lines, three ovarian cancer lines, three colon cancer lines and three prostate cancer lines are examined for cell surface expression of DR5 and susceptibility to TRA-8-induced apoptosis *in vitro* 7 of 9 breast cancer lines, 3 of 3 ovarian cancer lines, 3 of 3 colon cancer lines and 3 of 3 prostate cancer lines expressed variable levels of cell surface DR5 Of 9 breast cancer lines, three are very susceptible, three are intermediate and three are resistant to TRA-8-mediated apoptosis All three ovarian cancer lines are very susceptible One of three colon cancer lines is very susceptible, while two have intermediate sensitivity Two of three prostate cancer lines have intermediate sensitivity and one is resistant

Table 2 Expression and function of DR5 in human cancer cells

Cell line	Origin	Expression[1]	Susceptibility[2]
2LMP	breast	+	++++
LCC6	breast	+++	++++
MB468	breast	+++	+++
MB231	breast	++	+
ZR-75-1	breast	+++	++
SKBR3	breast	+	++
MB453	breast	++	+
BT474	breast	+	-
DY36T2	breast	-	-
Caov-3	ovary	+	+++
OVCAR-3	ovary	++	++++
Skov-3	ovary	+	+++
WiDR	colon	++	++++
HST29	colon	++	+++
T84	colon	+	++
PC3	prostate	+++	++
LnCap	prostate	+++	+
Du-145	prostate	+++	+

Note [1]determined by flow cytometry, cells are stained with 20 μg/ml TRA-8 and compared to control antibody [2]determined by ATPLite assay ++++ over 80% killing, +++ killing between 60-80%, ++ killing between 40-60%, + killing between 20-40%, - no killing

iii) Combined cytotoxicity of TRA-8 with adriamycin In several breast cancer lines, the effect of adriamycin on TRA8-induced apoptosis is examined High doses of adriamycin exhibited an additive effect. However, in some of TRA-8 resistant lines, low doses of adriamycin synergistically enhance TRA-8-induced apoptosis

iv) In vitro and in vivo binding activity of TRA-8 to human cancer cells. Using radioisotope labeled TRA-8 The binding activity of TRA-8 to a breast cancer line is examined *in vitro* and *in vivo* in SCID mice implanted with tumor The *in vitro* binding activity to cancer cells is estimated as a Kd value of 3 nM, which is constant with our previous estimation using ELISA, and at least 50-fold higher than soluble TRAIL *In vivo*, TRA-8 localized to implanted tumor tissues

15 2 Therapy of chronic lympholytic leukemia in NOD/SCID mice with TRA-8

Chronic lympholytic leukemia (CLL) is a common form of B cell malignancy Most malignant B cells in CLL are of the mature phenotype and are resistant to many apoptosis stimuli DR5 expression and function in the B cells of five patients with CLL is examined All patients had high counts of peripheral B cells as shown by more than 95% CD19+ B cells in PBMC Compared to normal primary B cells, the CLL B cells of all patients had higher levels of cell surface DR5 and are more susceptible to TRA-8 induced apoptosis *in vitro* Interestingly, the CLL B cells are also sensitive to bisindolemaleimide VIII (BisVIII) induced cytotoxicity Following combined treatment with TRA-8 and BisVIII, nearly 50% of CLL B cells are killed while normal B cells remained unresponsive (Fig 18) Transfer of CLL B cells into NOD/SCID mice resulted in about 25%-30% CD19+ B cells repopulated in the spleen of recipient mice at five days after transfer However, three doses of 100 μg TRA-8 treatment completely eliminated CLL B cells of four out of five patients in the spleen of the recipient SCID mice Thus, TRA-8 alone or in concert with other substances is active as a therapeutic agent for chronic lympholytic leukemia.

Example 16 cDNA Cloning

(1) Determination of the N-terminal amino acid sequences of the heavy and light chains of TRA-8

In order to obtain cDNAs of the heavy and light chains of TRA-8, the N-terminal amino acid sequences of the heavy and light chains of TRA-8 and cloned TRA-8 genes are determined by known techniques

Ten µg of the solution containing the anti-human DR5 antibody TRA-8 is subjected to SDS-polyacrylamide gel electrophoresis ("SDS-PAGE"), using a gel concentration of 12% w/v, 100 V constant voltage, for 120 minutes After electrophoresis, the gel is immersed in transfer buffer 25 mM Tris-HCl (pH 9 5), 20% methanol, 0 02% v/v SDS for 5 minutes After this time, the protein content of the gel is transferred to a polyvinylidene difluoride membrane ("PVDF membrane", pore size 0 45 um, Millipore, Japan), presoaked in transfer buffer, using a blotting apparatus (KS-8451, Marysol) under conditions of 10 V constant voltage, 4°C, for 14 hours

After this time, the PVDF membrane is washed with washing buffer 25 mM NaCl, 10 mM sodium borate buffer (pH 8 0), then stained in a staining solution (50% v/v methanol, 20% v/v acetic acid and 0 05% w/v Coomassie Brilliant Blue) for 5 minutes to locate the protein bands The PVDF membrane is then destained with 90% v/v aqueous methanol, and the bands corresponding to the heavy chain, the band with the lower mobility and light chain, the band with the higher mobility previously located on the PDVF membrane are excised and washed with deionized water

The N-terminal amino acid sequence of the heavy and light chains are determined by the Edman automated method (Edman, P , et al , (1967), Eur J Biochem , 1, 80) using a gas-phase protein sequencer (PPSQ-10, Shimadzu Seisakusyo, K. K.)

The N-terminal amino acid sequence of the band corresponding to the heavy chain is determined to be

Glu-Val-Met-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-Lys-Pro-Gly-Gly-Ser-Leu-Lys-Leu (SEQ ID No 4 of the Sequence Listing),

and that of the band corresponding to the light chain is determined to be

Asp-Ile-Val-Met-Thr-Gln-Ser-His-Lys-Phe-Met-Ser-Thr-Ser-Val-Gly-Asp-Arg-Val-Ser (SEQ ID No 5 of the Sequence Listing)

Comparison of these amino acid sequences with the database of amino acid sequence of antibodies produced by Kabat et al (Kabat E A , et al , (1991), in "Sequences of Proteins of Immunological Interest Vol II," U S Department of Health and Human Services) revealed that the heavy chain (γ1 chain) and the light chain (k chain) of TRA-8 belonged to subtypes 3d and 1, respectively

(2) cDNA cloning

Based on above findings, oligonucleotide primers are synthesized which would be expected to hybridize with portions of the 5'-untranslated regions and the very ends of the 3'-translated regions of the genes belonging to these mouse subtypes Then, cDNAs encoding the heavy and light chains of TRA-8 are cloned by the following combination of reverse transcription and PCR (RT-PCR)

a) Template

The total RNA of TRA-8 hybridoma (ATCC No PTA-1428) is extracted by using TRIzol Reagent (GIBCO BRL) The template for the PCR reaction used cDNA that is obtained by using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the instruction manual provided with the kit

b) PCR Primers

The following oligonucleotide primers are synthesized for the PCR

5'-cagcactgaa cacggacccc-3' (H5NCS1 SEQ ID No 6 of the Sequence Listing),

5'-aaaggtaatt tattgagaag-3' (H5NCS2 SEQ ID No 7 of the Sequence Listing),

5'-cctcaccatg aacttcgggc-3' (H5SS1 SEQ ID No 8 of the Sequence Listing),

5'-ctgttgtatg cacatgagac-3' (H5SS2 SEQ ID No 9 of the Sequence Listing),

5'-gaagtgatgc tggtggagtc-3' (H5CS1 SEQ ID No 10 of the Sequence Listing),

5'-agtgtgaagt gatgctggtg-3' (H5CS2 SEQ ID No 11 of the Sequence Listing),

5'-tttaccagga gagtgggaga g-3' (H3CR SEQ ID No 12 of the Sequence Listing),

5'-tgcagagaca gtgaccagag-3' (H3VR SEQ ID No 13 of the Sequence Listing),

5'-tgttcaggac cagcatgggc-3' (L5NCS1 SEQ ID No 14 of the Sequence Listing),

5'-aagacatttt ggattctaac-3' (L5NCS2 SEQ ID No 15 of the Sequence Listing),

5'-tatcatgaag tctttgtatg-3' (L5SS1 SEQ ID No 16 of the Sequence Listing),

5'-gatggagaca cattctcagg-3' (L5SS2 SEQ ID No 17 of the Sequence Listing),

5'-gacattgtga tgacccagtc-3' (L5CS SEQ ID No 18 of the Sequence Listing),

5'-ttaacactca ttcctgttga-3' (L3CR SEQ ID No 19 of the Sequence Listing), and

5'-gactgggtca tcacaatgtc-3' (LCSR SEQ ID No 20 of the Sequence Listing)

Unless otherwise specified, all oligonucleotides in these Examples are synthesized by Pharmacia Biotech All oligonucleotides are stored at –20°C after being dissolved in distilled water

c) PCR reaction

Composition of the PCR reaction solution

template cDNA, 5 µl of total 33 µl reaction

primer 1, 10 pmol,

primer 2, 10 pmol,

10 x concentrated PCR buffer (provided with the kit), 10 µl,

dNTPs (each 2 5 mM), 4 µl, and

Taq polymerase (Promega), 5 units

Sterile distilled water is added to the solution to a total volume of 100 µl Unless otherwise specified, dNTPs are an equimolar mixture of dATP, dCTP, dGTP and dTTP (2 5 mM each)

The PCR reaction is conducted as follows The solution is first heated at 94°C for 2 minutes, after which a cycle of heating to 94°C for 30 sec, 52°C for 1 minute and 72°C for 3 minutes, is repeated 40 times After completion of this procedure, the reaction solution is heated at 72°C for 10 minutes

The amplified DNA fragments, thus obtained, are separated on a 1% agarose gel containing 0 25 µg/ml ethidium bromide The bands determined to contain the desired DNA fragments are cut out using a razor blade and the DNA is recovered therefrom using the Gene Clean kit (BIO101) The DNA fragment is cloned using pGEM-T Easy vector (Promega) This is performed as follows

The DNA fragment recovered from the PCR reaction solution, together with 50 ng of pGEM-T Easy vector (provided with the kit), is mixed with 1 µl of 10 X ligase reaction buffer (6 mM Tris-HCl (pH 7 5), 6 mM magnesium chloride, 5 mM sodium chloride, 7 mM β-mercaptoethanol, 0 1 mM ATP, 2 mM DTT, 1 mM spermidine, and 0 1 mg/ml bovine serum albumin), to which 4 units of T4 DNA ligase (1 µl) has been added The total volume of the mixture is adjusted to 10 µl with sterile deionized water, and the resulting ligase solution is incubated at 14°C for 15 hours After this time, 2 µl of the ligase reaction solution is added to 50 µl of competent *E coli* strain JM109 (provided with the kit and brought to competence in accordance with the instruction manual) to which 2 µl of 0 5 M β-mercaptoethanol had been added, and the resulting mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 5 minutes Next, 500 µl of medium containing 2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM potassium chloride, 1 mM magnesium chloride, and 20 mM glucose (hereinafter referred to as "SOC" medium) is added to the culture, and the mixture is incubated for 1 hour at 37°C with shaking After this time, the culture is spread on an L-broth agar plate (1%

v/v tryptone, 0 5% w/v yeast extract, 0 5% w/v sodium chloride, 0 1% w/v glucose, and 0 6% w/v bacto-agar (Difco)), containing 100 µg/ml Ampicillin resistant colonies appearing on the plate are selected and scraped off with a platinum transfer loop, and cultured in L-broth medium containing 100 µg/ml ampicillin at 37°C, overnight, with shaking at 200 r p m After incubation, the cells are harvested by centrifugation, from which plasmid DNA is prepared by the alkali method. The obtained plasmid is designated as plasmid pH62 for heavy chain of TRA-8 or pL28 for light chain of TRA-8 The transformant *E coli* strains harboring these plasmid, designated as *E coli* JM109/pH62 and *E coli* JM109/pL28 were deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and were accorded the accession numbers FERM BP-7560 and FERM BP-7561, respectively The nucleotide sequences of these DNAs encoding the heavy chain and the light chain of TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The nucleotide sequences of the heavy and light chains of TRA-8 are given as SEQ ID No 21 and No 22 of the Sequence Listing, respectively The amino acid sequences of the heavy and light chains of TRA-8 are given as SEQ ID No 23 and No 24 of the Sequence Listing, respectively The N-terminal amino acid sequences of the heavy and light chains of TRA-8 established in above matched perfectly Furthermore, when the amino acid sequences of the heavy and light chains are compared with the database of amino acid sequences of antibodies, it is established that, for the heavy chain, nucleotide Nos 58 to 414 in SEQ ID No 21 constituted the variable region, while nucleotide Nos 415 to 1392 in SEQ ID No 21 constituted the constant region For the light chain, nucleotide Nos 64 to 387 in SEQ ID No 22 constituted the variable region, while nucleotide Nos 388 to 702 in SEQ ID No 22 constituted the constant

region The locations and sequences of the CDRs are also elucidated by comparing the homologies with the database The amino acid sequences of CDR 1, CDR2, and CDR3 of heavy chain of TRA-8 are shown in SEQ ID No 25, No 26, and No 27, respectively The amino acid sequences of CDR1, CDR2, and CDR3 of light chain of TRA-8 are shown in SEQ ID No 28, No 29, and No 30, respectively

Example 17 Designing a Humanized Version of the TRA-8 Antibody

(1) Molecular modeling of a variable region of TRA-8

Molecular modeling of the variable region of TRA-8 is performed by the method generally known as homology modeling (Methods in Enzymology, 203, 121-153, (1991)) The primary sequences of variable regions of human immunoglobulin registered in the Protein Data Bank (Nuc Acid Res 28, 235-242 (2000)), for which the three-dimensional structures derived from x-ray crystallography are available, are compared with the framework regions of TRA-8 determined above As a result, 1NCD and 1HIL are selected as having the highest sequence homologies to the framework regions for the light and heavy chains of TRA-8, respectively Three-dimensional structures of the framework regions are generated by combining the coordinates of 1NCD and 1HIL which correspond to the light and heavy chains of TRA-8, to obtain the "framework model" Using the classification defined by Chothia et al, the CDRs of TRA-8 are classified as follows, $CDRL_1$, $CDRL_2$, $CDRH_1$ and $CDRH_2$ belong to canonical classes 2,1,1,3, respectively, while $CDRL_3$ does not belong to any specific canonical classes The CDR loops of $CDRL_1$, $CDRL_2$, $CDRH_1$, $CDRH_2$ are fixed to the conformations inherent to their respective canonical classes, and integrated into the framework model $CDRL_3$ is assigned the conformation of cluster 8A, according to the classification of Thornton et al (J Mol Biol, 263, 800-815, (1996)), and $CDRH_3$ is classified into k(8)C using the H3 rule (FEBS letter 455,188-197(1999)) Then representative conformations for $CDRL_3$ and $CDRH_3$ are integrated into the framework model

Finally, energy calculations are carried out to eliminate unfavorable inter-atomic contacts, in order to obtain a probable molecular model of TRA-8's

variable region in terms of energy The above procedure is performed using the commercially available common molecular modeling system ABM (Oxford Molecular Limited, Inc) For the molecular model obtained, the accuracy of the structure is further evaluated using the software, PROCHECK (J Appl Cryst (1993), 26, 283-291)

(2) Designing the amino acid sequences for humanized TRA-8

Construction of humanized TRA-8 antibodies is performed by the method generally known as CDR grafting (Proc Natl Acad. Sci USA 86, 10029-10033 (1989)) The acceptor antibody is chosen based on the amino acid homology in the framework region. The sequences of framework region in TRA-8 are compared with all the human framework sequences in the Kabat database of amino acid sequences of antibodies (Nuc Acid Res 29, 205-206 (2001)) As a result, mAB58'CL antibody is selected as an acceptor due to the highest sequence homology of 80% for the framework region. The amino acid residues in the framework region for mAb58'CL are aligned with that for TRA-8 and the positions where different amino acids are used are identified The location of those residues are analyzed using the three dimensional model of TRA-8 constructed above and the donor residues which should be grafted on the acceptor are chosen by the criteria given by Queen et al (Proc Natl Acad. Sci USA 86, 10029-10033 (1989)) Humanized TRA-8 sequences are constructed as described in the following example by transferring several donor residues into acceptor antibody, mAb58'CL

Example 18 Construction of an Expression Vector for the Heavy Chain of the Humanized Antibody

(1) Construction of plasmid carrying the heavy chain variable region DNA of Humanized TRA-8

In order to determine the activity of humanized TRA-8, the plasmid carrying the heavy chain of humanized TRA-8 is constructed as follows However, it is appreciated the humanization of TRA-8 is not limited to these examples

As shown in SEQ ID No 31 of the Sequence Listing, humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 84th amino acid (serine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, glycine, glycine, asparagine, alanine, valine, leucine, and valine, respectively

The plasmid carrying DNA encoding heavy chain variable region of humanized TRA-8 (SEQ ID No 31 of the Sequence Listing) are constructed as follows

PCR is used to construct the following DNA sequences, each of which comprised described above

The following 12 oligonucleotides are synthesized

5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32),

5'- tctgaagtaa tgctggtgga gtctgggggа ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctc cacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

5'- tccagaccct tccctggtgc ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtaa ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42), and

5'- gaccgatggg cccttggtgg aggctgagga gactgtgacc agggtccctt ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43)

The following 2 PCR primers are synthesized as described above

5'- ttggataagc ttggcttgac -3' (P1, SEQ ID No 44), and

5'- gaccgatggg cccttggtgg a -3' (P2, SEQ ID No 45)

The synthesis of DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,

oligonucleotide B, 10 pmol,

oligonucleotide C, 10 pmol,

oligonucleotide D, 10 pmol,

oligonucleotide E, 10 pmol,

oligonucleotide F, 10 pmol,

oligonucleotide G, 10 pmol,

oligonucleotide H, 10 pmol,

oligonucleotide I, 10 pmol,

oligonucleotide J, 10 pmol,

oligonucleotide K, 10 pmol,

oligonucleotide L, 10 pmol,

oligonucleotide primer P1, 2 μM,

oligonucleotide primer P2, 2 μM,

10 X Pyrobest buffer II, 10 µl,

dNTP mix, 8 µl,

Pyrobest DNA polymerase, 0 5 µl, and

Redistilled water to a final volume of 50 µl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

An equal volume of phenol-chloroform (50% v/v phenol saturated with water, 48% v/v chloroform, 2% v/v isoamyl alcohol) is added to 200 µl of each of the PCR products, and vigorously mixed for 1 minute After this time, the mixture is centrifuged at 10,000 X g, and the aqueous layer is recovered and mixed with an equal volume of chloroform-isoamyl alcohol (96% v/v chloroform and 4% v/v isoamyl alcohol), which is again vigorously at 10,000 X g and the aqueous layer is recovered. The series of steps recited in this paragraph is referred to, hereafter, as "phenol extraction")

Ethanol precipitation is then performed on the recovered aqueous layer As used and referred to herein, "ethanol precipitation" consists of adding, with mixing, a one tenth volume of 3M sodium acetate (pH 5 2) and 2 5 volumes of 100% ethanol to the solution to be treated, and freezing the mixture using dry ice The resulting mixture is then centrifuged at 10,000 X g to recover DNA as a precipitate

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 µg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The DNA band corresponding to humanized TRA-8 DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 µl of distilled water

The resulting, each extracted DNA is cloned using pGEM-T Easy vector (Promega) as follows

The DNA fragment recovered from the PCR reaction, 5 µl,

10 X Taq polymerase buffer, 1 µl,

dNTP mixture, 1 µl

Taq polymerase (5 unit/ml), 1 µl, and

redistilled water to a final volume of 10 µl

After the above each solution is reacted at 70°C for 30 minutes, each DNA solution and pGEM-T Easy vector are ligated using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) using the manufacturer's protocol

After 4 hours incubation at 15°C, 2 µl of the incubated reaction solution is mixed with 100 µl of competent *E coli* strain JM109 at a cell density of 1-2 x 10^9 cells/ml (Takara Shuzo Co , Ltd), and the mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 1 minutes Then, 500 µl of SOC medium (2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM w/v potassium chloride, 1 mM magnesium chloride, and 20 mM glucose) is added the mixture, which is incubated for a further hour, with shaking Transformant strains are then isolated, and plasmid DNA is prepared from the strains as described in "Molecular Cloning A Laboratory Manual" The nucleotide sequences of these DNAs encoding the heavy chain of humanized TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pHB14 (the plasmid carrying cDNA encoding the heavy chain of humanized TRA-8) The transformant *E coli* strain harboring these plasmid, designated as E coli JM109/pHB14 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7556

(2) Construction of expression plasmids carrying the heavy chain variable region DNA of Humanized TRA-8

Recombinant expression vectors for animal cells are constructed by inserting the DNA encoding the heavy chain of humanized TRA-8 (cloned in above) as follows

One μg of plasmid pSRHHH3 (European patent application EP 0-909-816-A1) carrying the heavy chain variable region of humanized anti-Fas monoclonal antibody HFE7A and human IgG1 constant region genomic DNA, an expression vector for mammalian cells, is digested with the restriction enzymes HindIII and ApaI, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The vector DNA bands containing human IgG1 constant region genomic DNA without the heavy chain variable region of humanized HFE7A are cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water and then dephosphorylated using CIP The resulting digested, dephosphorylated plasmid (100 ng) is ligated with 1 μg of the pHB14 DNA fragment containing the DNA encoding the heavy chain variable region of humanized TRA-8, which had also been digested with HindIII and ApaI, using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) The ligation mixture is then used to transform E coli JM109, which is then plated on LB agar plates containing 50 μg/ml ampicillin

The transformants obtained by this method are cultured in 2 ml of liquid LB medium containing 50 μg/ml ampicillin at 37°C overnight, and plasmid DNA is subsequently extracted from the resulting culture by the alkaline-SDS method.

The extracted plasmid DNA is digested with HindIII and ApaI, and subjected to 3% w/v agarose gel electrophoresis to confirm the presence or absence of the insert of the DNA encoding the heavy chain variable region of

humanized TRA-8 The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems) The resulting expression plasmid carrying cDNA encoding the heavy chain of humanized TRA-8 is designated pHB14-1

Example 19 Construction of an Expression Vector for the Light Chain of the Humanized Antibody

(1) Construction of vectors for the light chains of humanized versions of TRA-8 antibody

As shown in SEQ ID No 46 of the Sequence Listing, in humanizing the amino acid sequence of the light chain of the mouse anti-human DR5 antibody TRA-8, 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd (serine), 60th amino acid (aspartic acid), 63rd amino acid (threonine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine),and 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, serine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine respectively The resulting sequence is designated LM2

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized TRA-8

DNA coding for the LM2 polypeptide chain (SEQ ID No 46 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig

light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- gtccccccaca gatgcagaca aagaacttgg agattgggtc atcacaatgt caccagtgga -3' (HKSPR11, SEQ ID No 49),

5'- ccaagttctt tgtctgcatc agtaggagac agggtcacca tcacctgc -3' (HKCDF11, SEQ ID No 50),

5'- agtgtgccgg gtggatgccc agtaaatcag tagtttagga gctttccctg gtttctg -3' (HKCDR12, SEQ ID No 51),

5'- tgggcatcca cccggcacac tggggtccca agcaggttta gtggcagt -3' (HKCDF22, SEQ ID No 52),

5'- ataactacta tattgctgac agtaataggt tgcaaaatcc tccggctgca gactagagat ggt -3' (HKCDR22, SEQ ID No 53), and

5'- cagcaatata gcagctatcg gacgttcggt caaggcacca aggtggaaat caaacggact gtg -3' (HKCF12, SEQ ID No 54)

2) Construction of plasmid pCR3 1/M2-1 (cloning of humanized TRA-8 light chain)

LM2-DNA fragment as defined in SEQ ID No 55 of the Sequence Listing coding for the amino acid sequence as defined in SEQ ID No 46 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM2-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA (European patent application EP 0 909 816 A1), 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR11, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase (PerkinElmer), 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F2-DNA fragment coding for a portion of FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$ and is prepared under the following conditions

Composition of the reaction solution

plasmid pL28 DNA, 25 ng

oligonucleotide primer HKCDF11, 50 pmol

oligonucleotide primer HKCDR12, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F3-DNA fragment coding for CDRL2, FRL_3, and a portion of $CDRL_3$ is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA(European patent application EP 0 909 816 A1), 25 ng

oligonucleotide primer HKCDF22, 50 pmol

oligonucleotide primer HKCDR22, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F4-DNA fragment coding for $CDRL_3$, FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer HKCF12, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM2-DNA in which above described LM2-F1-DNA, LM2-F2-DNA, LM2-F3-DNA and LM2-F4-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM2-F1-DNA prepared in the first step PCR,

Gel fragment of LM2-F2-DNA prepared in the first step PCR,

Gel fragment of LM2-F3-DNA prepared in the first step PCR,

Gel fragment of LM2-F4-DNA prepared in the first step PCR

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 0 µl

10×PCR buffer, 5 0 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM2-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (Invitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/M2-1 (the plasmid carrying cDNA encoding the light chain variable region of humanized TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/M2-1 containing LM2-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM2-DNA fragment, that have been digested with the restriction enzymes Hind III and EcoR I, are ligated

using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM2-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M2-1-4 carrying a fusion fragment of the variable region of the humanized LM2 TRA-8 light chain and the constant region of human Igκ chain is obtained. The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M2-1-4 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7563

3) Construction of plasmid pSR/M2-1 (expression plasmid for humanized LM2 TRA-8 light chain)

The obtained plasmid pHSG/M2-1-4 carrying a fusion fragment of the variable region of the humanized LM2 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0-909-816-A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M2-1-4 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from

the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer, Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized TRA-8 is designated pSR/M2-1

Example 20 Production of Humanized Antibody

Transfection of COS-7 cells, i e , a cell line derived from a monkey kidney, with the expression plasmids for the humanized TRA-8 heavy chain and the humanized TRA-8 light chain obtained above is conducted by FUGENE6 transfection reagent methods (Boehringer Mannheim Biochemica) according to the instruction manual provided with the kit

COS-7 cells (American Type Culture Collection No CRL-1651) are grown to semi-confluent (3×10^6 cells/dish) in a culture dish (culture area 57 cm^2, Sumitomo Bakelite) containing Dulbecco's Modified Eagle medium (hereinafter referred to as "D-MEM", Gibco BRL) supplemented with 10% fetal calf serum (hereinafter abbreviated as "FCS", Moregate)

In the meantime, 10 µg/dish (total 5 dishes) of the humanized DR5 heavy chain expression plasmid DNA (pHA15-1) and 10 µg/dish of the humanized DR5 light chain expression plasmid DNA prepared by the alkaline-SDS method and cesium chloride density gradient centrifugation are mixed, and then precipitated with ethanol, followed by suspending in 5 µl/dish of dH_2O

After 15 µl/dish of FUGENE6 Transfection regent is mixed with 180 µl/dish D-MEM without FCS, this FUGENE solution (185 µl/dish) is mixed with 5 µl/dish DNA solution containing 10 µg/dish of the humanized DR5 heavy chain expression plasmid DNA and 10µg/dish of the humanized DR5 light chain expression plasmid DNA After 15 minutes incubation at room temperature, the obtained plasmid suspension (200 µl) is added to the previously prepared COS-7 plates After incubating in 5% CO_2 at 37°C for 24 hours, the culture medium is changed with D-MEM without FCS After incubating in 5% CO_2 at 37°C for 72 hours, the culture supernatant is recovered to purify the expression products in

the supernatant fluids By the method as described above, COS-7 cells are transfected with each of the following plasmid combinations

(A) no plasmid DNA

(B) cotransfection of pHB14-1 and pSR/M2-1

The culture is then centrifuged (1,000 r p m , 5 minutes) and collected the supernatant The supernatant is centrifuged again (9,800 r p.m., 15 minutes) and filtrated with 0 45 μm filter (ADVANTEC TOYO DISMIC-25cs, Cat # 25CS045 AS) The purification of IgG from the filtrates are achieved using Protein G-POROS affinity chromatography (Applied Biosystems) under the following conditions

HPLC BioCAD 700E (Applied Biosystems)

column ProteinG-ID sensor cartridge (column size 2 1 mmID x 30 mm LD, bed volume 0 1 ml, Cat # 2-1002-00, Applied Biosystems)

elution buffer 0 1M Glycine-HCl (pH 2 5)

neutralization buffer 1M Tris-HCl (pH 8 5)

detection 280 nm

flow rate 1 ml/min

fraction size 0 5 ml/0 5 min

fraction tube 1 5 ml polypropylene microtube

temperature 4°C

After all the filtrates are applied to column, 30 ml of PBS (Sigma, Cat # 1000-3) is used to wash column When the elution buffer is applied, fraction collector started Each fraction microtube previously contained 55 μl of 1M NaCl, 110 μl of neutralization buffer and 74 μl of 2 mg/ml bovine serum albumin (Sigma, Cat # A-7030) in PBS The fractions from No 8 through No 10 are collected and dialyzed against 1 liter PBS (pH 7 5) at 4°C for 1 day using Slide-A lyzer (Pierce, Cat # 66450) The dialysis buffer is changed twice

Verification of the expression of the humanized antibodies and quantitative assay of the expression products in the culture supernatant fluids prepared is performed by ELISA with an antibody against anti-human IgG

To each well of a 96-well plate (MaxiSorp, Nunc), 100 µl of goat anti-human IgG Fc specific polyclonal antibody (Kappel) dissolved at the final concentration of 0 5 µg/ml in adsorption buffer (0 05 M sodium hydrogencarbonate, 0 02% sodium azide, pH 9 6) is added and the plate is incubated at 37°C for 2 hours to cause adsorption of the antibody Then, the plate is washed with 350 µl of PBS(-) containing 0 05% Tween-20 (BioRad) (hereinafter referred to as "PBS-T") five times To the wells after washing, the culture supernatant diluted with D-MEM containing 10% FCS is added and incubated at 37°C for 2 hours After washing again with PBS-T, 100 µl of alkaline phosphatase-labeled goat anti-human IgG Fc specific polyclonal antibody (Jackson Immuno Research Lab) diluted 10,000-fold with PBS-T is added to each well and incubated at 37°C for 2 hours After washing again with PBS-T, a substrate solution of p-nitrophenyl phosphate obtained from Alkaline Phosphatase Substrate kit (Bio Rad) is added according to the instruction manual provided with the kit. After incubating at 37°C for 0 5 to 1 hour, the absorbance at 405 nm is measured In the present experiments, human plasma immunoglobulin G subclass 1 (IgG1) (Biopure AG) diluted with D-MEM containing 10% FCS to certain concentrations is used as concentration reference samples of the humanized DR5 antibodies contained in the culture supernatant fluids

As a result, the expression and purified products in the culture supernatant are detected specifically with the anti-human IgG antibody The amount of human IgG antibody is 8 96 µg (800 µl)

Example 21 Apoptosis-inducing activity of Humanized Antibody

Jurkat cells (ATCC No TIB-152), are used to examine the apoptosis-inducing activity of the purified humanized TRA-8 antibody

Jurkat cells cultured in RPMI1640 medium with 10% FCS (Gibco BRL) at 37°C for 3 days in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) at 50 µl per well The humanized TRA-8 prepared in Example 20 are adjusted to have the concentration of the final product of interest of 100 ng/ml with RPMI1640 medium containing 10%

FCS by estimating their concentrations in the fluids according to the method described in Example 20 Each of the solutions of the expression products thus adjusted to 100 ng/ml is used to produce serial dilutions by repeating serial 2-fold dilution with RPMI1640 containing 10% FCS Each of the diluted humanized TRA-8 solution is added to each well at 50 µl per well After reacting at 37°C for 12 hours, 50 µl of 25 µM PMS (phenazine methosulfate, Sigma Chemical Co) containing 1 mg/ml XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt, Sigma Chemical Co) is added (final concentrations of 250 µg/ml for XTT and 5 µM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells, and "c" is the measurement of a well with no antibody added

As a result, the expression product prepared in Example 20 (humanized TRA-8) is demonstrated to induce apoptosis in cells of T lymphoma cell line expressing human DR5 antigen.

Example 22 Reactivity of TRA-8 to various DR5 molecules

In order to determine the reactivity of TRA-8 to various DR5 molecules, the reactivity of TRA-8 is examined using activated lymphocytes as follows

First, peripheral blood samples are taken from a human (30 ml), marmoset (3 ml), and cynomolgus monkey (20 ml) The blood samples had 1 ml of heparin (Novoheparin, Novo) added to them and the samples are then slowly layered over an equal volume of Ficoll-Paque PLUS solution ((Amersham Pharmacia Biotech) specific gravity 1 077 for all except cynomolgus monkey, which had a specific gravity of 1 072) and centrifuged at 1,700 r p m for 30 minutes in order to obtain a fraction of peripheral blood mononuclear cells This mononuclear cell fraction is washed twice with Hanks' balanced salt solution

and then suspended in RPMI 1640 medium with 10% v/v FCS to a cell density of 1 x 10^6 cells/ml Phytohemagglutinin-P (PHA-P, Sigma Chemicals, Co) is added to the resulting suspension to a final concentration of 5 µg/ml and the sample incubated at 37°C under 5% v/v CO_2 for 24 hours After this time, the cells are recovered by centrifugation, washed and resuspended in RPMI 1640 medium containing 10% v/v FCS Then, to activate the recovered cells, interleukin-2 (Amersham Pharmacia Biotech) is added to the suspension to a final concentration of 10 units/ml, and this is incubated at 37°C under 5% v/v CO_2 for 72 hours

An amount of the activated preparation calculated to contain 1 x 10^6 activated lymphocyte cells is placed in a test tube and either suspended in 50 µl of 0 5, 1, 5, 10 µg/ml of TRA-8 in PBS or 50 µl of PBS alone The resulting suspension is allowed to stand on ice for 1 hour, after which the cells are washed 3 times with aliquots of 500 µl of PBS and then suspended in 50 µl of 20 µg/ml FITC-labeled anti-mouse IgG antibody (Bioresource) in PBS Using the cells suspended in 500 µl of PBS as controls, the fluorescence intensities are measured, using a flow cytometer (FACSCalibur, Becton Dickinson)

Distributions of cell numbers by fluorescence intensity are obtained and the proportions of the numbers of the stained cells to those of total cells are calculated Further, each Kd value is calculated using the concentration of TRA-8 and the proportions of the numbers of the stained cells to those of total cells Each frequency of reactivity to activated lymphocytes of human, marmoset, and cynomologus monkey is almost same Accordingly, TRA-8 is able to bind a wide range of primate DR5 including human against which TRA-8 is originally prepared

Example 23 Escalating dose study of TRA-8 in marmosets

An escalating dose preliminary toxicity study of TRA-8 is performed using 1 male and 1 female marmoset Three sets of single intravenous dosing, which are separated by a 7-day withdrawal period, are carried out. The dose of TRA-8 is set at 50, 250 and 1250 µg/body Forty-eight hours after each treatment, blood is collected from the femoral vein and the plasma is prepared

Plasma aspartate aminotransferase and alanine aminotransferase activities are measured using an analyzer (FUJI DRI-CHEM Fuji Film Medical Co , Ltd) All blood is taken without any anesthetization As a result, no evidences indicating hepatic injury are noted in plasma biochemical examination after each treatment

Example 24 ***In vitro*** **and** ***in vivo*** **pharmacological studies of TRA-8 against cancer cells**

In order to determine whether TRA-8 has the therapeutic efficacy in anti-cancer therapy, *in vitro* killing activity of TRA-8 using various cancer cell lines is examined as follows

Various cancer cells (2-8 x 10^3 cells/50 µl) cultured in RPMI1640 medium (for Jurkat), DMEM medium (for HCT-116), MEM-R (for WiDr), or DMEM-F12 (for COL2-Jck) obtained from Gibco BRL with 10% FCS (Gibco BRL) at 37°C in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) TRA-8 are adjusted to have the concentration of the final product of interest of 100 ng/ml with medium containing 10% FCS The TRA-8 solution (100 ng/ml) is used to produce serial dilutions by repeating serial 2-fold dilution with medium containing 10% FCS Each of the diluted TRA-8 solution is added to each well at 50 µl per well and incubated at 37°C After reacting at 37°C for 72 hours, 50 µl of 25 µM PMS (phenazine methosulfate, Sigma Chemical Co) containing 1 mg/ml XTT is added (final concentrations of 250 µg/ml for XTT and 5 µM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells, and "c" is the measurement of a well with no antibody added.

The results are shown in Table 3, below

Table 3

Cells	ED50 (μg/ml)
Jurkat	0 001 - 0 01
HCT-116	0 004 - 0 02
WiDr	0 007 - 0 03
COL2-Jck	2.28

Various cancer cell lines are strongly induced apoptosis by TRA-8 under the *in vitro* conditions

Furthermore, the *in vivo* anti-tumor effect of TRA-8 in nude mice transplanted with WiDr cells is determined, because TRA-8 is not cross-reactive with murine DR5

TRA-8 anti-human DR5 antibody is administered to nude mice bearing human xenografts that express the human DR5 molecule The mice used were 6 week-old BALb/c nude/nude mice (female, from Clea Japan Inc), which were transplanted with human colon cancer cell lines WiDr (5 mm^3) At one day after tumor transplantation, these transplanted mice are daily treated with the intraarticular injection of TRA-8 (5 μg/body) to 14 times WiDr tumor growth is daily determined by the size of the tumor mass The results are shown in Table 4, below

Table 4

	8 days	11 days	15 days	18 days	22 days	25 days
Control (PBS)	196	249	469	584	833	1193
SD	±55	±77	±149	±230	±274	±419
TRA-8	158	97	155	195	365	530
SD	±78	±30	±60	±58	±91	±135

In this model, while all untreated animals exhibited visible tumor growth, tumor growth in TRA-8 treated animals is inhibited as demonstrated by the size of tumor This result indicated that TRA-8 is effective in the elimination of tumor cells *in vivo*

Example 25 Combination study of TRA-8

Human prostate cancer cell line PC-3 is obtained from American Tissue Culture Collection (ATCC) and maintained in F-12K Nutrient Mixture

(21127-022, Gibco BRL) containing 10% fetal bovine serum (FBS, Hyclone), 1% L-Glutamine-200 mM (25030-149, Gibco BRL) and 0 5% Penicillin Streptomycin Solution (P-7539, Sigma) RPMI1640 medium (MED-008, IWAKI) supplemented with 10% FBS and 0 5% Penicillin Streptomycin Solution is used in the following experiment. Exponentially growing PC-3 cells are collected by trypsinization and washed twice with fresh medium. The cells are then counted, resuspended in fresh medium at a density of 5 x 10^4 cells/ml and distributed in triplicate into flat-bottomed 96 well plates (3598, Corning-Coster) in a total volume of 100 µl/well one day before the start of the experiment A representative anti-cancer drug, Paclitaxel (169-18611, Wako) dissolved in dimethylsulfoxide (10 mg/ml) is diluted in fresh medium and then added to the 96-well plates containing the cells at 50 µl/well. The final concentrations of dimethylsulfoxide are less than 0 1% After incubation for 24 hr at 37°C in 5% CO_2 atmosphere, TRA-8 diluted in fresh medium is added to the wells After incubation for a further 24 hr, 50 µl of Minimum Essential Medium (11095-098, Gibco BRL) containing 1 mg/ml of XTT and 25 mM of PMS is added to the wells and the plates are incubated for 6 hr OD450 is then measured by SPECTRA MAX 250 (Molecular Devices) and the cell viability is calculated as follows

Cell viability (%) = (OD450 for the well containing cells treated with Taxol and/or TRA-8 (agent(s)) - OD450 for the well containing neither cells nor agent) x 100 / (OD450 for the well containing cells with no agent –OD450 for the well containing neither cells nor agent)

The result of the above assay for TRA-8 combined with a representative anti-cancer drug, Paclitaxel, is followed Paclitaxel reduced the cell viability of PC-3 cells but more than 40% of the signals indicating viable cancer cells still remained at concentrations of up to 200 nM Notably, the addition of 0 1 ng/ml of TRA-8 greatly decreased the cell viability of the cancer cells, up to 10%, even though no reduction in cell viability is seen after a single application of TRA-8 at this concentration This result clearly indicates that TRA-8 exhibited anti-cancer activity synergistically when combined with other anti-cancer drugs

Example 26 Analysis of Other Type Humanized Antibodies of TRA-8

(1) Designing Humanized Antibodies

Construction of a humanized version of TRA-8 is performed by the method generally known as CDR grafting mAB58'CL antibody is used as an acceptor as described in Reference Example 2 and the CDR regions of TRA-8 antibody is grafted on the acceptor In the framework region, some amino acids are grafted on the acceptor from either TRA-8 or human consensus sequences by the criteria given by Queen et al (Proc Natl Acad Sci USA 86, 10029-10033, (1989)) and humanized TRA-8 sequences are constructed as described hereinbelow

(2) Construction of Plasmid Carrying the Heavy Chain Variable Region DNA of Other Types Humanized or Mouse TRA-8

As shown in SEQ ID No 56 of the Sequence Listing, H1 type-humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 3rd amino acid (methionine), the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 84th amino acid (serine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, glutamine, arginine, alanine, glycine, glycine, asparagine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 59 of the Sequence Listing, H3 type-humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, glycine, glycine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 60 of the Sequence Listing, H4 type-humanization of the amino acid sequences of the heavy chain of the mouse

anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 61 of the Sequence Listing, the plasmid carrying the heavy chain variable region DNA of chimeric TRA-8 is designated as "M type" In addition, humanized TRA-8 described in Example 17 and 18 is designated as "H2 type"

The plasmids carrying DNA encoding heavy chain variable region of humanized or chimeric TRA-8 are constructed as follows

PCR is used to construct the following DNA sequences, each of which comprised described above

The following 24 oligonucleotide are synthesized

5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32),

5'- tctgaagtaa tgctggtgga gtctgggggga ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- tctgaagtac agctggtgga gtctgggggga ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B2, SEQ ID No 57),

5'- tctgaagtaa tgctggtgga gtctgggggga ggcttagtaa agcctggagg gtccctgaaa ctctcctgtg cagcctctgg -3' (B3, SEQ ID No 66),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag actccagaga agaggctgga gtgggttgca accattagta -3' (C2, SEQ ID No 64),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- tatctgcaaa tgagcagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E2, SEQ ID No 62),

5'- tatctgcaaa tgagcagtct gagatctgag gacacggcta tgtattactg tgcaagaagg ggtgactcta tgattacgac -3' (E3, SEQ ID No 67),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctccacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5'- ggactactgg ggccaaggga ccactctcac agtctcctca gcctccacc aagggcccat cggtc -3' (F2, SEQ ID No 68),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagct gtacttcaga gtggacacct gtagctgttg -3' (H2, SEQ ID No 58),

5'- tttcagggac cctccaggct ttactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H3, SEQ ID No 69),

5'- tccagaccct tccctggtgc ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- tccagcctct tctctggagt ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I2, SEQ ID No 65),

5'- ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtaa ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K2, SEQ ID No 63),

5'- ccttcttgca cagtaataca tagccgtgtc ctcagatctc agactgctca tttgcagata cagggtgttc ttggcattgt -3' (K3, SEQ ID No 70),

5'- gaccgatggg cccttggtgg aggctgagga gactgtgacc agggtccctt ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43) and

5'- gaccgatggg cccttggtgg aggctgagga gactgtgaga gtggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L2, SEQ ID No 71)

The following 2 PCR primers are synthesized as described above

5'- ttggataagc ttggcttgac -3' (P1, SEQ ID No 44), and

5'- gaccgatggg cccttggtgg a -3' (P2, SEQ ID No 45)

The synthesis of H1 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H1 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B2, 10 pmol,
oligonucleotide C, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E, 10 pmol,
oligonucleotide F, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H2, 10 pmol,
oligonucleotide I, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K, 10 pmol,
oligonucleotide L, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C

for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The DNA bands corresponding to H1 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The synthesis of H3 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H3 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B, 10 pmol,
oligonucleotide C, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E2, 10 pmol,
oligonucleotide F, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H, 10 pmol,
oligonucleotide I, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K2, 10 pmol,
oligonucleotide L, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,

10 X Pyrobest buffer II, 10 µl,

dNTP mix, 8 µl,

Pyrobest DNA polymerase, 0 5 µl, and

Redistilled water to a final volume of 50 µl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 µg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The DNA bands corresponding to H3 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 µl of distilled water

The synthesis of H4 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H4 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,

oligonucleotide B, 10 pmol,

oligonucleotide C2, 10 pmol,

oligonucleotide D, 10 pmol,

oligonucleotide E2, 10 pmol,

oligonucleotide F, 10 pmol,

oligonucleotide G, 10 pmol,

oligonucleotide H, 10 pmol,

oligonucleotide I2, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K2, 10 pmol,
oligonucleotide L, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The DNA bands corresponding to H4 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit. After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The synthesis of M type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of chimeric TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The M type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B3, 10 pmol,

oligonucleotide C2, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E3, 10 pmol,
oligonucleotide F2, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H3, 10 pmol,
oligonucleotide I2, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K3, 10 pmol,
oligonucleotide L2, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The DNA bands corresponding to M type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The resulting, each extracted DNA (H1 type, H3 type, H4 type, and M type) is cloned using pGEM-T Easy vector (Promega) as follows

The DNA fragment recovered from the PCR reaction (H1, H3, H4 or M), 5 µl,

10 X Taq polymerase buffer, 1 µl,

dNTP mixture, 1 µl

Taq polymerase (5 unit/ml), 1 µl, and

redistilled water to a final volume of 10 µl.

After the above each solution is reacted at 70°C for 30 minutes, each DNA solution and pGEM-T Easy vector are ligated using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) using the manufacturer's protocol

After 4 hours incubation at 15°C, 2 µl of the incubated reaction solution is mixed with 100 µl of competent *E coli* strain JM109 at a cell density of 1-2 x 10^9 cells/ml (Takara Shuzo Co , Ltd.), and the mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 1 minute Then, 500 µl of SOC medium (2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM w/v potassium chloride, 1 mM magnesium chloride, and 20 mM glucose) is added the mixture, which is incubated for a further hour, with shaking Transformant strains are then isolated, and plasmid DNA is prepared from the strains as described in "Molecular Cloning A Laboratory Manual" The nucleotide sequence of this DNA encoding the heavy chain of humanized or mouse TRA-8 is confirmed by the dideoxy method, respectively (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmid is designated pHA15 (the plasmid carrying cDNA encoding the H1-type heavy chain of humanized TRA-8), pHC10 (the plasmid carrying cDNA encoding the H3-type heavy chain of humanized TRA-8), pHD21 (the plasmid carrying cDNA encoding the H4-type heavy chain of humanized TRA-8), and pM11 (the plasmid carrying cDNA encoding the heavy chain of chimeric TRA-8) The transformant *E coli* strains harboring these plasmid, designated as *E coli* JM109/pHA15, *E coli* JM109/pHC10, *E coli* JM109/pHD21, and *E coli* JM109/pM11 were deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science

and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7555, FERM BP-7557, FERM BP-7558, and FERM BP-7559, respectively

(3) Construction of Expression Plasmids Carrying the Heavy Chain Variable Region DNA of Several Types Humanized or Mouse TRA-8

Recombinant expression vector for animal cells are constructed by inserting the DNA encoding the heavy chain of H1 type, H3 type, and H4 type humanized or M type chimeric TRA-8 (cloned in above) as follows

One μg of plasmid pSRHHH3 (European patent application EP 0 909 816 A1) carrying the heavy chain variable region of humanized anti-Fas monoclonal antibody HFE7A and human IgG1 constant region genomic DNA, an expression vector for mammalian cells, is digested with the restriction enzymes HindIII and ApaI, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The vector DNA bands containing human IgG1 constant region genomic DNA without the heavy chain variable region of humanized HFE7A are cut out using a razor blade and eluted from the gel using Geneclean Spin Kit After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water and then dephosphorylated using CIP The resulting digested, dephosphorylated plasmid (100 ng) is ligated with 1 μg of the DNA fragment of pHA15, pHC10, pHD21, or pM11 containing the DNA encoding the heavy chain variable region of humanized or chimeric TRA-8, which had also been digested with HindIII and ApaI, using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) The ligation mixture is then used to transform *E coli* JM109, which is then plated on LB agar plates containing 50 μg/ml ampicillin

The transformants obtained by this method are cultured in 2 ml of liquid LB medium containing 50 μg/ml ampicillin at 37°C overnight, and plasmid

DNA is subsequently extracted from the resulting culture by the alkaline-SDS method

The extracted plasmid DNA is digested with HindIII and ApaI, and subjected to 3% w/v agarose gel electrophoresis to confirm the presence or absence of the insert of the DNA encoding the heavy chain variable region of humanized or chimeric TRA-8 The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems) The resulting expression plasmids carrying cDNA encoding the heavy chain of humanized or chimeric TRA-8 were designated pHA15-1, pHC10-3, pHD21-1, and pM11-1, respectively

(4) Construction of Vectors for the Humanized Light Chains

(4 1) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM1 type)

As shown in SEQ ID No 72 of the Sequence Listing, other humanization (LM1 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 3rd amino acid (valine), 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd (serine), 60th amino acid (aspartic acid), 63rd amino acid (threonine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine),and 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with glutamine, proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, serine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine respectively The resulting sequence is designated LM1

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM1 type, SEQ ID No 72 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized TRA-8 (LM1 type)

DNA coding for the LM1 polypeptide chain (SEQ ID No 72 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain (LM1 type) and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), HKCDF11 (SEQ ID No 50), HKCDR12 (SEQ ID No 51), HKCDF22 (SEQ ID No 52), HKCDR22 (SEQ ID No 53), and HKCF12 (SEQ ID No 54)

The following oligonucleotide primers are synthesized for PCR

5'- gtcccccaca gatgcagaca aagaacttgg agattgggtc atctgaatgt caccagtgga -3' (HKSPR12, SEQ ID No 77)

2) Construction of plasmid pCR3 1/LM1-2 (cloning of humanized TRA-8 light chain type LM1)

LM1-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 72 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM1-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA, 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR12, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

amplıTaq DNA polymerase (PerkınElmer), 2 5 unıts

The reactıon solutıon havıng the above compositıon ıs adjusted to a final volume of 50 µl by addıng redıstılled water and used ın PCR.

PCR thermal condıtıons Heatıng at 94°C for 2 mınutes, after whıch a thermal cycle of 94°C for 1 mınute, 55°C for 1 mınute and 72°C for 2 mınutes, repeated 30 tımes, followed by heatıng at 72°C for 10 mınutes

LM1-F2-DNA fragment codıng for a portıon of FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$ ıs prepared under the followıng condıtıons

Compositıon of the reactıon solutıon

plasmıd pL28 DNA, 25 ng

olıgonucleotıde prımer HKCDF11, 50 pmol

olıgonucleotıde prımer HKCDR12, 50 pmol

dNTPs cocktaıl, 5 µl

10×PCR buffer, 5 µl

amplıTaq DNA polymerase, 2 5 unıts

The reactıon solutıon havıng the above compositıon ıs adjusted to a final volume of 50 µl by addıng redıstılled water and used ın PCR.

PCR thermal condıtıons Heatıng at 94°C for 2 mınutes, after whıch a thermal cycle of 94°C for 1 mınute, 55°C for 1 mınute and 72°C for 2 mınutes, repeated 30 tımes, followed bv heatıng at 72°C for 10 mınutes

LM1-F3-DNA fragment codıng for CDRL2, FRL_3, and a portıon of $CDRL_3$ ıs prepared under the followıng condıtıons

Compositıon of the reactıon solutıon

plasmıd pSRPDHH DNA, 25 ng

olıgonucleotıde prımer HKCDF22, 50 pmol

olıgonucleotıde prımer HKCDR22, 50 pmol

dNTPs cocktaıl, 5 µl

10×PCR buffer, 5 µl

amplıTaq DNA polymerase, 2 5 unıts

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM1-F4-DNA fragment coding for $CDRL_3$, FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng
oligonucleotide primer HKCF12, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor

b) Second step PCR

LM1-DNA in which above described LM1-F1-DNA, LM1-F2-DNA, LM1-F3-DNA and LM1-F4-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM1-F1-DNA prepared in the first step PCR,
Gel fragment of LM1-F2-DNA prepared in the first step PCR,

Gel fragment of LM1-F3-DNA prepared in the first step PCR,
Gel fragment of LM1-F4-DNA prepared in the first step PCR
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 µl
10×PCR buffer, 5 0 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM1-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit. The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 (LM1 type) are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM1-2 (the plasmid carrying cDNA encoding the light chain variable region of humanized TRA-8 (LM1 type) and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM1-2 containing LM1-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM1-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 µg/ml chloramphenicol (final

concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM1-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M1-2-2 carrying a fusion fragment of the variable region of the humanized LM1 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M1-2-2 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7562

3) Construction of plasmid pSR/LM1-2 (expression plasmid for humanized LM1 TRA-8 light chain)

The obtained plasmid pHSG/M1-2-2 carrying a fusion fragment of the variable region of the humanized LM1 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M1-2-2 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM1 TRA-8 is designated pSR/LM1-2

(4 2) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM3 type)

As shown in SEQ ID No 73 of the Sequence Listing, other humanization (LM3 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine), 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine, respectively The resulting sequence is designated LM3

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM3 type, SEQ ID No 73 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM3 TRA-8

DNA coding for the LM3 polypeptide chain (SEQ ID No 73 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- atctagttct cagagatgga gacagacaca atcctgctat gggtgctgct gctctgggtt ccagg -3' (MOD1F1, SEQ ID No 78),

5'- cagcacccat agcaggattg tgtctgtctc catctctgag aactagatga gaggatgctt cttaagctt -3' (MOD1R1, SEQ ID No 79),

5'- ctccactggt gacattgtga tgacccaatc tccaagttct ttgtctgcat ctgtgggggga cagggtc -3' (MOD1F22, SEQ ID No 80),

5'- acttggagat tgggtcatca caatgtcacc agtggagcct ggaacccaga gcag-3' (MOD1R22, SEQ ID No 81),

5'-accatcacct gcaaggccag tcaggatgtg ggtactgctg tagcctggta ccaacagaaa ccaggaa -3' (MOD1F3, SEQ ID No 82),

5'- tacagcagta cccacatcct gactggcctt gcaggtgatg gtgaccctgt cccccacaga tgcagacaaa ga -3' (MOD1R3, SEQ ID No 83),

5'- aagcacccaa actcctcatc tattgggcat ccacccggca cactggggtc ccagataggt ttacaggcag t -3' (MOD1F42, SEQ ID No 84),

5'- cccagtgtgc cgggtggatg cccaatagat gaggagtttg ggtgcttttc ctggtttctg ttggtaccag gc -3' (MOD1R4, SEQ ID No 85),

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcaacc tat -3'(MOD1F5, SEQ ID No 86),

5'- actagagatg gtgagggtga agtctgtccc agacccactg cctgtaaacc tatctgggac -3' (MOD1R52, SEQ ID No 87),

5'- tactgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3' (MOD1F6, SEQ ID No 88),

5'- cgtccgatag ctgctatatt gctgacagta ataggttgca aaatcctccg gctgcac -3' (MOD1R6, SEQ ID No 89)

5'- aaacggactg tggctgcacc atctgtcttc atcttcccgc catctgatga g -3' (MOD1F7, SEQ ID No 90),

5'- gaagatgaag acagatggtg cagccacagt ccgtttgatt tccaccttgg tgccttgacc gaa -3' (MOD1R7, SEQ ID No 91), and

5'- agatttcaac tgctcatcag atggcgggaa (LR17, SEQ ID No 101)

2) Construction of plasmid pCR3 1/LM3-3-44 (cloning of humanized TRA-8 light chain type LM3)

LM3-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 73 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM3-F31B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1 $CDRL_1$, FRL_2, and $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol
oligonucleotide primer MOD1R1, 5 pmol
oligonucleotide primer MOD1F22, 5 pmol
oligonucleotide primer MOD1R22, 5 pmol
oligonucleotide primer MOD1F3, 5 pmol
oligonucleotide primer MOD1R3, 5 pmol
oligonucleotide primer MOD1F42, 5 pmol
oligonucleotide primer MOD1R4, 5 pmol
oligonucleotide primer MOD1F5, 5 pmol
oligonucleotide primer MOD1R52, 5 pmol
oligonucleotide primer MOD1F6, 5 pmol
oligonucleotide primer MOD1R6, 5 pmol
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer MOD1R7, 5 pmol
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer LR17, 50 pmol
dNTPs cocktail, 5 µl
10× PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 ul by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM3-F31C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

The template plasmids, pSRPDHH, is obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer MOD1F7, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light. The respective DNA bands thus detected are excised with a razor

b) Second step PCR

LM3-DNA in which above described LM3-F31B-DNA, and LM3-F31C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM3-F31B-DNA prepared in the first step PCR,
Gel fragment of LM3-F31C-DNA prepared in the first step PCR,
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 µl
10× PCR buffer, 5 0 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM3-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized LM3 TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM3-3-44 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM3 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM3-3-44 containing LM3-DNA fragment is digested with the restriction enzymes Hind III and EcoR I.

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM3-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 µg/ml chloramphenicol (final

concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM3-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M3-3-22 carrying a fusion fragment of the variable region of the humanized LM3 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M3-3-22 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7564

3) Construction of plasmid pSR/LM3-3-44-10 (expression plasmid for humanized LM3 TRA-8 light chain)

The obtained plasmid pHSG/M3-3-22 carrying a fusion fragment of the variable region of the humanized LM3 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M3-3-22 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by

DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM3 TRA-8 is designated pSR/LM3-3-44-10

(4.3) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM4 type)

As shown in SEQ ID No 74 of the Sequence Listing, other humanization (LM4 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, threonine, glutamine, valine and threonine respectively The resulting sequence is designated LM4

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM4 type) (SEQ ID No 74 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM4 TRA-8

DNA coding for the LM4 polypeptide chain (SEQ ID No 74 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79), MOD1F22 (SEQ ID No 80),

MOD1R22 (SEQ ID No 81), MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1F5 (SEQ ID No 86), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), and MOD1R7 (SEQ ID No 91), LR17 (SEQ ID No 101), the following oligonucleotide primers are synthesized for PCR.

5'- ttctgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3' (MOD1F62, SEQ ID No 92)

5'- cgtccgatag ctgctatatt gctgacagaa ataggttgca aaatcctccg gctgcag -3' (MOD1R62, SEQ ID No 93)

2) Construction of plasmid pCR3 1/LM4-5-3 (cloning of humanized TRA-8 light chain type LM4)

LM4-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 74 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM4-F41B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol
oligonucleotide primer MOD1R1, 5 pmol
oligonucleotide primer MOD1F22, 5 pmol
oligonucleotide primer MOD1R22, 5 pmol
oligonucleotide primer MOD1F3, 5 pmol
oligonucleotide primer MOD1R3, 5 pmol
oligonucleotide primer MOD1F42, 5 pmol
oligonucleotide primer MOD1R4, 5 pmol
oligonucleotide primer MOD1F5, 5 pmol
oligonucleotide primer MOD1R52, 5 pmol
oligonucleotide primer MOD1F62, 5 pmol

oligonucleotide primer MOD1R62, 5 pmol
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer MOD1R7, 5 pmol
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer LR17, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM4-F41C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

The template plasmids, pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 μg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM4-DNA in which above described LM4-F41B-DNA, and LM4-F41C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution.

Gel fragment of LM4-F41B-DNA prepared in the first step PCR,
Gel fragment of LM4-F41C-DNA prepared in the first step PCR,
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 μl
10×PCR buffer, 5 0 μl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 ul by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM4-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit. The nucleotide sequences of these DNAs encoding the light chain of humanized LM4 TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al., (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM4-5-3 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM4 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM4-5-3 containing LM4-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM4-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphemcol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphemcol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM4-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M4-5-3-1 carrying a fusion fragment of the variable region of the humanized LM4 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M4-5-3-1 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7565

3) Construction of plasmid pSR/LM4-5-3-3 (expression plasmid for humanized LM4 TRA-8 light chain)

The obtained plasmid pHSG/M4-5-3-1 carrying a fusion fragment of the variable region of the humanized LM4 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III

and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M4-5-3-1 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E. coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM4 TRA-8 is designated pSR/LM4-5-3-3

(4 4) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM5 type)

As shown in SEQ ID No 75 of the Sequence Listing, other humanization (LM5 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, valine and threonine respectively The resulting sequence is designated LM5

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM5 type) (SEQ ID No 75 of the Sequence Listing) is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM5 TRA-8

DNA coding for the LM5 polypeptide chain (SEQ ID No 75 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79), MOD1F22 (SEQ ID No 80), MOD1R22 (SEQ ID No 81), MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), and LR17 (SEQ ID No 101), the following oligonucleotide primers are synthesized for PCR

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcagat tat -3' (MOD1F52, SEQ ID No 94)

5'- ttctgtcagc aatatagcag ctatcggacg ttcggtggag gcaccaaggt ggaaatc -3' (MOD1F63, SEQ ID No 95)

5'- cgtccgatag ctgctatatt gctgacagaa ataatctgca aaatcctccg gctgcag -3' (MOD1R63, SEQ ID No 96)

5'- gaagatgaag acagatggtg cagccacagt ccgtttgatt tccaccttgg tgccttccacc gaa-3' (MOD1R72, SEQ ID No 102)

2) Construction of plasmid pCR3 1/LM5-3-42 (cloning of humanized TRA-8 light chain type LM5)

LM5-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 75 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM5-F51B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1, $CDRL_1$, FRL_2, $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol

oligonucleotide primer MOD1R1, 5 pmol

oligonucleotide primer MOD1F22, 5 pmol

oligonucleotide primer MOD1R22, 5 pmol

oligonucleotide primer MOD1F3, 5 pmol

oligonucleotide primer MOD1R3, 5 pmol

oligonucleotide primer MOD1F42, 5 pmol

oligonucleotide primer MOD1R4, 5 pmol

oligonucleotide primer MOD1F52, 5 pmol

oligonucleotide primer MOD1R52, 5 pmol

oligonucleotide primer MOD1F63, 5 pmol

oligonucleotide primer MOD1R63, 5 pmol

oligonucleotide primer MOD1F7, 50 pmol

oligonucleotide primer MOD1R72, 5 pmol

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer LR17, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM5-F51C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions The template plasmids, pSRPDHH, is obtained by following the description in an European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer MOD1F7, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light. The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM5-DNA in which above described LM5-F51B-DNA, and LM5-F51C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM5-F51B-DNA prepared in the first step PCR,

Gel fragment of LM5-F51C-DNA prepared in the first step PCR,

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 0 µl

10×PCR buffer, 5 0 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM5-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized LM5 TRA-8 are confirmed by the dideoxy method (Sanger, F S, et al, (1977), Proc Natl Acad Sci USA, 74 5463-5467) using DNA analyzer

The resulting plasmids are designated pCR3 1/LM5-3-42 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM5 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM5-3-42 containing LM5-DNA fragment is digested with the restriction enzymes Hind III and EcoR I.

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM5-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM5-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M5-3-27 carrying a fusion fragment of the variable region of the humanized LM5 TRA-8 light chain and the constant region of human Igκ chain is obtained

3) Construction of plasmid pSR/LM5-3-27-1 (expression plasmid for humanized LM5 TRA-8 light chain)

The obtained plasmid pHSG/M5-3-27 carrying a fusion fragment of the variable region of the humanized LM5 TRA-8 light chain and the constant

region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M5-3-27 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer, Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM5 TRA-8 is designated pSR/LM5-3-27-1

(4 5) Construction of an Expression Vector for the Light Chain of the Humanized Antibody(chimera type)

The sequence shown in SEQ ID No 76 of the Sequence Listing, the amino acid sequence of the light chain of chimera type TRA-8, is designated LM6

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM6 type) (SEQ ID No 75 of the Sequence Listing) is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM6 TRA-8

DNA coding for the LM6 polypeptide chain (SEQ ID No 75 of the Sequence Listing), each of which is a fusion of the variable region of mouse anti-DR5 antibody TRA-8 light chain (LM6 type) and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- tgatgtggac atgaatttgt gagactgggt catcacaatg tcaccagtgg a -3' (HKSPR13, SEQ ID No 97),

5'- tgggttccag gctccactgg tgacattgtg atgacccagt ctcacaaatt c -3' (MVF11, SEQ ID No 98),

5'- aagacagatg gtgcagccac agcccgtttg atttccagct tggtgcctc -3' (MVR11, SEQ ID No 99), and

5'- aagctggaaa tcaaacgggc tgtggctgca ccatctgtct tcatc -3'(MCF11, SEQ ID No 100)

2) Construction of plasmid pCR3 1/LM6-1-16 (cloning of humanized TRA-8 light chain type LM6)

LM6-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 75 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM6-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA, 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR13, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

ampliTaq DNA polymerase (PerkinElmer), 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM6-F2-DNA fragment coding for a portion of FRL_1, $CDRL_1$, FRL_2, $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

plasmid pL28 DNA, 25 ng

oligonucleotide primer MVF11, 50 pmol

oligonucleotide primer MVR12, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM6-F3-DNA fragment coding for a portion of FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer MCF11, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 μg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM6-DNA in which above described LM6-F1-DNA, LM6-F2-DNA, and LM6-F3-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM6-F1-DNA prepared in the first step PCR,
Gel fragment of LM6-F2-DNA prepared in the first step PCR,
Gel fragment of LM6-F3-DNA prepared in the first step PCR,
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 μl
10×PCR buffer, 5 0 μl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 μl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM6-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (Invitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 are confirmed by the dideoxy method using a DNA analyzer

The resulting plasmids are designated pCR3 1/LM6-1-16 (the plasmid carrying cDNA encoding the light chain variable region of mouse TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM6-1-16 containing LM6-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM6-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM6-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M6-1-4-1 carrying a fusion fragment of the variable region of the mouse TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M6-1-4-1 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7566

3) Construction of plasmid pSR/LM6-1-4-6(expression plasmid for chimera type LM6 TRA-8 light chain)

The obtained plasmid pHSG/LM6-1-4-1 carrying a fusion fragment of the variable region of the mouse TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/LM6-1-4-1 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd.) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method. The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer

The resulting expression plasmid carrying cDNA encoding the light chain of TRA-8 (chimera type) is designated pSR/LM6-1-4-6

(5) Production of several types-Humanized or chimeric TRA-8 Antibody

Transfection of COS-7 cells is conducted by FUGENE6 transfection reagent methods (Boehringer Mannheim Biochemica) according to the instruction manual provided with the kit

COS-7 cells (American Type Culture Collection No CRL-1651) are grown to semi-confluent (3 x 10^6 cells/dish) in a culture dish (culture area 57 cm^2, Sumitomo Bakelite) containing Dulbecco's Modified Eagle medium (hereinafter referred to as "D-MEM", Gibco BRL) supplemented with 10% fetal calf serum (hereinafter abbreviated as "FCS", Moregate)

In the meantime, 10 μg/dish (total 5 dishes) of the humanized DR5 heavy chain expression plasmid DNA (pHA15-1) and 10 μg/dish of the humanized DR5 light chain expression plasmid DNA prepared by the alkaline-SDS method and cesium chloride density gradient centrifugation are mixed, and then precipitated with ethanol, followed by suspending in 5 μl/dish of dH_2O

After 15 μl/dish of FUGENE6 Transfection regent is mixed with 180 μl/dish D-MEM without FCS, this FUGENE solution (185 μl/dish) is mixed with 5 μl/dish DNA solution containing 10 μg/dish of the humanized DR5 heavy chain expression plasmid DNA and 10 μg/dish of the humanized DR5 light chain expression plasmid DNA After 15 minutes incubation at room

temperature, the obtained plasmid suspension (200 µl) is added to the previously prepared COS-7 plates After incubating in 5% CO_2 at 37°C for 24 hours, the culture medium is changed with D-MEM without FCS After incubating in 5% CO_2 at 37°C for 72 hours, the culture supernatant is recovered to purify the expression products in the supernatant fluids By the method as described above, COS-7 cells are transfected with each of the following plasmid combinations

(A) cotransfection of pHA15-1 and pSR/LM1-2 (H1L1)

(B) cotransfection of pHB14-1 and pSR/M2-1 (H2L2)

(C) cotransfection of pHB14-1 and pSR/LM3-3-44-10 (H2L3)

(D) cotransfection of pHB14-1 and pSR/LM4-5-3-3 (H2L4)

(E) cotransfection of pHC10-3 and pSR/M2-1 (H3L2)

(F) cotransfection of pHC10-3 and pSR/LM3-3-44-10 (H3L3)

(G) cotransfection of pHC10-3 and pSR/LM4-5-3-3 (H3L4)

(H) cotransfection of pHD21-1 and pSR/LM5-3-27-1 (H4L5)

(I) cotransfection of pM11-1 and pSR/LM6-1-4-6 (Chimera)

The culture is then centrifuged (3,500 r p m., 15 minutes) and collected the supernatant The supernatant is filtrated with 0 45 µm filter (ADVANTEC TOYO DISMIC-25cs, Cat # 25CS045 AS) The purification of IgG from the filtrates are achieved using Protein G-POROS affinity chromatography (Applied Biosystems) under the following conditions

HPLC system BioCAD 700E (Applied Biosystems)

column ProteinG-ID sensor cartridge

(column size 2 1 mmID x 30 mm LD, bed volume 0 1 ml, Applied Biosystems)

elution buffer 0 1 M Glycine-HCl (pH 2 5)

neutralization buffer 1 M Tris-HCl (pH 8 5)

detection 280 nm

flow rate 1 ml/min

fraction size 0 5 ml/0 5 min

fraction tube 1 5 ml polypropylene microtube

temperature 4°C

After all the filtrates are applied to column, 50 ml of PBS (Sigma, Cat # 1000-3) is used to wash column When the elution buffer is applied, fraction collector started Each fraction microtube previously contained 55 µl of 1 M NaCl, 110 µl of neutralization buffer and 74 µl of 2 mg/ml bovine serum albumin (Sigma, Cat # A-7030) in PBS The fractions from No 7 through No 8 are collected

Verification of the expression of the humanized antibodies and quantitative assay of the expression products in the culture supernatant fluids prepared is performed by ELISA with an antibody against anti-human IgG

To each well of a 96-well plate (MaxiSorp, Nunc), 100 µl of goat anti-human IgG Fc specific polyclonal antibody (Kappel) dissolved at the final concentration of 0 5 µg/ml in adsorption buffer (0 05 M sodium hydrogencarbonate, 0 02% sodium azide, pH 9 6) is added and the plate is incubated at 37°C for 2 hours to cause adsorption of the antibody Then, the plate is washed with 350 µl of PBS-T five times To the wells after washing, the culture supernatant diluted with D-MEM containing 10% FCS is added and incubated at 37°C for 2 hours After washing again with PBS-T, 100 µl of alkaline phosphatase-labeled goat anti-human IgG Fc specific polyclonal antibody (Jackson Immuno Research Lab) diluted 10,000-fold with PBS-T is added to each well and incubated at 37°C for 2 hours After washing again with PBS-T, a substrate solution of p-nitrophenyl phosphate obtained from Alkaline Phosphatase Substrate kit (Bio Rad) is added according to the instruction manual provided with the kit After incubating at 37°C for 0 5 to 1 hour, the absorbance at 405 nm is measured. In the present experiments, human plasma immunoglobulin G subclass 1 (IgG1) (Biopure AG) diluted with D-MEM containing 10% FCS to certain concentrations is used as concentration reference samples of the humanized DR5 antibodies contained in the culture supernatant fluids

As a result, the expression and purified products in the culture supernatant are detected specifically with the anti-human IgG antibody The

final concentration of human IgG antibody is 44 03 µg/ml (H1L1), 39 8 µg/ml (H2L2), 26 7 µg/ml (H2L3), 41 0 ug/ml (H2L4), 39 3 µg/ml (H3L2), 24 7 µg/ml (H3L3), 21 5 µg/ml (H3L4), 16 7 ug/ml (H4L5) and 18 3 µg/ml (chimera), respectively

(6) Apoptosis-inducing activity of several types Humanized Antibody or Chimeric Antibody

Jurkat cells (ATCC No TIB-152), are used to examine the apoptosis-inducing activity of the purified humanized TRA-8 antibody

Jurkat cells cultured in RPMI1640 medium with 10% FCS (Gibco BRL) at 37°C for 3 days in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) at 50 µl per well The humanized TRA-8 prepared in this Example 26 are adjusted to have the concentration of the final product of interest of 100 ng/ml with RPMI1640 medium containing 10% FCS by estimating their concentrations in the fluids according to the method described in Example 26 Each of the solutions of the expression products thus adjusted to 100 ng/ml is used to produce serial dilutions by repeating serial 2-fold dilution with RPMI1640 containing 10% FCS Each of the diluted humanized TRA-8 solution (H1L1, H2L2, H2L3, H2L4, H3L3, H3L4 or H4L5) is added to each well at 50 µl per well After reacting at 37°C for 12 hours, 50 ul of 25 uM PMS containing 1 mg/ml XTT is added (final concentrations of 250 ug ml for XTT and 5 uM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula.

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells and "c" is the measurement of a well with no antibody added.

As a result, the tested humanized antibodies are demonstrated to induce apoptosis in cells of T lymphoma cell line expressing human DR5 antigen.

Furthermore, the apoptosis-inducing activity of humanized TRA-8 to PC-3 is examined by adding taxol according to the method described in Example 25

Human prostate cancer cell line PC-3 (ATCC No CRL-1435) is obtained from American Tissue Culture Collection (ATCC) and maintained in F-12K Nutrient Mixture (21127-022, Gibco BRL) containing 10% fetal bovine serum (FBS, Hyclone), 1% L-Glutamine-200 mM (25030-149, Gibco BRL) and 0 5% Penicillin Streptomycin Solution (P-7539, Sigma) RPMI1640 medium (MED-008, IWAKI) supplemented with 10% FBS and 0 5% Penicillin Streptomycin Solution is used in the following experiment. Exponentially growing PC-3 cells are collected by trypsinization and washed twice with fresh medium. The cells are then counted, resuspended in fresh medium at a density of 5 x 10^4 cells/ml and distributed in triplicate into flat-bottomed 96 well plates (3598 Corning-Coster) in a total volume of 100 ul/well one day before the start of the experiment A representative anti-cancer drug, Paclitaxel (169-18611, Wako) dissolved in dimethylsulfoxide (10 mg/ml) is diluted in fresh medium and then added to the 96-well plates containing the cells at 50 µl/well The final concentrations of dimethylsulfoxide are less than 0 1% After incubation for 24 hr at 37°C in 5% CO_2 atmosphere, humanized TRA-8 antibody (H1L1, H1L2 H2L3, H2L4, H3L2, H3L3, H3L4 or H4L5) diluted in fresh medium is added to the wells After incubation for a further 24 hr, 50 ul of Minimum Essential Medium (11095-098, Gibco BRL) containing 1 mg/ml of XTT and 25 mM of PMS is added to the wells and the plates are incubated for 6 hr OD450 is then measured by ARVO HTS 1420 Multilabel Counter (Wallac Berthold) and the cell viability is calculated as follows

Cell viability (%) = (OD450 for the well containing cells treated with Taxol and humanized TRA-8 (agent(s)) - OD450 for the well containing neither cells nor agent) x 100 / (OD450 for the well containing cells with no agent –OD450 for the well containing neither cells nor agent)

As a result, the tested humanized antibodies are demonstrated to induce apoptosis in human prostate cancer cells expressing human DR5 antigen

Any patents or publications mentioned in the specification are indicative of the level of those skilled in the art to which the invention pertains These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference

The present invention is not limited in scope by the above-referenced deposit or the embodiments disclosed in the examples which are intended as illustrations of a few aspects of the invention and any embodiments which are functionally equivalent are within the scope of this invention Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims

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Claims

1 1 An antibody which recognizes a TRAIL receptor DR5, wherein
2 said antibody has apoptosis-inducing activity to a cell expressing DR5 *in vivo*

1 2 An antibody which recognizes a TRAIL receptor DR5, and
2 which does not recognize TRAIL receptors DR4, DcR1, nor DcR2

1 3 The antibody of claims 1 or 2, wherein said antibody is
2 monoclonal

1 4 The antibody of claims 1, 2, or 3, wherein the antigen of said
2 antibody is human DR5

1 5 The antibody of claims 1, 2, 3, or 4, wherein said cell is synovial
2 cells of rheumatoid arthritis

1 6 The antibody of claims 1, 2, 3, or 4, wherein said cell is
2 malignant

1 7 A monoclonal antibody which is produced by mouse-mouse
2 hybridoma TRA-8 having ATCC Accession Number PTA-1428

1 8 A process of inhibiting cell proliferation comprising the step of
2 exposing cells expressing DR5 to a therapeutic quantity of an antibody capable
3 of binding thereto along with a suitable carrier for said antibody of any of claims
4 1 to 7

1 9 A composition of any of claims 1 to 7 comprising a therapeutic
2 amount of monoclonal antibody active against a DR5, and a pharmaceutically
3 acceptable carrier

10 The pharmacological composition of claim 9 wherein said therapeutic amount is 0 1-10,000 micrograms per dosage

11 The composition of claim 10 wherein said monoclonal antibody is present at a concentration from 0 2 nanograms to 2000 nanograms per milliliter of said carrier

12 Use of an antibody of any of claims 1 to 7 for preparing a therapeutic for selective apoptosis of abnormal or dysregulated cells

13 A mobilized antibody interacting with a tumor necrosis factor ligand receptor selected from a group consisting of DR4, DR5, DrR1, DrR2 and OPG, said antibody inducing apoptosis in a cell expressing said receptor

14 A mobilized antibody capable of selectively binding an agonistic or antagonistic tumor necrosis factor ligand receptor epitope

15 A process of treating an apoptosis related disease comprising the steps of

exposing a target tissue expressing the apoptosis related disease to a therapeutic quantity of a first antibody according to claim 1 or 13

16 The process of claim 15 further comprising the step of administering a pharmaceutically effective amount of a therapeutic agent that operates synergistically with said first antibody

17 The process of claim 16 wherein said therapeutic agent is a bisindolylmaleimide

18 The process of claim 15 further comprising the steps of

exposing said tissue to a therapeutic quantity of a second antibody according to claim 13 wherein said first and said second antibodies interact synergistically with said tissue

19 The method of claim 15 further comprising the steps of exposing said tissue to a sensitizer before or during exposing said tissue to said first antibody

20 A fusion protein comprising an antigenic TRAIL receptor amino acid sequence having at least ten bases, coupled to an immunoglobulin protein or fragment thereof capable of eliciting an immune response within a subject.

21 A method of gene therapy comprising the steps of transfecting target cells with a vector comprising an expressible TRAIL receptor nucleic acid sequence, expressing on said cells a TRAIL receptor encoded for by said TRAIL receptor nucleic acid sequence, and exposing said cells to an antibody selective for binding said TRAIL receptor

22 The antibody of any of claims 1 to 7 wherein said antibody is humanized

23 A nucleotide sequence comprising a coding sequence for a selectively heavy chain immunoglobulin of an antibody capable of recognizing DR5

24 The nucleotide sequence of claim 23 depicted as nucleotides 1-1398 of SEQ ID NO 21

25 An amino acid sequence comprising a coding sequence for a TRA-8 heavy chain immunoglobulin of an antibody capable of recognizing DR5

26 The amino acid sequence of claim 25 wherein said coding sequence contains a complete open reading frame and is depicted as residues 1-464 of SEQ ID NO 23

27 A nucleotide sequence comprising a coding sequence for TRA-8 light chain immunoglobulin of an antibody capable of recognizing DR5

28 The nucleotide sequence of claim 27 depicted as nucleotides 1-705 of SEQ ID NO 22

29 An amino acid sequence comprising a coding sequence for a TRA-8 light chain immunoglobulin of an antibody capable of recognizing DR5

30 The amino acid sequence of claim 29 wherein said coding sequence contains a complete open reading frame and is depicted as residues 1-234 of SEQ ID NO 24

31 An antibody selective for human DR5 comprising a humanized heavy chain immunoglobulin and a humanized light chain immunoglobulin.

32 An amino acid sequence comprising a coding sequence for humanized heavy chain immunoglobulin of an antibody according to claim 30

33 The amino acid sequence of claim 32 depicted as residues 1-119 of SEQ ID NO 31

34 An amino acid sequence comprising a coding sequence for humanized TRA-8 light chain immunoglobulin of an antibody according to claim 30

35 The amino acid sequence of claim 34 depicted as residues 1-213 of SEQ ID NO 46

36 A nucleotide sequence comprising a coding sequence for humanized light chain immunoglobulin of an antibody according to claim 30

37 The nucleotide sequence of claim 36 depicted as nucleotides 1-711 of SEQ ID NO 55

38 A vector comprising

(a) the nucleotide sequence of at least one of claims 23 or 28, and

(b) a regulatory element operably linked to said nucleotide sequence such that said nucleotide sequence is transcribed and translated in a host

39 The vector of claim 38 selected from the group consisting of a virus and a plasmid

40 The vector of claim 39 wherein the virus is selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a herpes virus and a lentivirus

41 A plasmid comprising a nucleic acid sequence encoding a heavy or light chain humanized immunoglobulin of an antibody according to claim 31

42 A host cell transformed with the vector of claim 38

43 A host cell transformed with the plasmid of claim 41

44 The host cell of claim 43 wherein the plasmid is selected from the group consisting of pHB14, pHB14-1, pHSG/M2-1-4, pCR3 1/M2-1 and pSR/M2-1

45 A host cell producing a humanized antibody of claim 22

46 A process for producing a humanized DR5 antibody comprising the steps of

(a) transforming a host with a first vector encoding at least one of a humanized immunoglobulin light chain or humanized immunoglobulin heavy chain and a second vector encoding the light chain or heavy chain if either is absent from said first vector; and

(b) incubating the transformed host for a predetermined period of time

such that the human DR5 antibody is generated.

47 The process of claim 46 wherein the host is selected from the group consisting of eukaryotic cells and prokaryotic cells

48 The process of claim 46 wherein said eukaryotic cell is a mammalian cell

49 The process of claim 47 wherein the mammalian cell is *in vivo*

50 The process of claim 48 wherein the mammalian cell is *ex vivo*

51 The process of claim 48 wherein the mammalian cell is *in vitro*

52 A process for inhibiting cell proliferation comprising the step of contacting a target cell with a pharmaceutically effective amount of a humanized DR5 antibody

53 The process of claim 52 wherein said target cell is a human cancer cell selected from the group consisting of breast, ovary colon, hematopoietic, prostate, lymphatic, lung glioma and liver

54 The process of claim 52 further comprising the step of contacting the target cell with a pharmaceutically effective amount of a therapeutic agent

55 The process of claim 54 wherein the therapeutic agent is an anti-cancer compound

56 The process of claim 55 wherein the anti-cancer compound is selected from the group consisting of Paclitaxel, Taxol, cycloneximide, carboplatin, chlorambucil, cisplatin, colchicine, cyclophosphamide, daunorubicin, dactinomycin, diethylstilbestrol doxorubicin, etoposide, 5-fluorouracil, floxuridine, melphalan, methotrexate, mitomycin, 6-mercaptopurine, teniposide, 6-thioguanine, vincristine and vinblastine

57 The process of claim 52 wherein the target cell is a human cell

58 The process of claim 52 wherein the target cell is a non-human cell originating in an animal selected from the group consisting of non-human primate, rat mouse rabbit, goat, sheep cow, pig, horse and chicken.

59 The process of claim 52 wherein the target cell is *in vivo*

60 The process of claim 52 wherein the target cell is *ex vivo*

61 The process of claim 52 wherein the target cell is *in vitro*

62 A commercial kit for inducing cell death comprising a humanized antibody selective for human DR5 packaged in a suitable container together with instructions for use as a therapeutic effective against expressing DR5

63 A process for increasing NFκB levels in a cell comprising the step of

administering a pharmaceutically effective amount of a DR5 selectively recognizing antibody

FIG - 1A

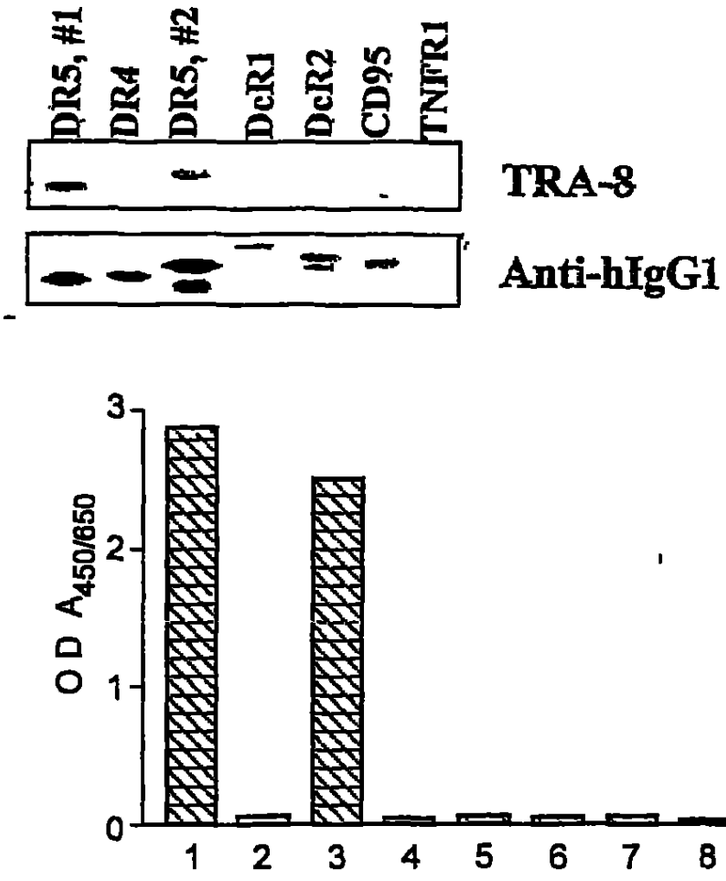

DR5, #1
DR4
DR5, #2
DcR1
DcR2
CD95
TNFR1
TRA-8
Anti-hIgG1
O D $A_{450/650}$
0
1
2
3
1
2
3
4
5
6
7
8

FIG - 1C

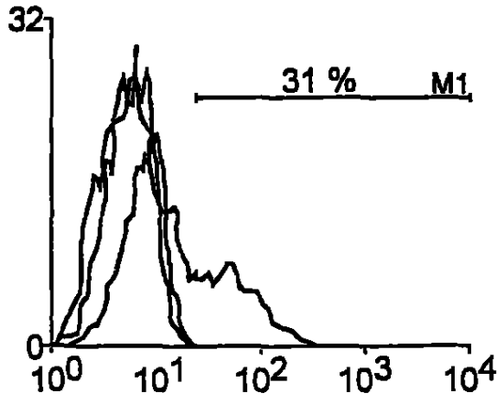

32
0
31 %
M1
10^0
10^1
10^2
10^3
10^4

FIG - 1B-1

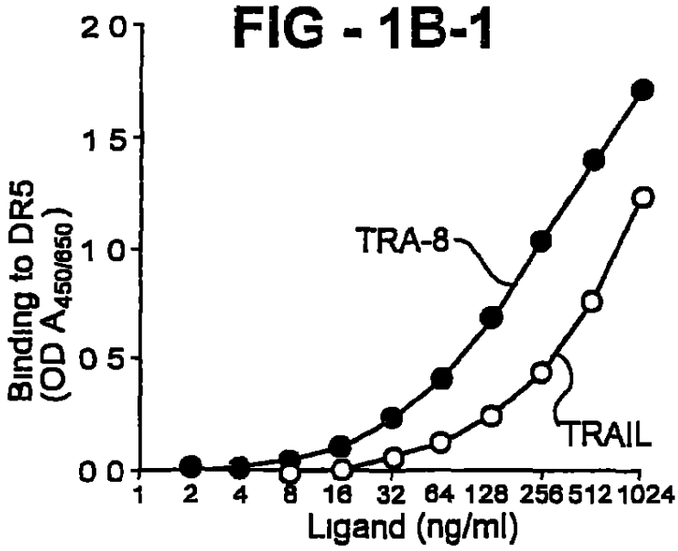
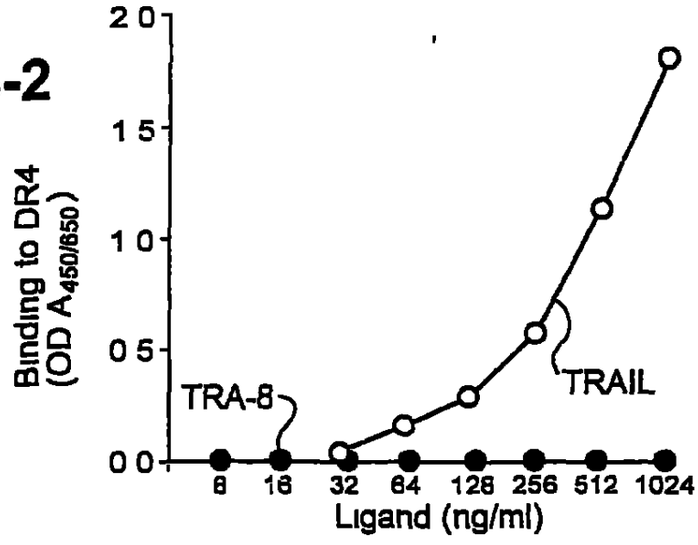


FIG - 1B-2



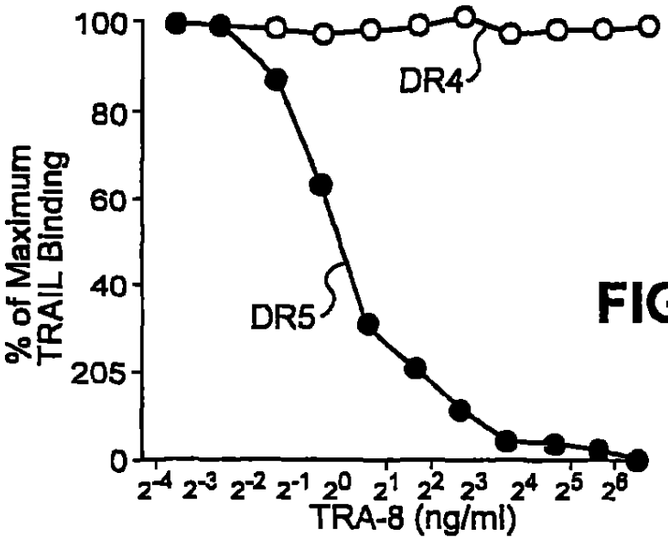


FIG - 1B-3

FIG - 1D-1

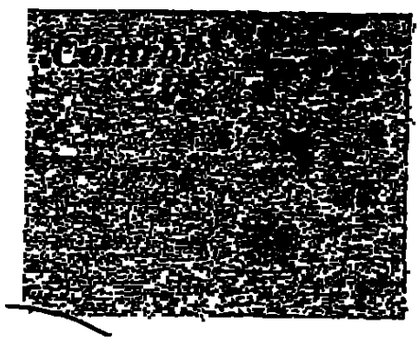


FIG - 1D-2

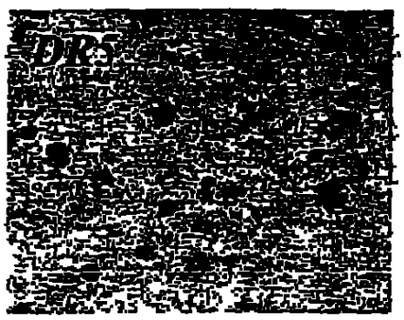


FIG - 1E

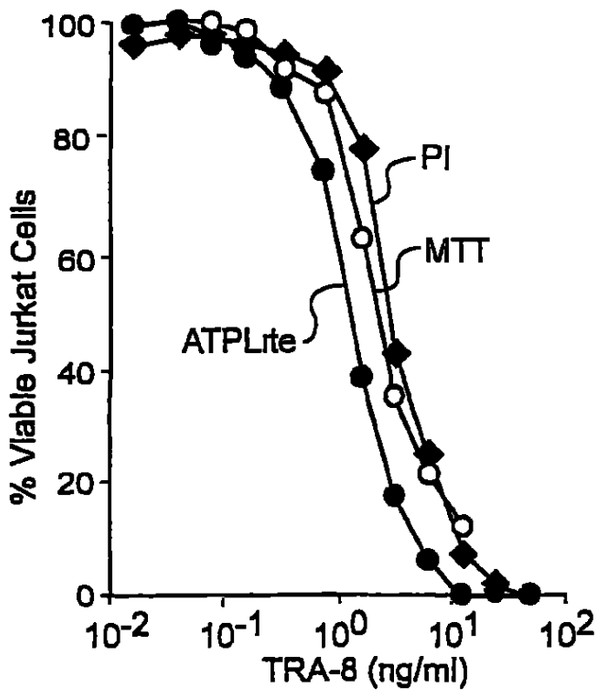


FIG - 1F

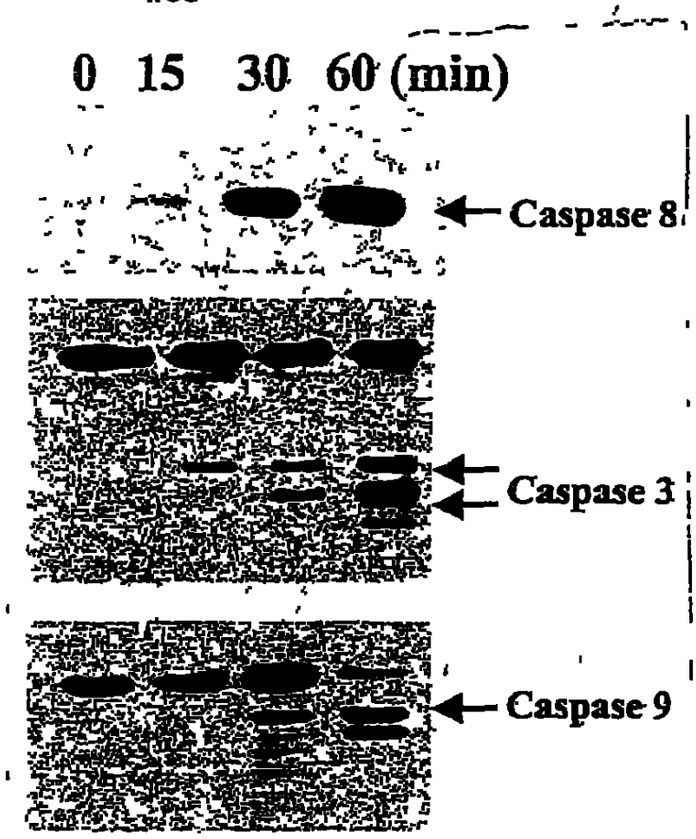

0 15 30 60 (min)
Caspase 8
Caspase 3
Caspase 9

FIG - 1G

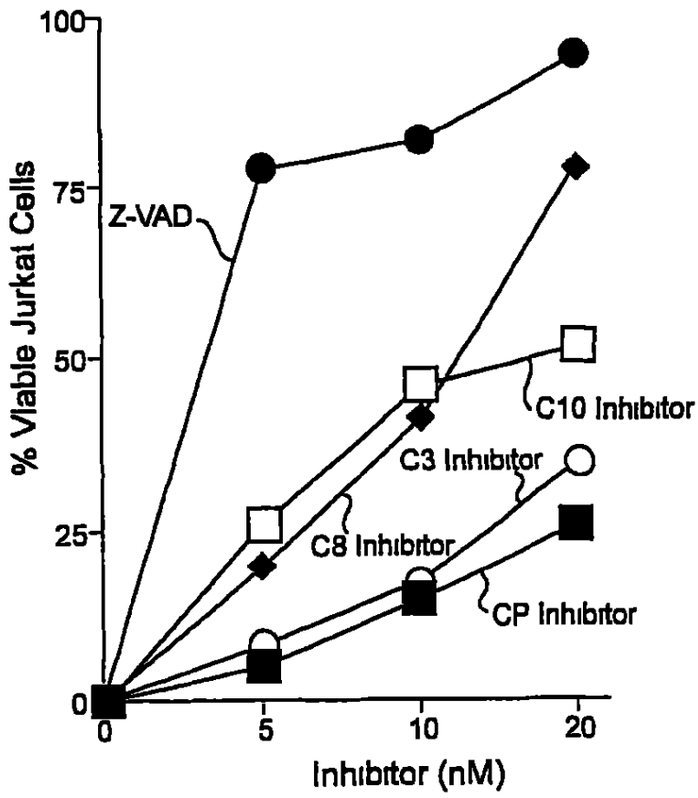

100
75
50
25
0
% Viable Jurkat Cells
Z-VAD
C10 Inhibitor
C3 Inhibitor
C8 Inhibitor
CP Inhibitor
0
5
10
20
Inhibitor (nM)

FIG - 2A-1

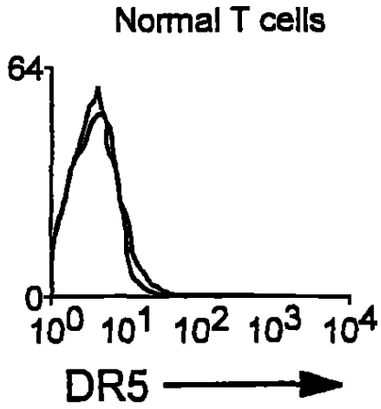


FIG - 2A-2

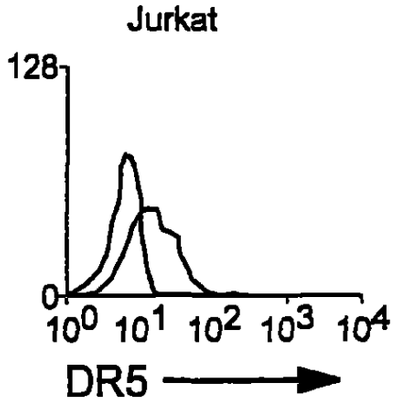
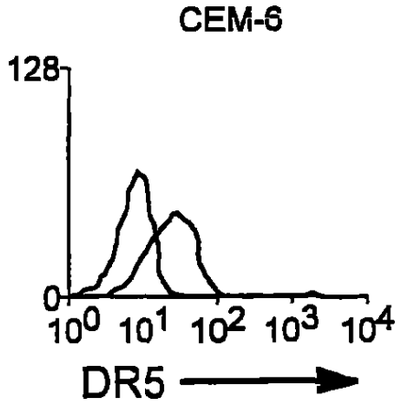


FIG - 2A-3



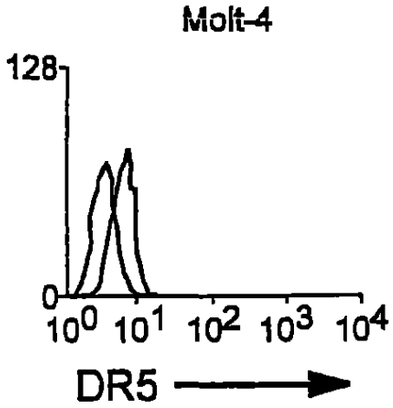


FIG - 2A-5

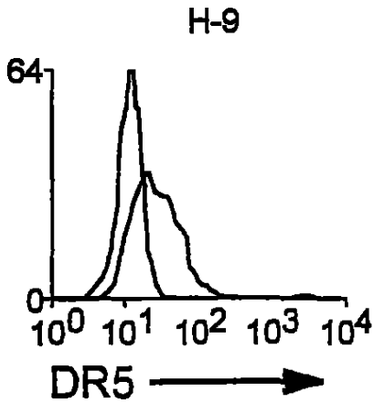


FIG - 2A-6



FIG - 2A-7

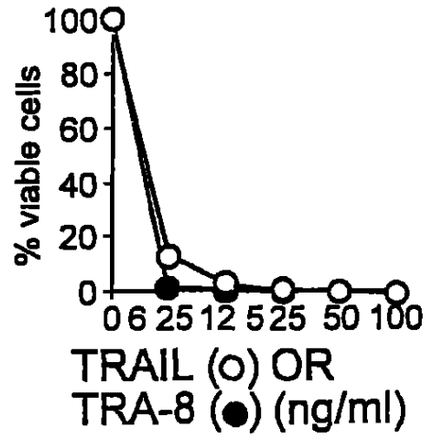


FIG - 2A-8

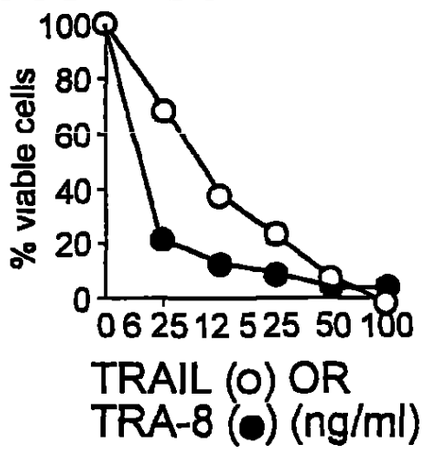


FIG - 2A-9

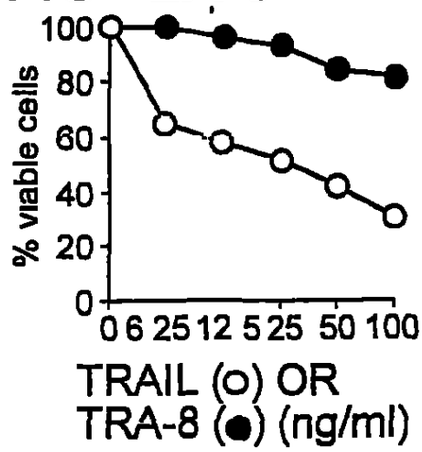


FIG - 2A-10

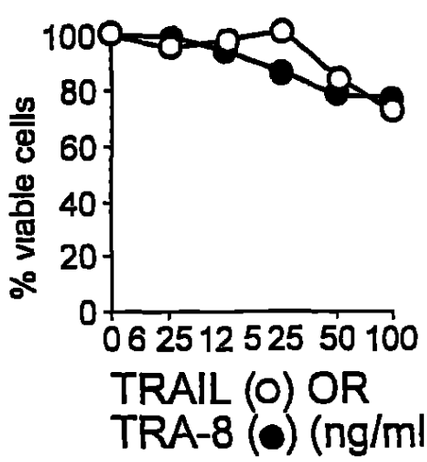


FIG - 2A'

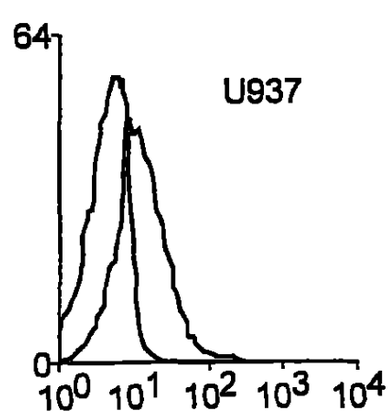


FIG - 2B-1

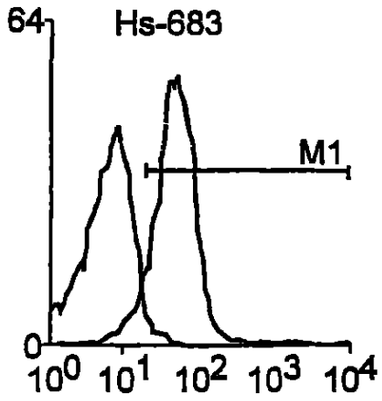


FIG - 2B-2

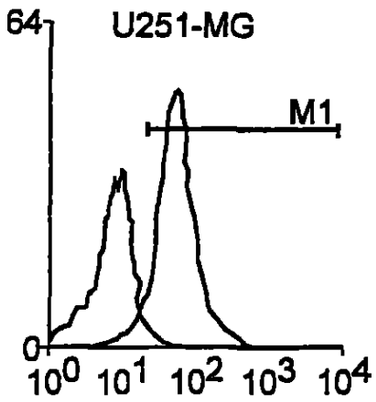


FIG - 2B-3

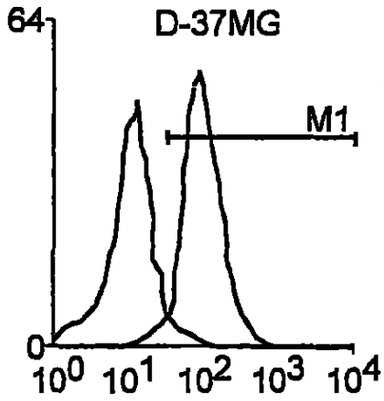


FIG - 2B-4

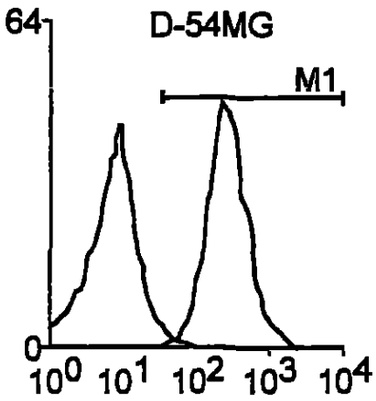


FIG - 2B-5

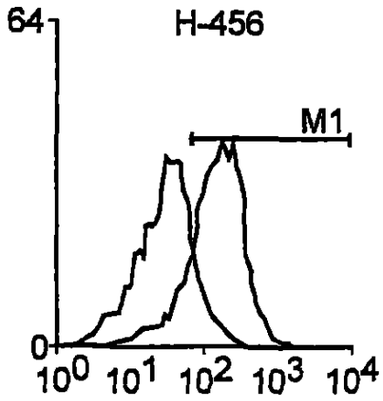


FIG - 2B-6

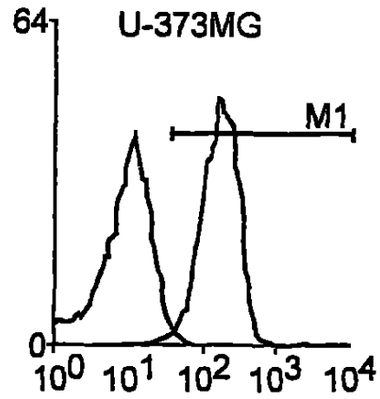


FIG - 2B-7

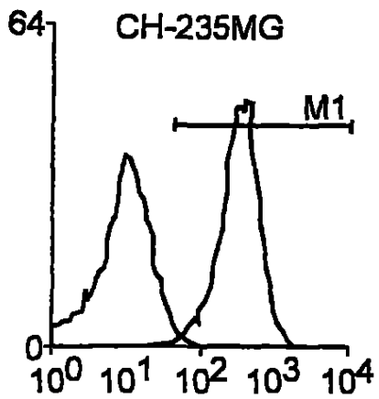


FIG - 2B-8

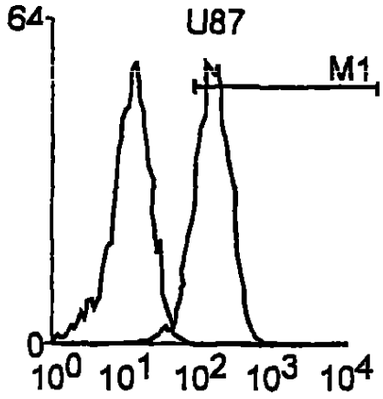


FIG - 2B-9

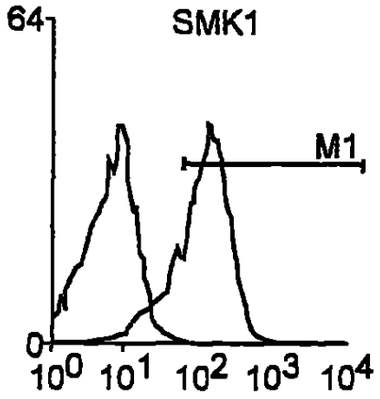


FIG - 2B-10

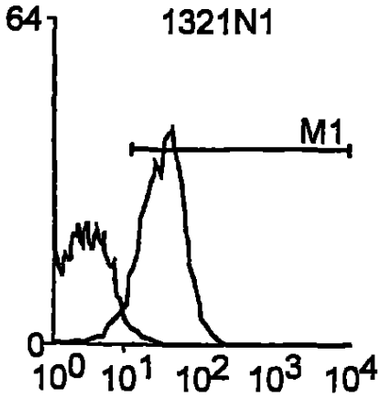


FIG - 2B'-1

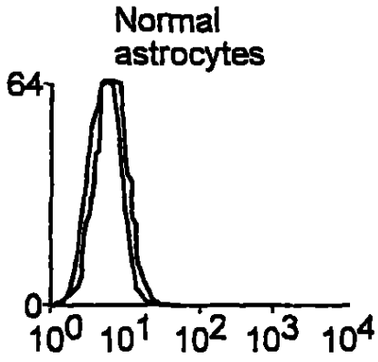


FIG - 2B'-2

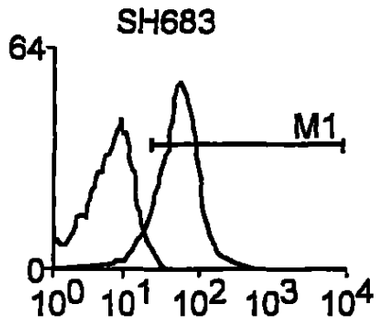


FIG - 2B'-3

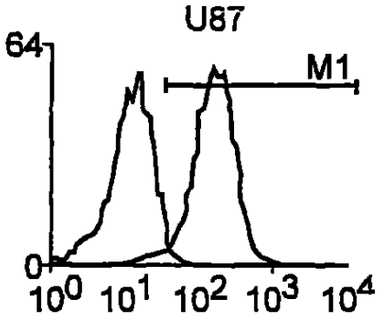


FIG - 2B'-4

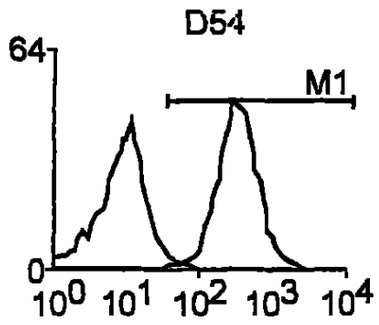


FIG - 2B'-5

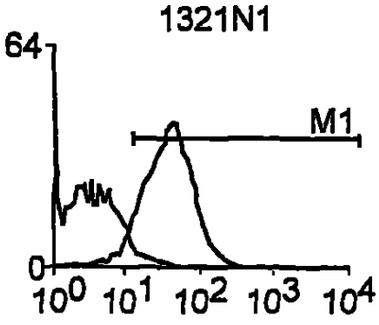


FIG - 2B'-6



FIG - 2B'-7

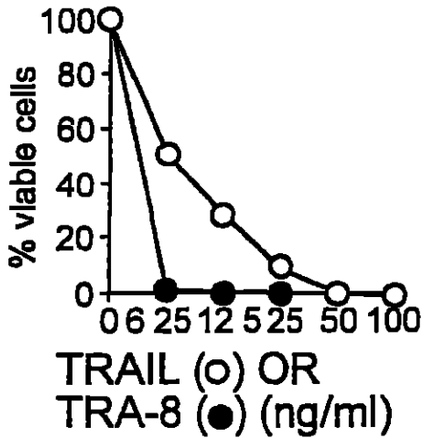


FIG - 2B'-8

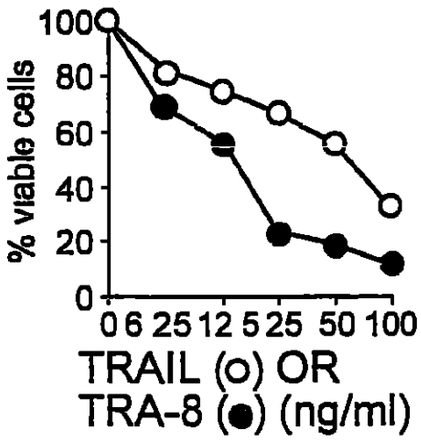


FIG - 2B'-9

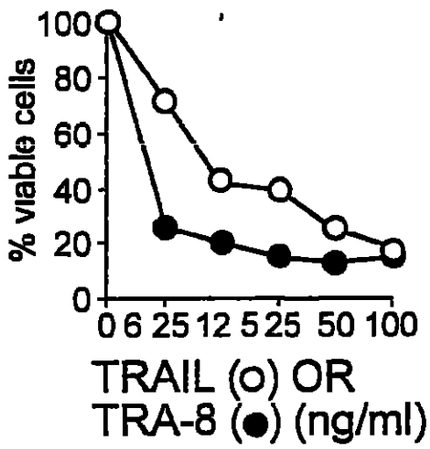


FIG - 2B'-10

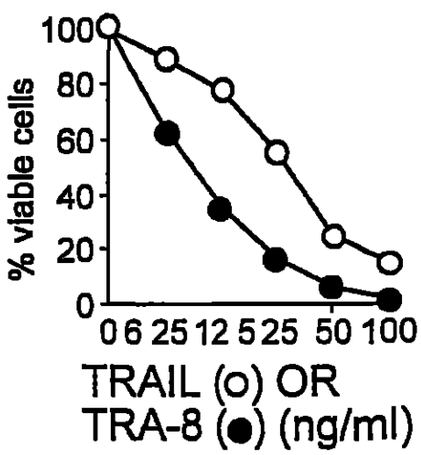


FIG - 2C-1

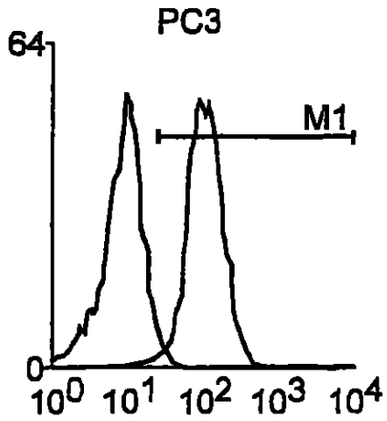


FIG - 2C-2

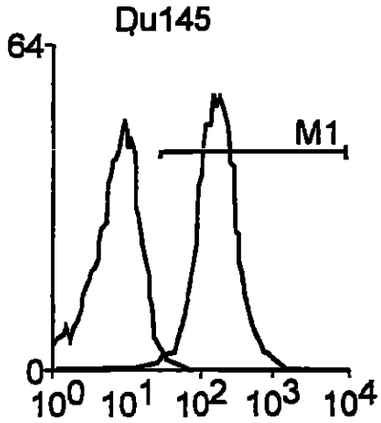


FIG - 2C-3

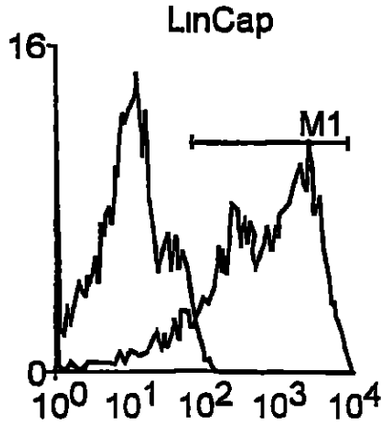


FIG - 2D-1

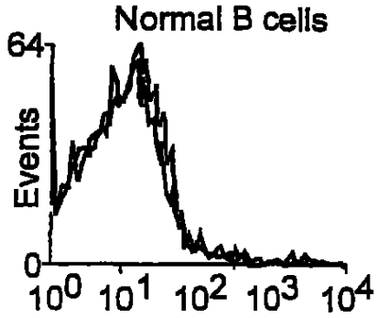


FIG - 2D-2

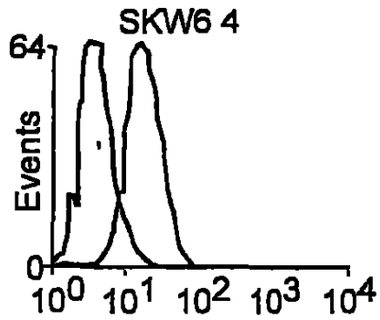


FIG - 2D-3

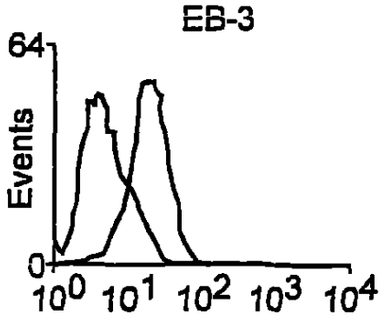


FIG - 2D-4

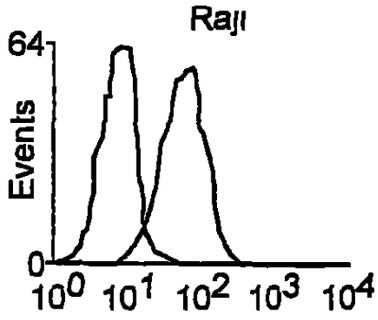


FIG - 2D-5

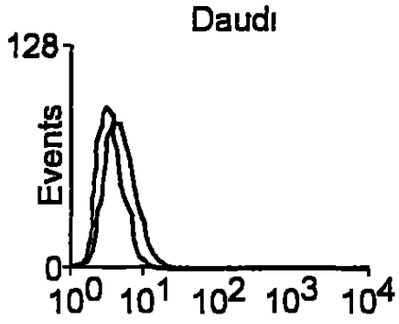


FIG - 2D-6



FIG - 2D-7

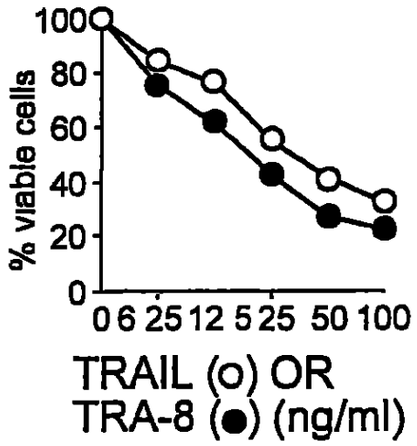


FIG - 2D-8

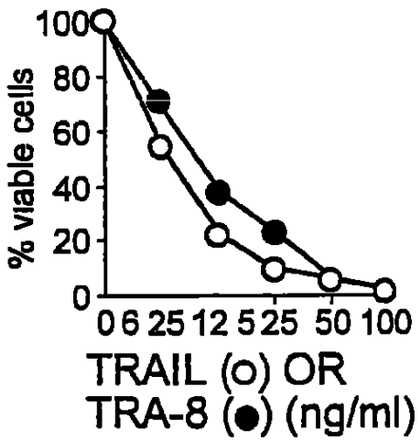


FIG - 2D-9

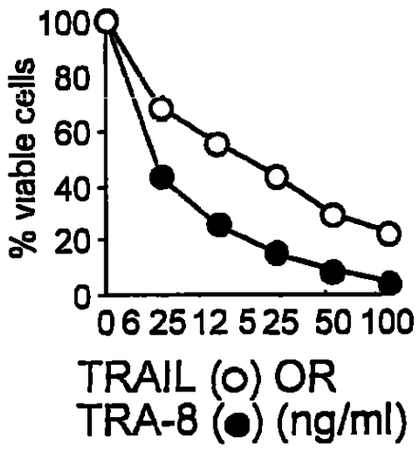


FIG - 2D-10

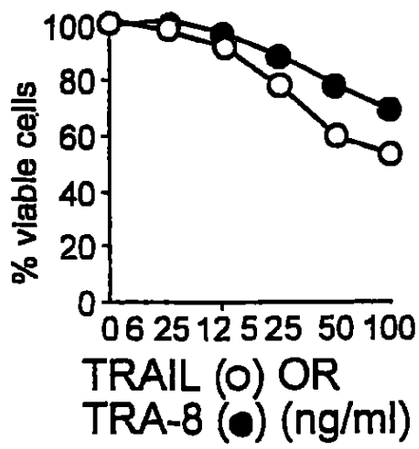


FIG - 3A'

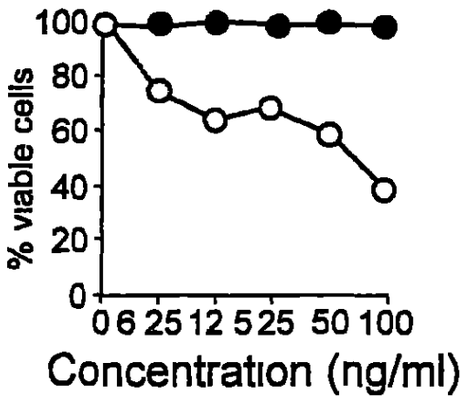


FIG - 3B-1

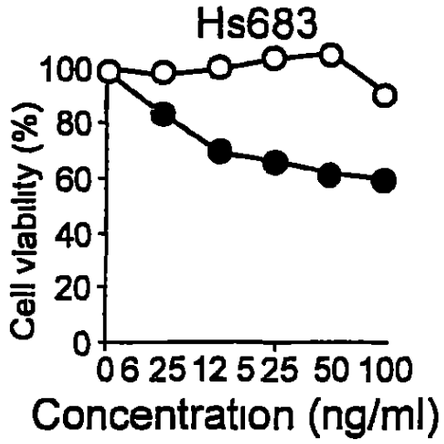


FIG - 3B-2

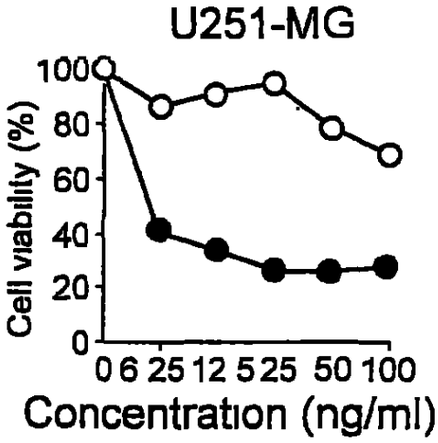


FIG - 3B-3

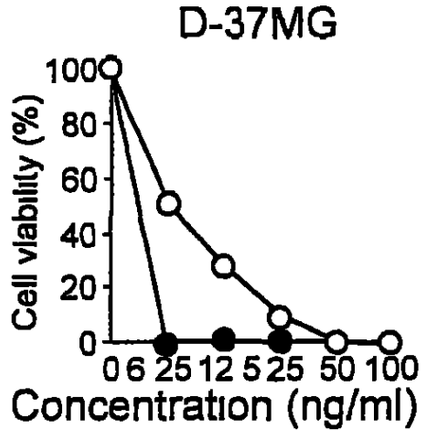


FIG - 3B-4

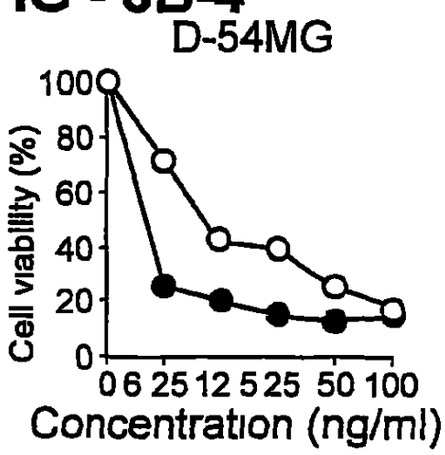


FIG - 3B-5

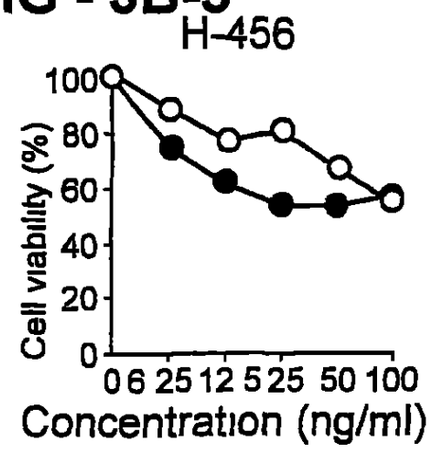


FIG - 3B-6

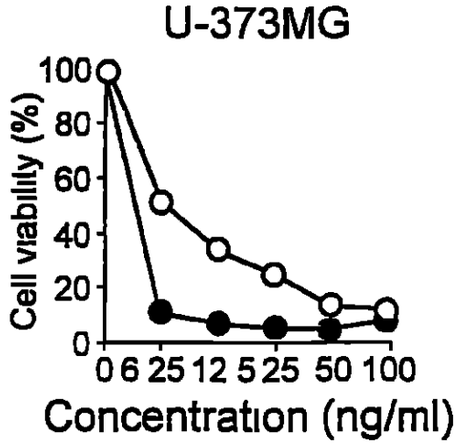


FIG - 3B-7

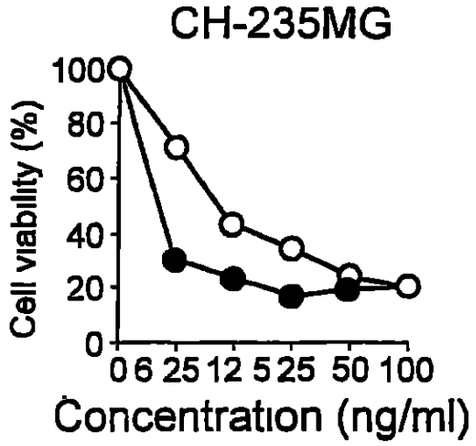


FIG - 3B-8

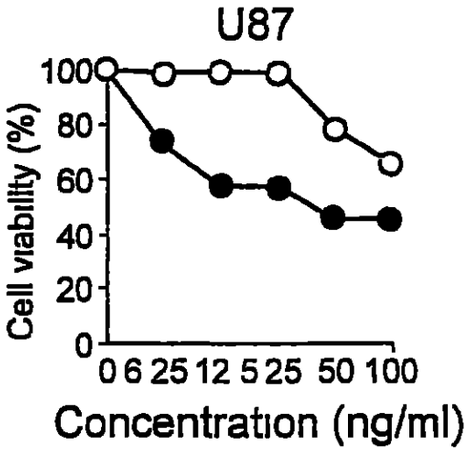


FIG - 3B-9

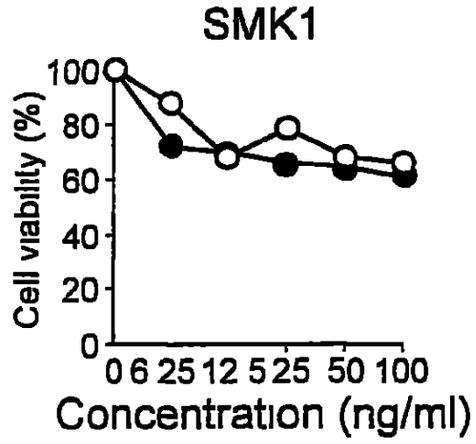


FIG - 3B-10

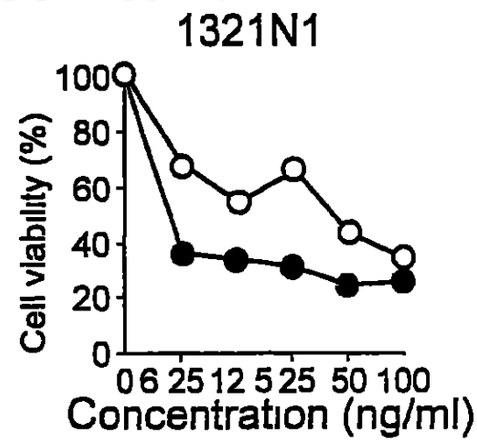


FIG - 3C-1

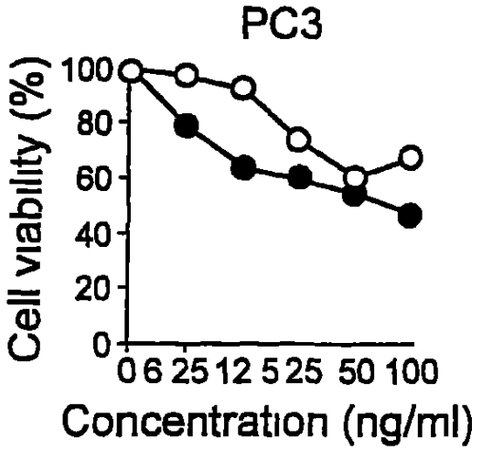


FIG - 3C-2

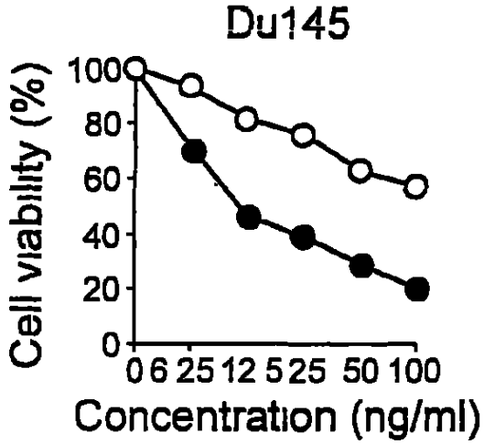
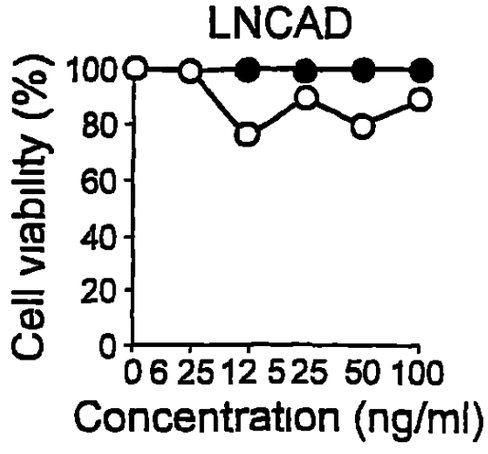


FIG - 3C-3



SUBSTITUTE SHEET (RULE 26)

FIG - 4A

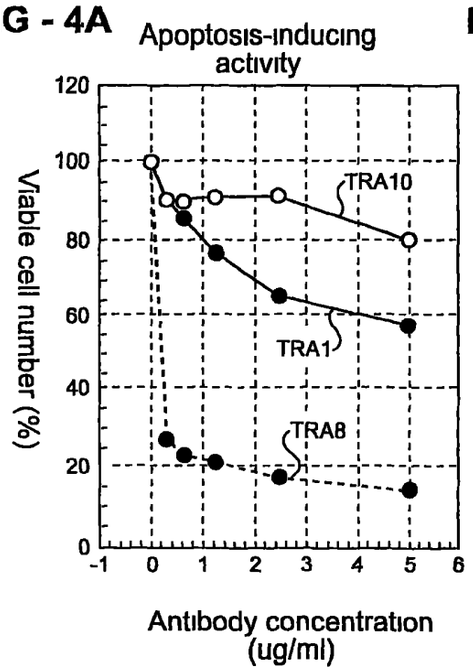


FIG - 4B

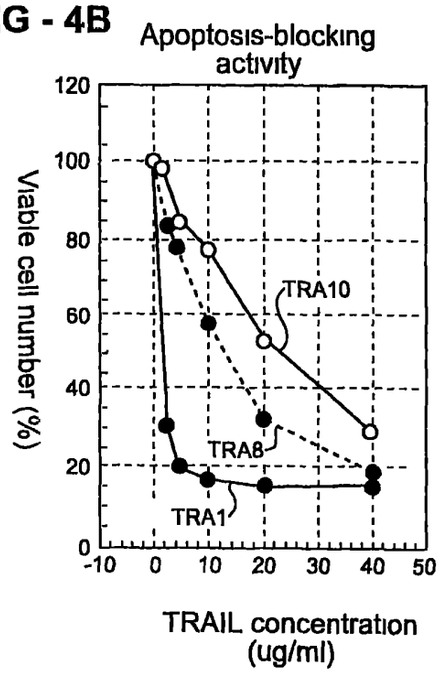


FIG - 5A

Normal tissue homogenates

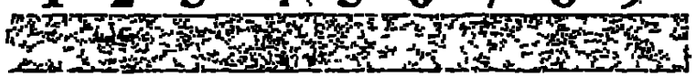


FIG - 5B

Cancer tissue homogenates

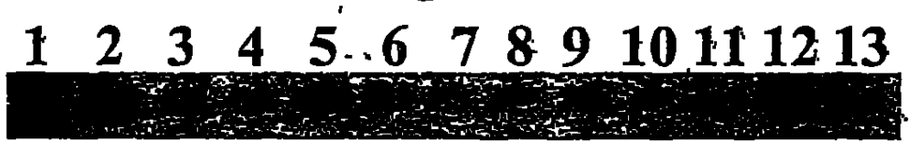


FIG - 5C

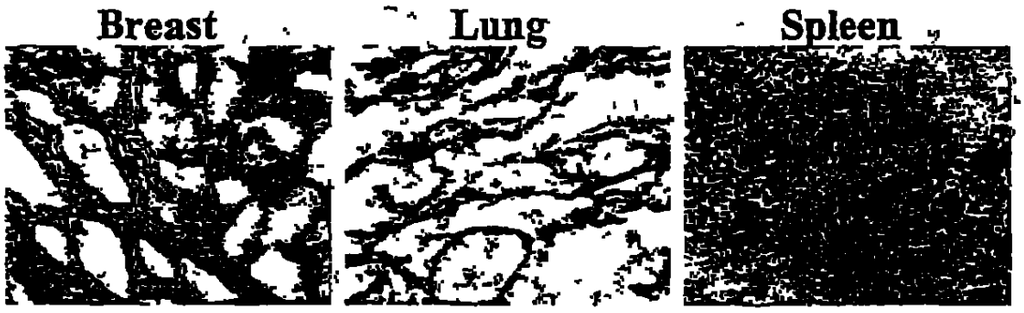


FIG - 5D

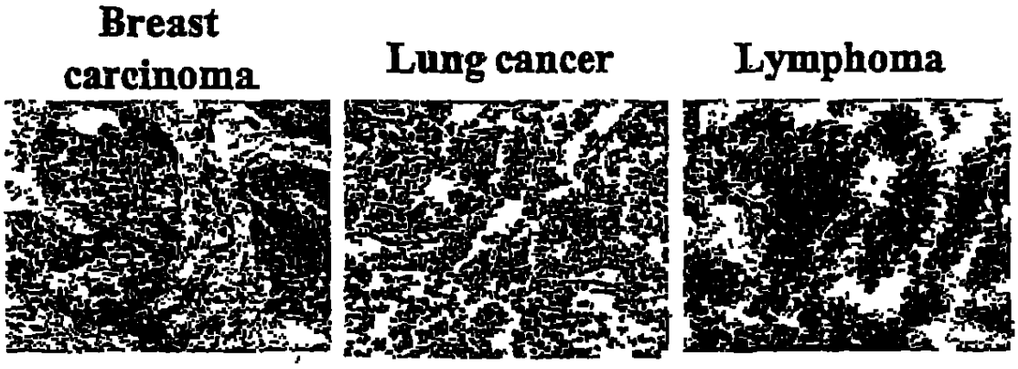


FIG - 6A

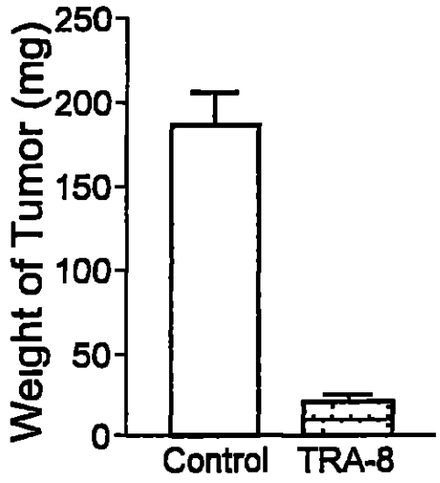


FIG - 6B

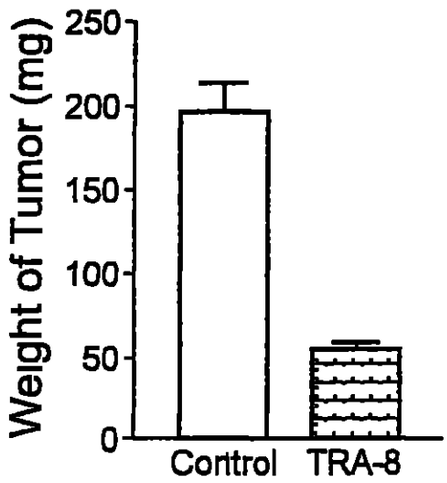


FIG - 6C

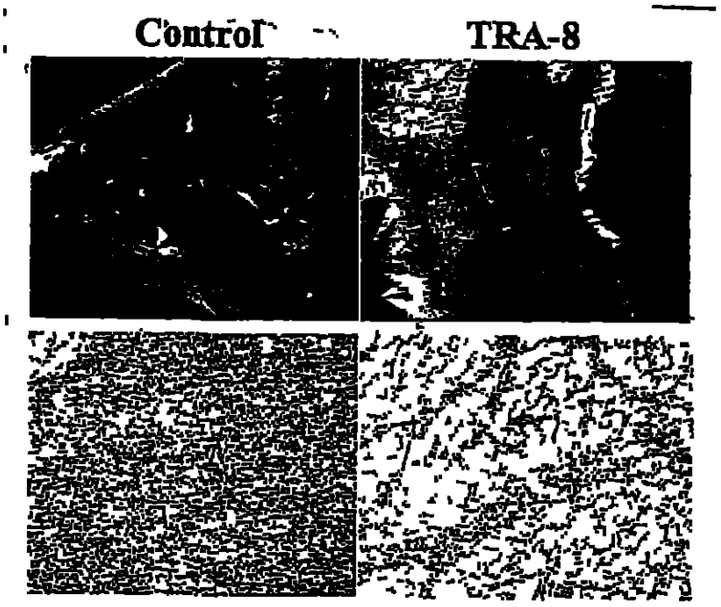


FIG - 6D

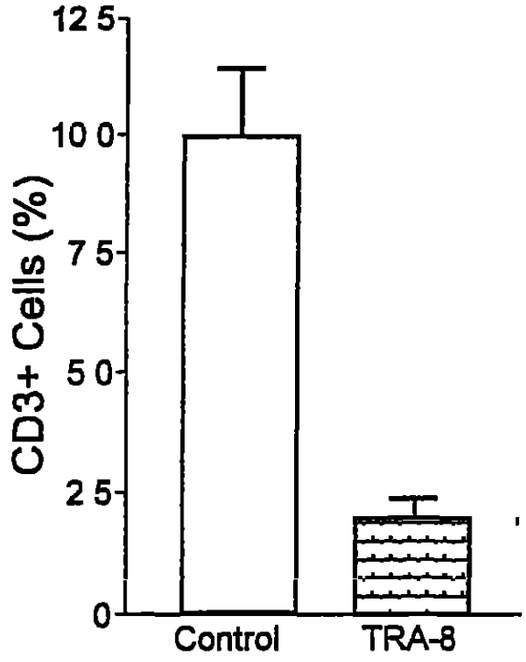


FIG - 6E

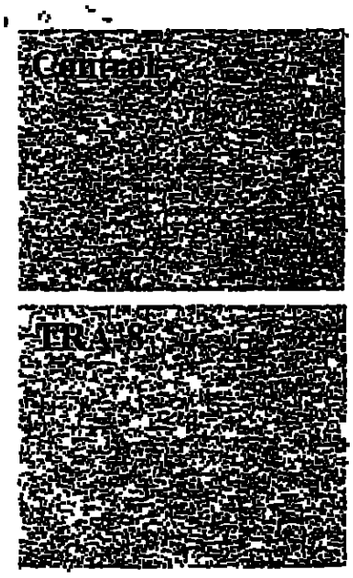


FIG - 7A-1

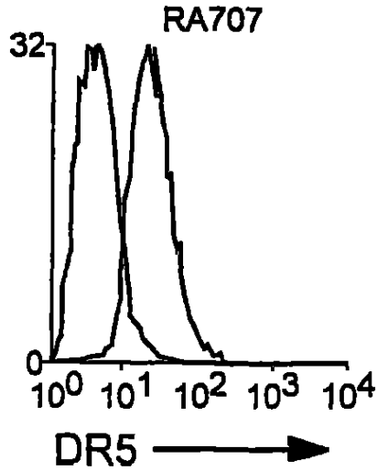


FIG - 7A-2

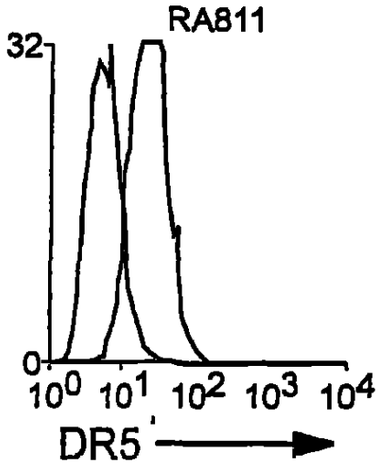


FIG - 7A-3

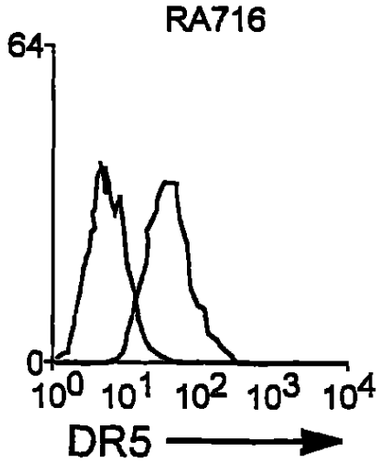


FIG - 7A-4

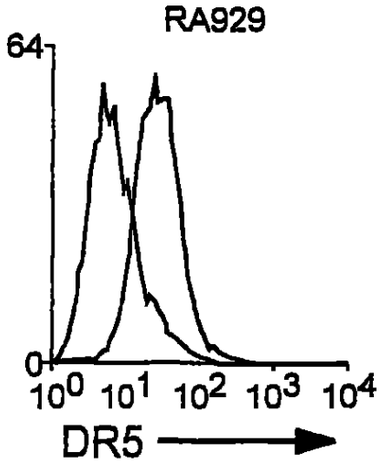


FIG - 7A-5

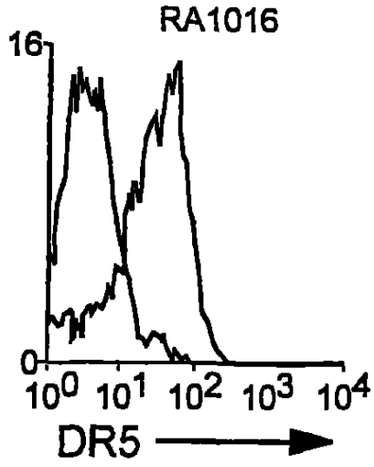


FIG - 7A-6

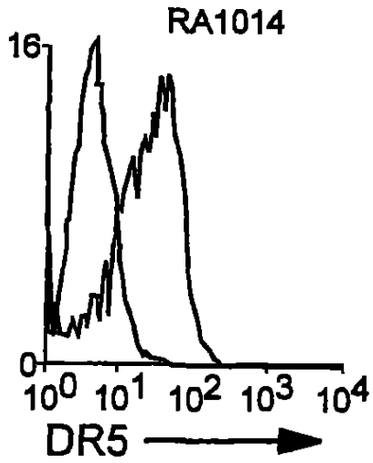


FIG - 7A-7

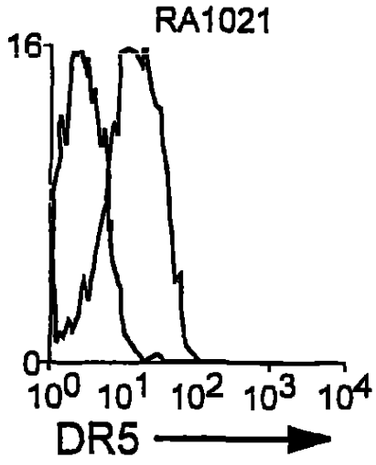


FIG - 7A-8

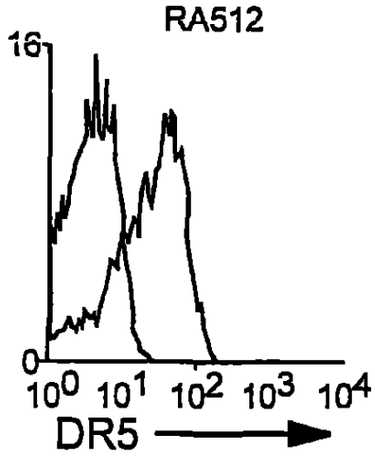


FIG - 7B-1

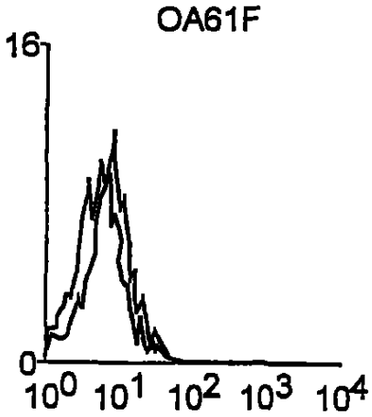


FIG - 7B-2

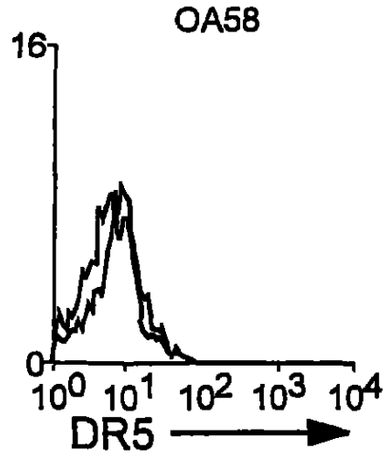


FIG - 7B-3

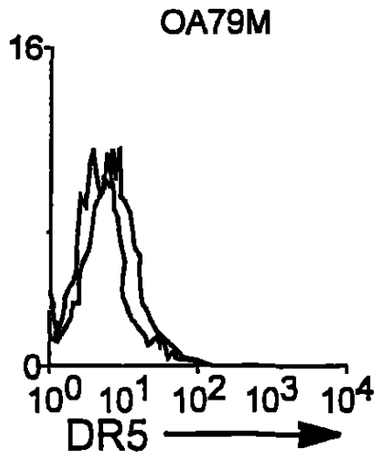


FIG - 7B-4

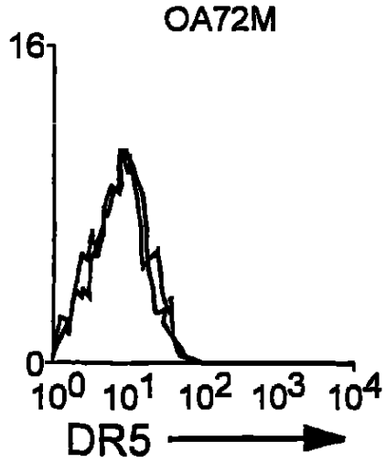


FIG - 7B-5

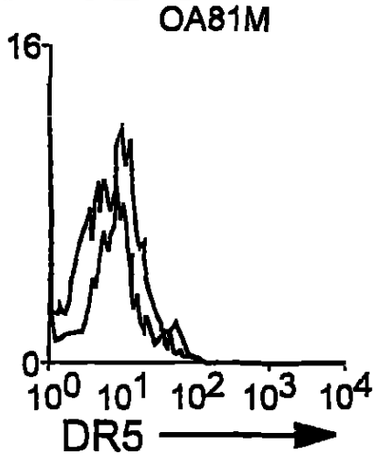


FIG - 7B-6

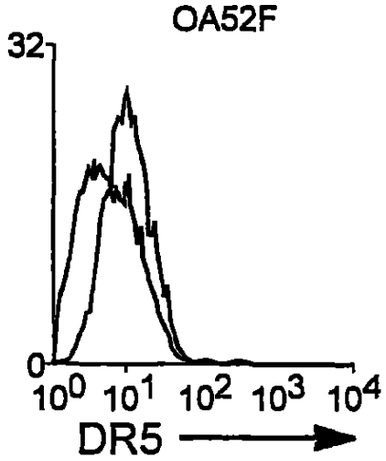


FIG - 7B-7

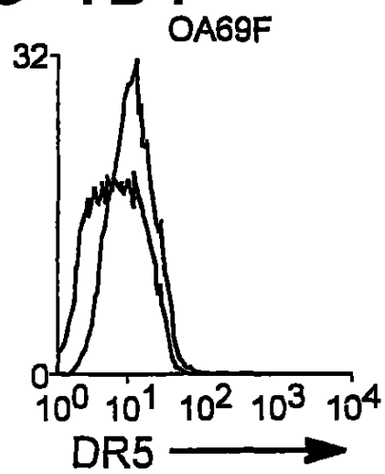


FIG - 8A-1

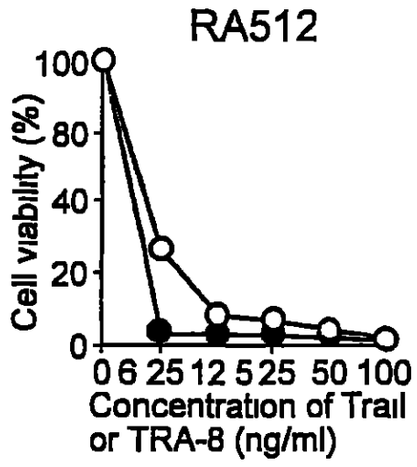


FIG - 8A-2

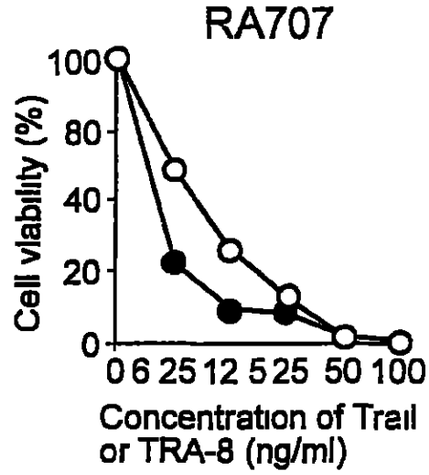


FIG - 8A-3

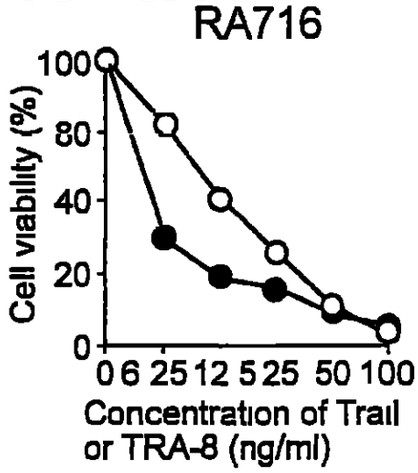


FIG - 8A-4

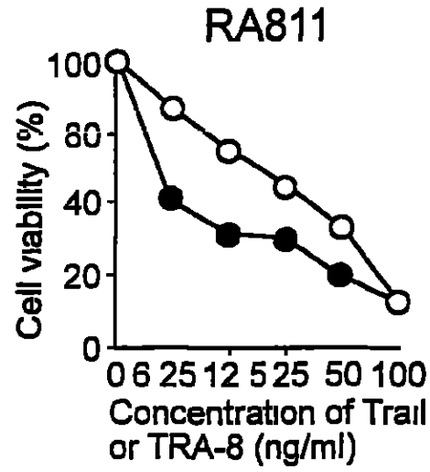


FIG - 8A-5

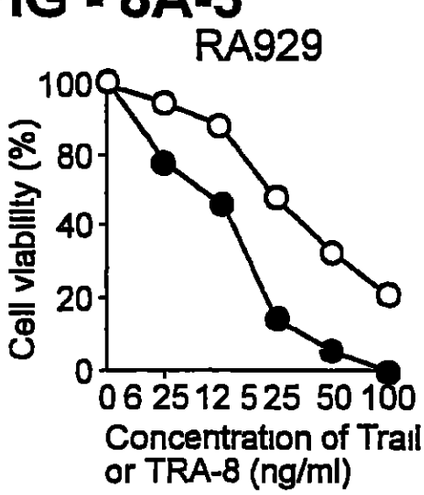


FIG - 8A-6

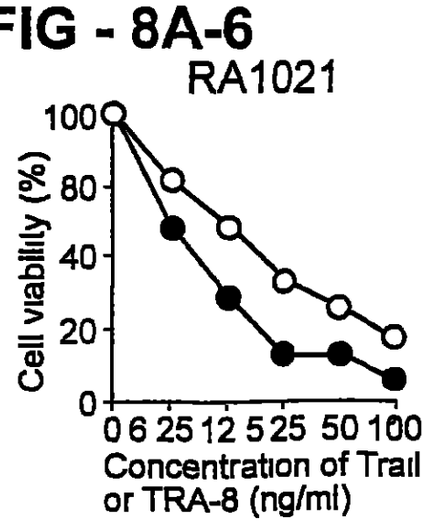


FIG - 8A-7

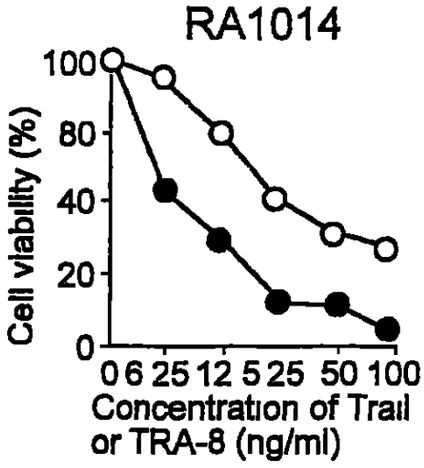


FIG - 8A-8

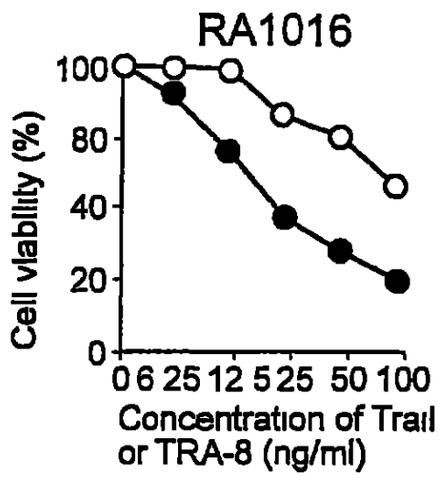


FIG - 8B-1

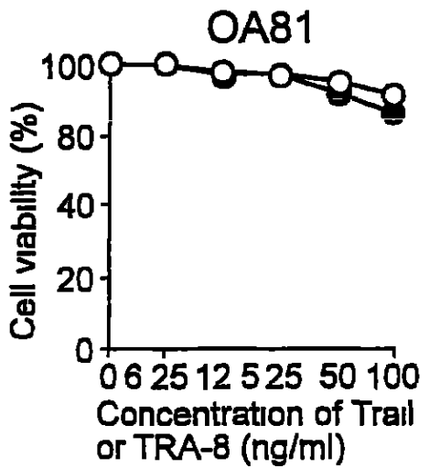


FIG - 8B-2

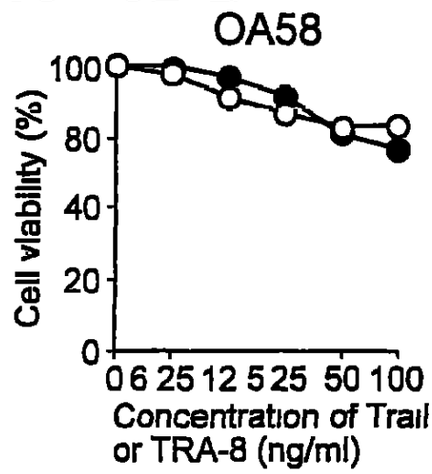


FIG - 8B-3

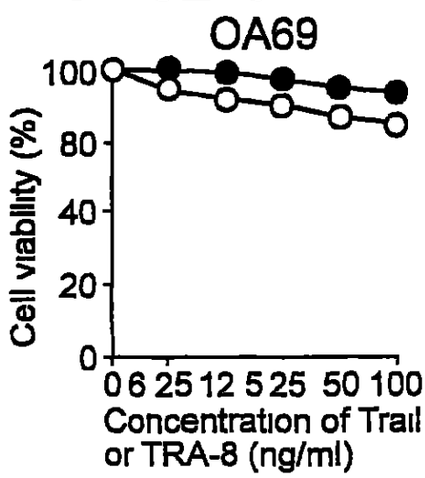


FIG - 8B-4

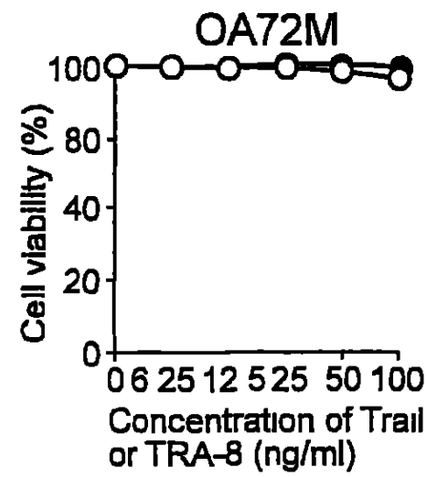


FIG - 9A

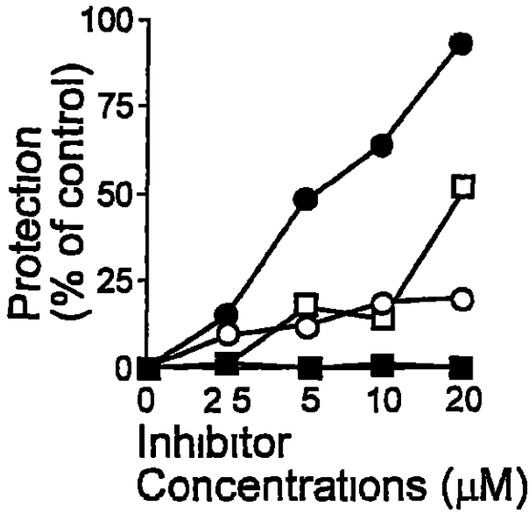


FIG - 9B

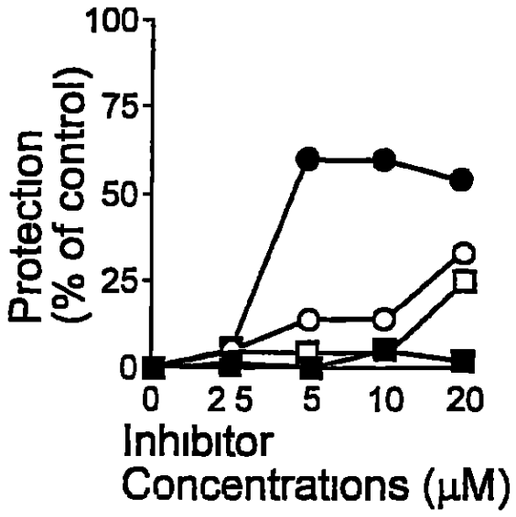


FIG - 9C

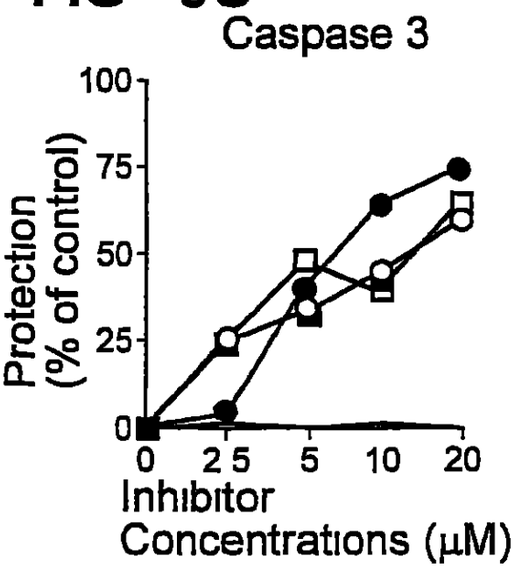


FIG - 9D

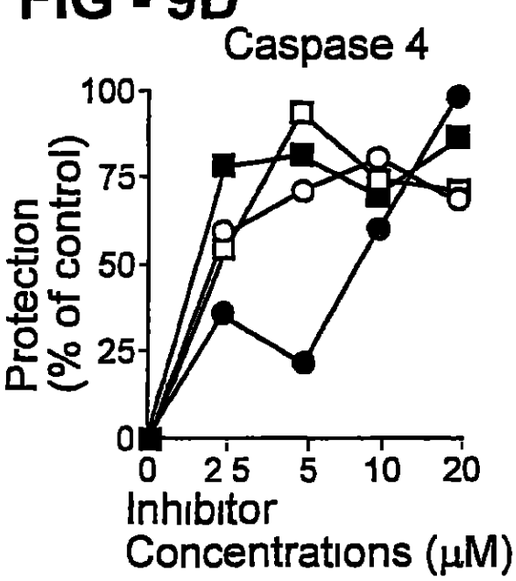


FIG - 9E

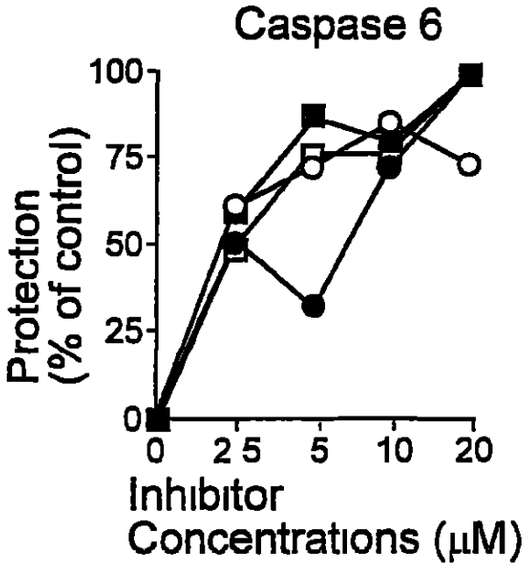


FIG - 9F

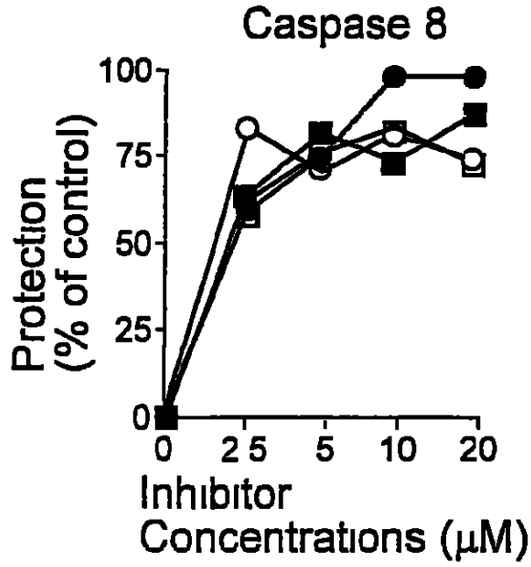


FIG - 9G

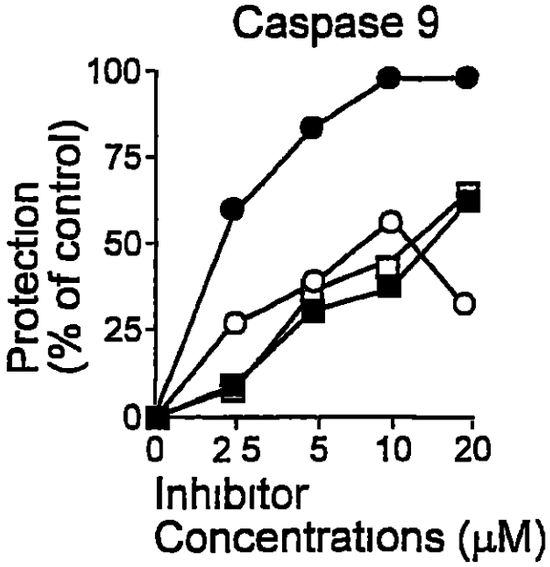


FIG - 9H

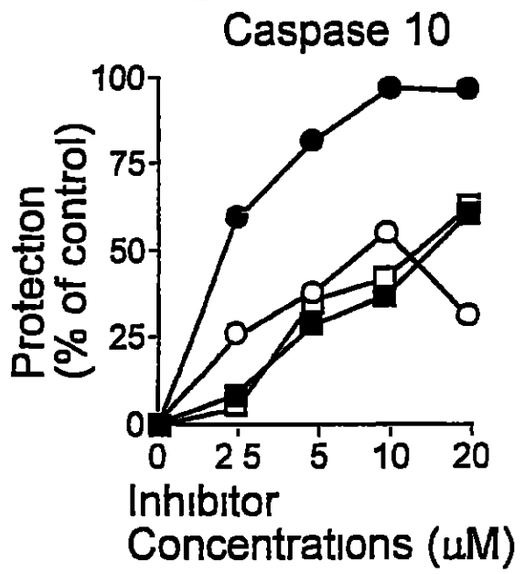


FIG - 10A

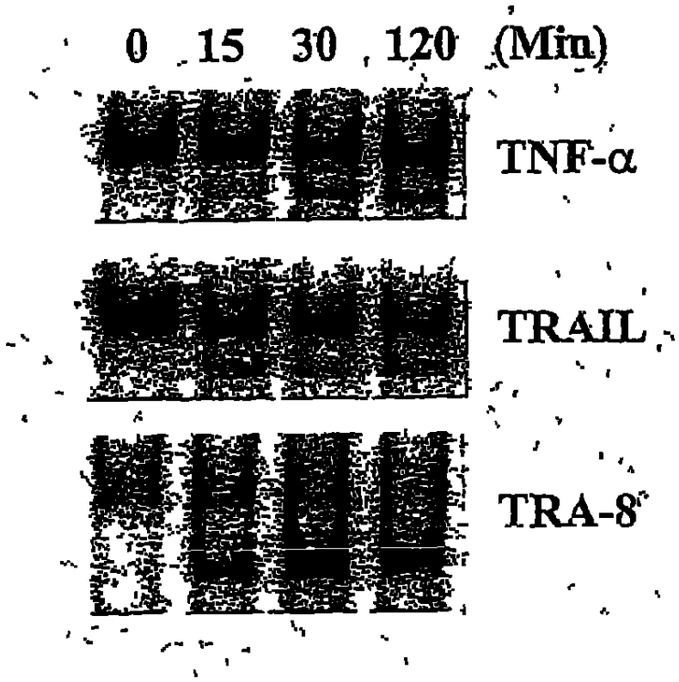


FIG - 10B

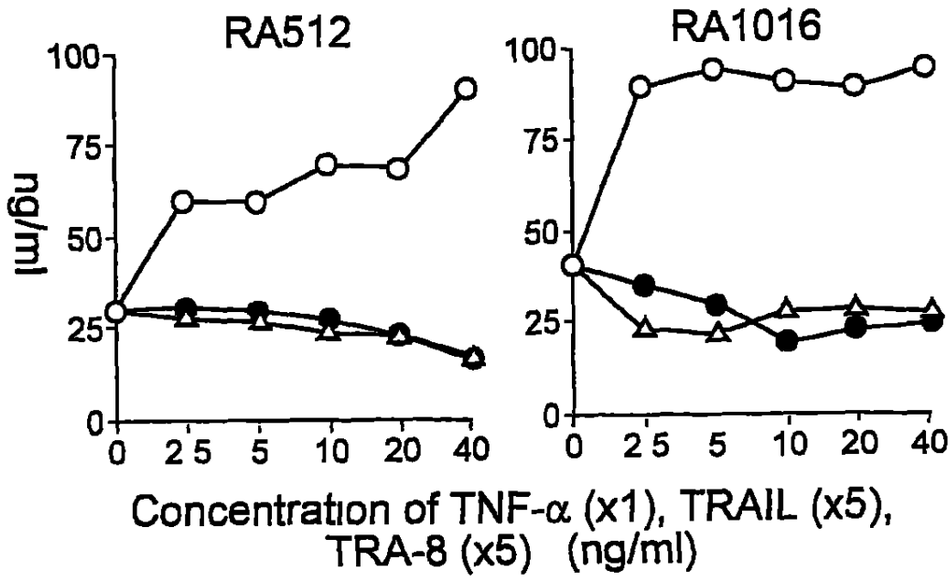


FIG - 10C

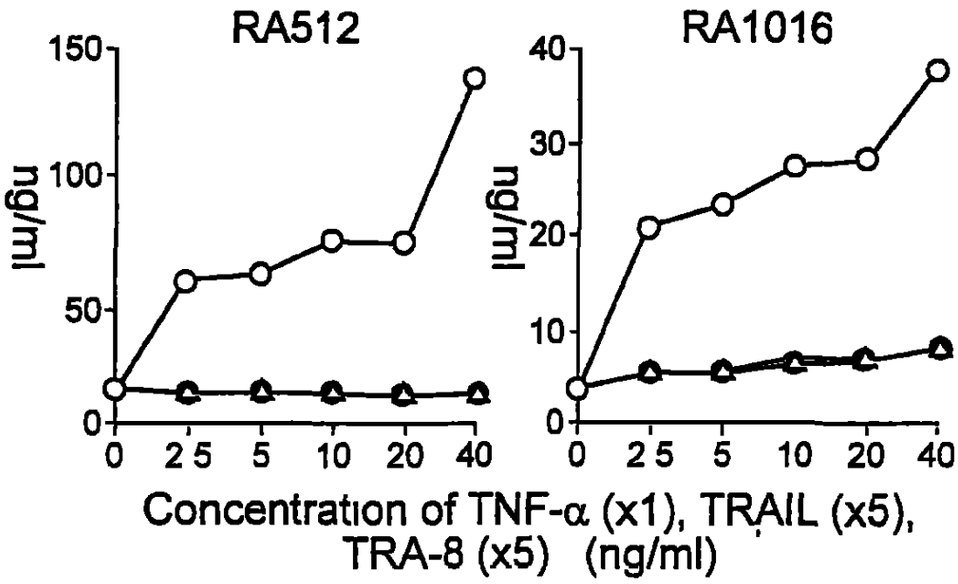


FIG - 11A

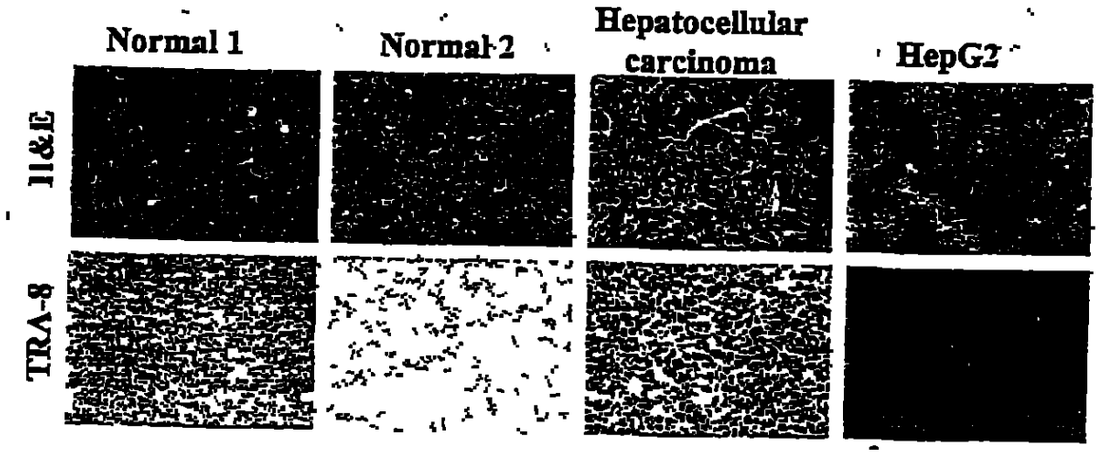


FIG - 11B-1

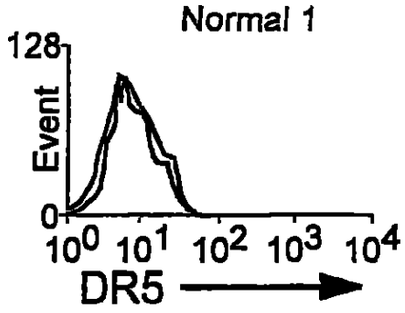


FIG - 11B-2

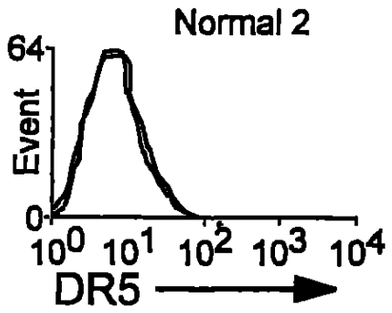


FIG - 11B-3

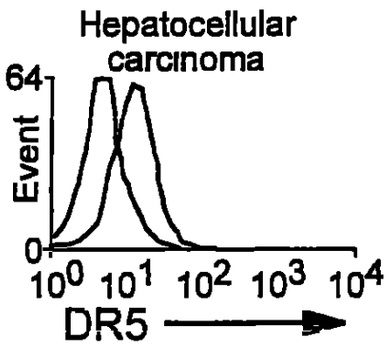


FIG - 11B-4

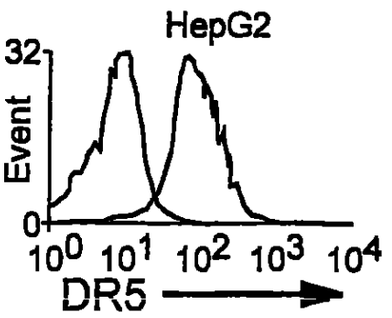


FIG - 11B-5

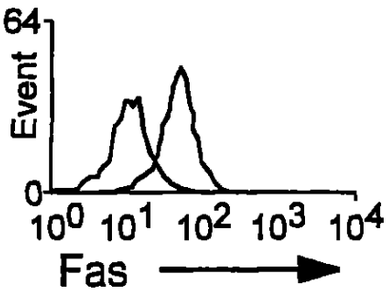


FIG - 11B-6

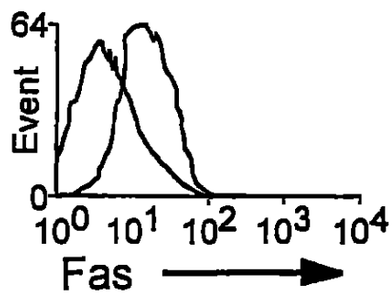


FIG - 11B-7

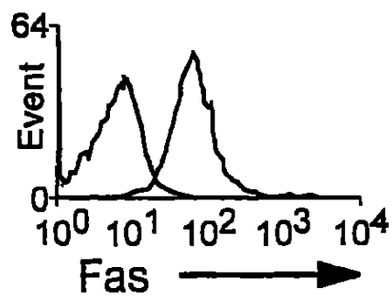


FIG - 11B-8

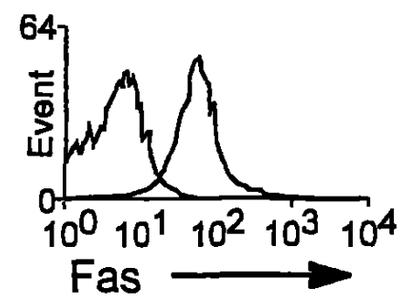


FIG - 12A

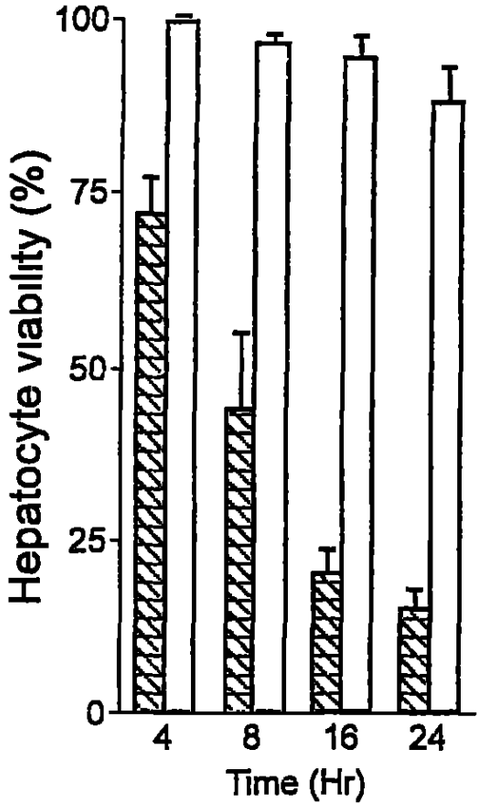


FIG - 12B

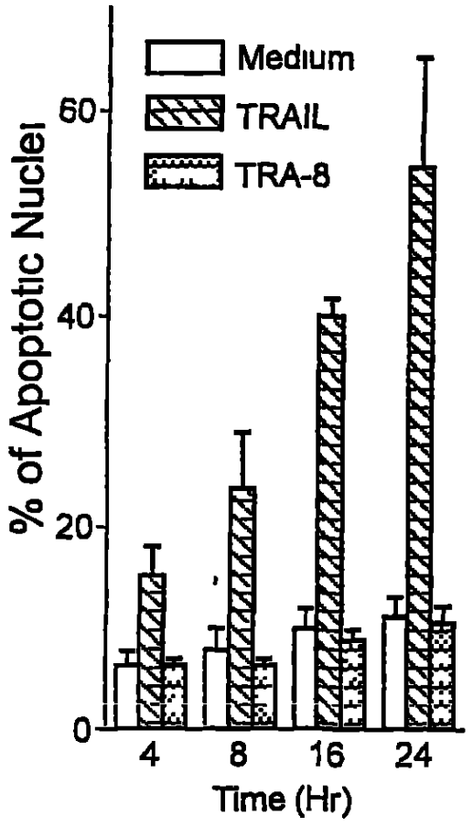


FIG - 12C

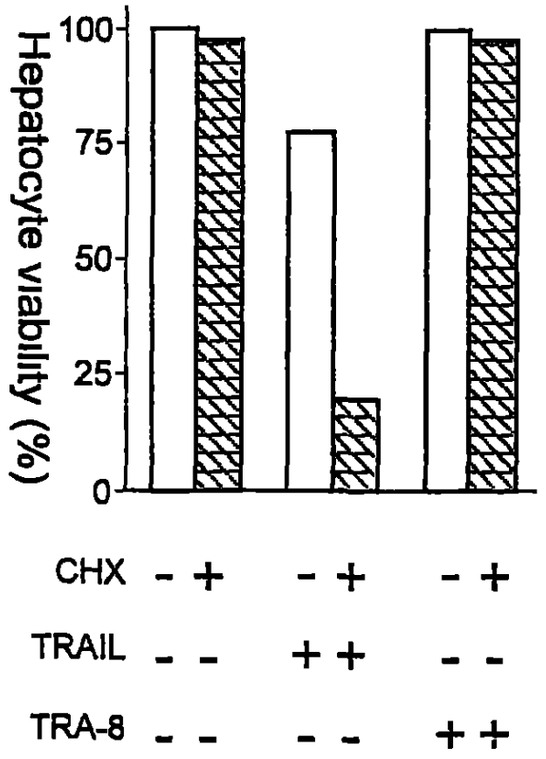


FIG - 12D-1

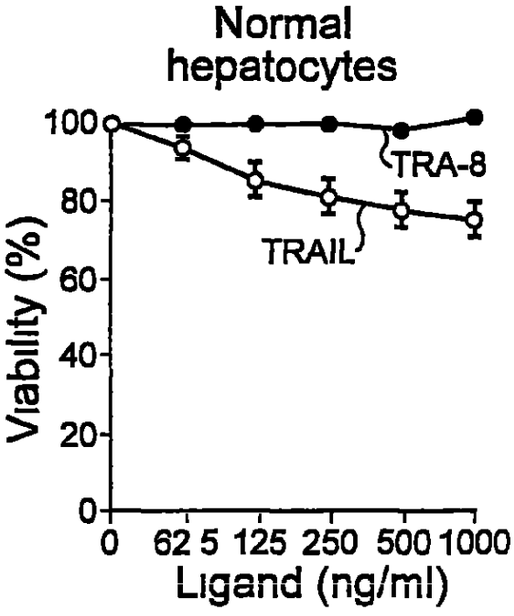


FIG - 12D-2

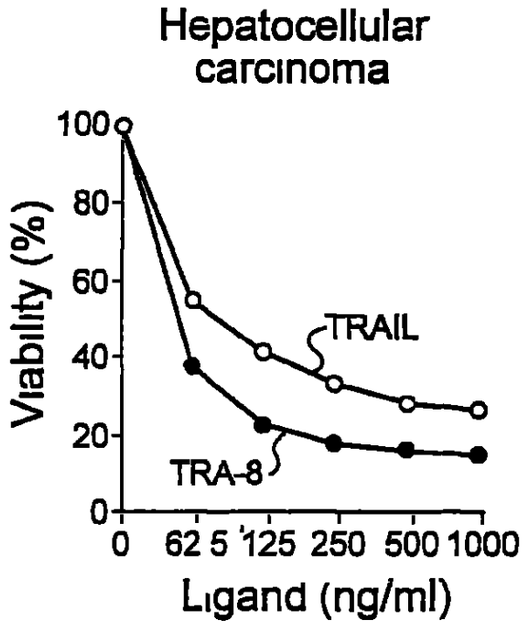


FIG - 12D-3

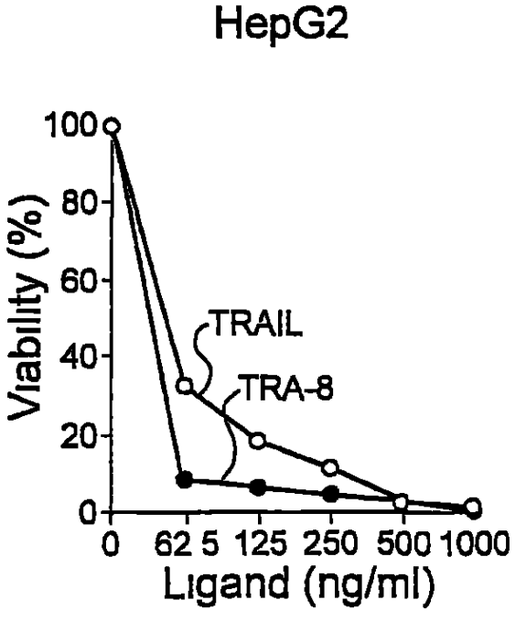
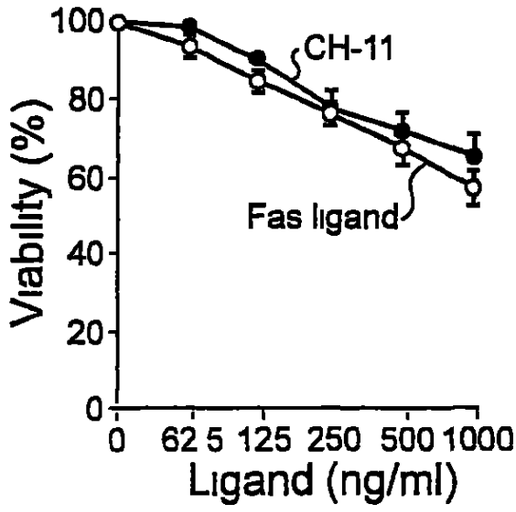


FIG - 12D-4



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FIG - 12D-5

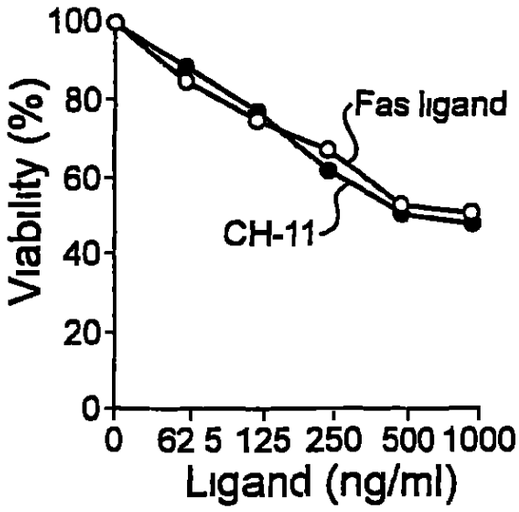


FIG - 12D-6

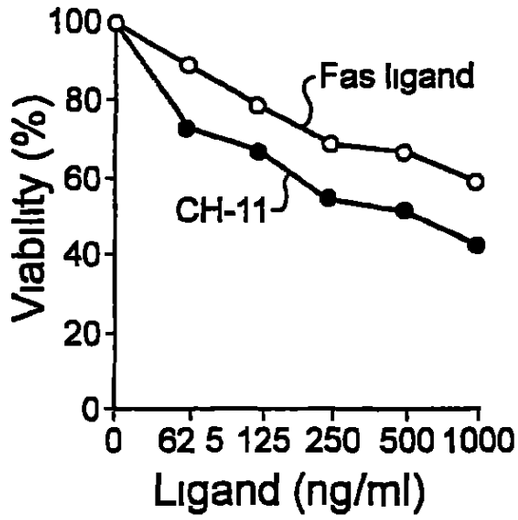


FIG - 13A

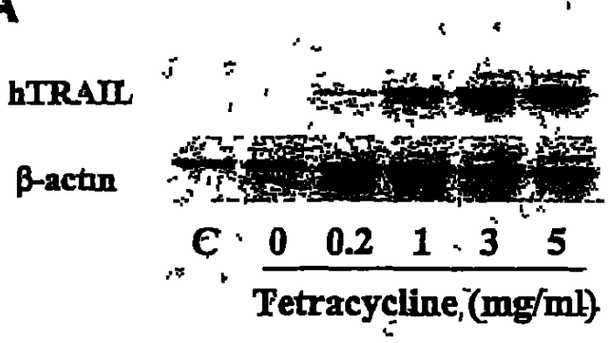


FIG - 13B

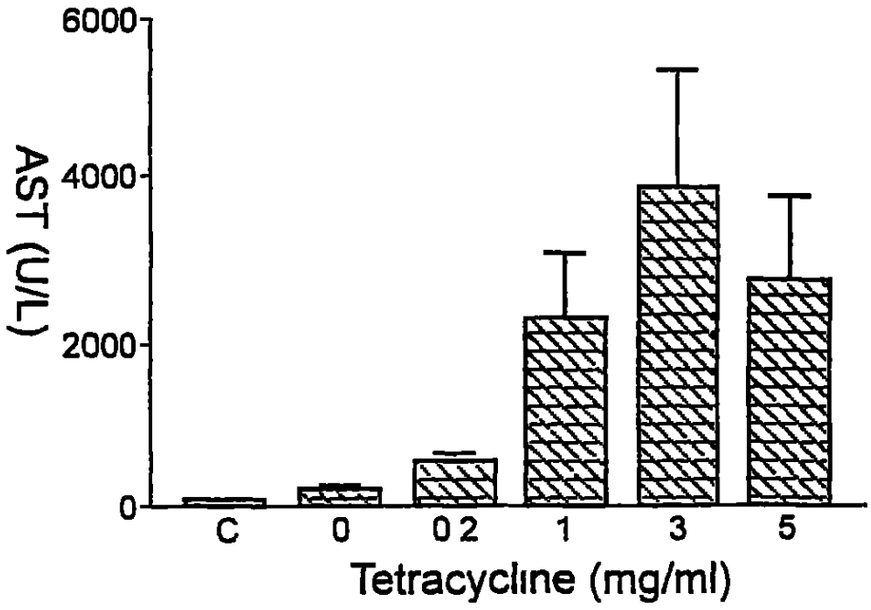


FIG - 13C-1

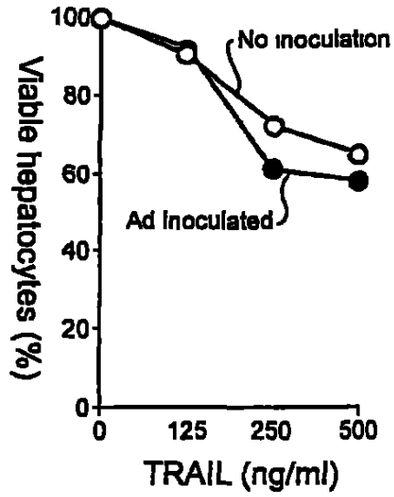
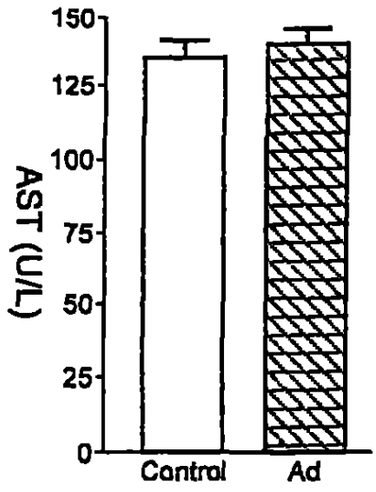


FIG - 13C-2



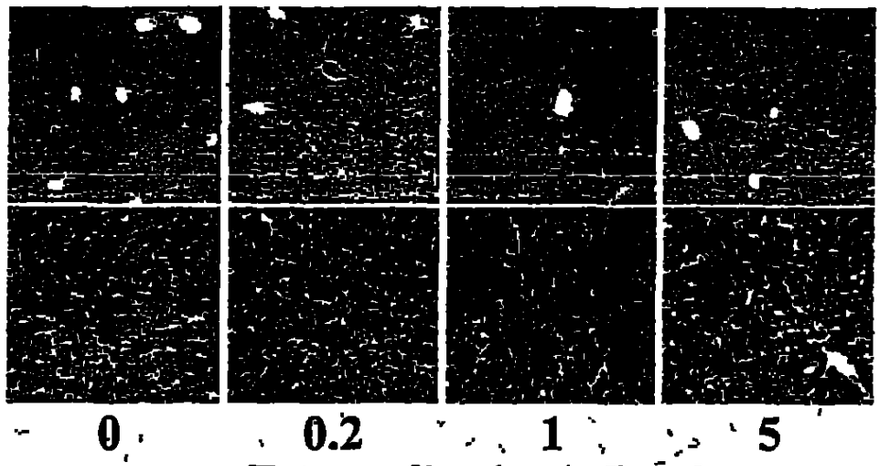


FIG - 13D

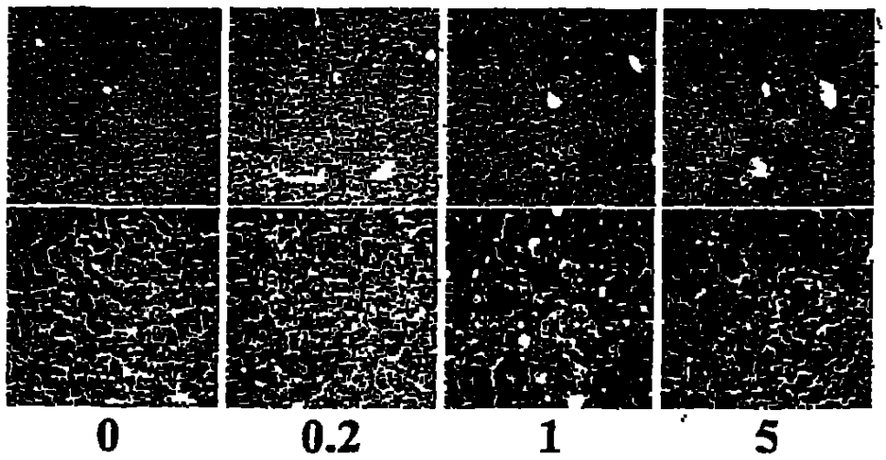


FIG - 13E

FIG - 14A

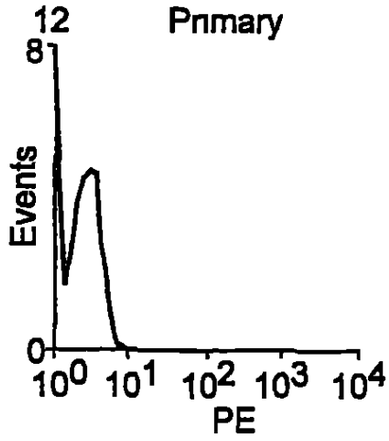


FIG - 14B

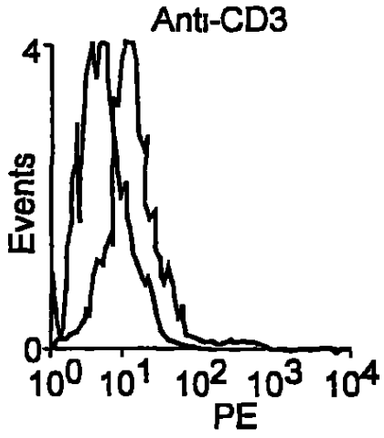


FIG - 14C

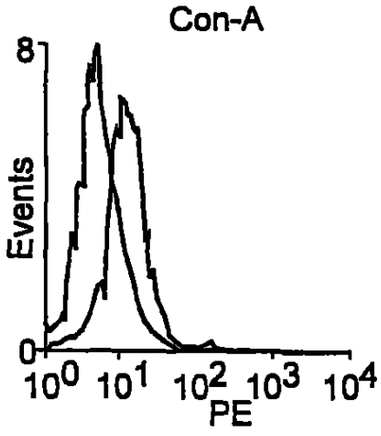


FIG - 14D

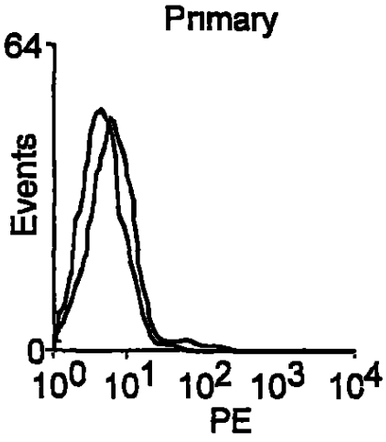


FIG - 14E

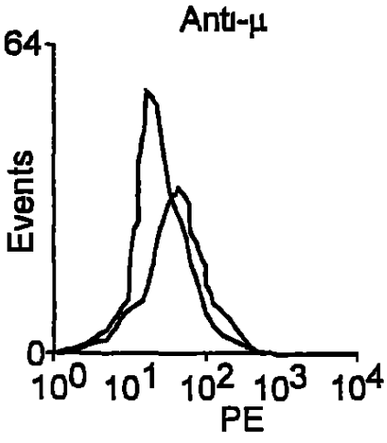


FIG - 14F

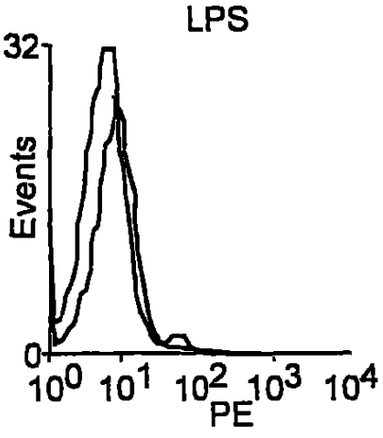


FIG - 15A

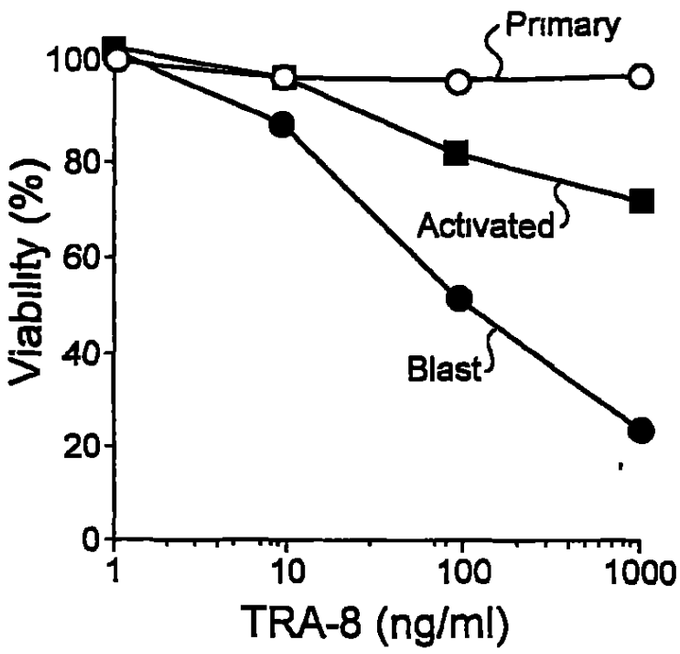


FIG - 15B

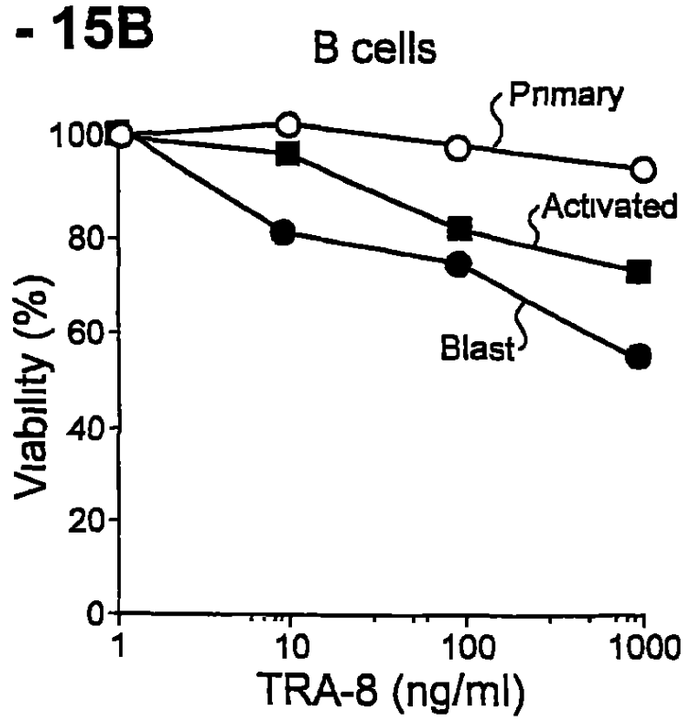


FIG - 16

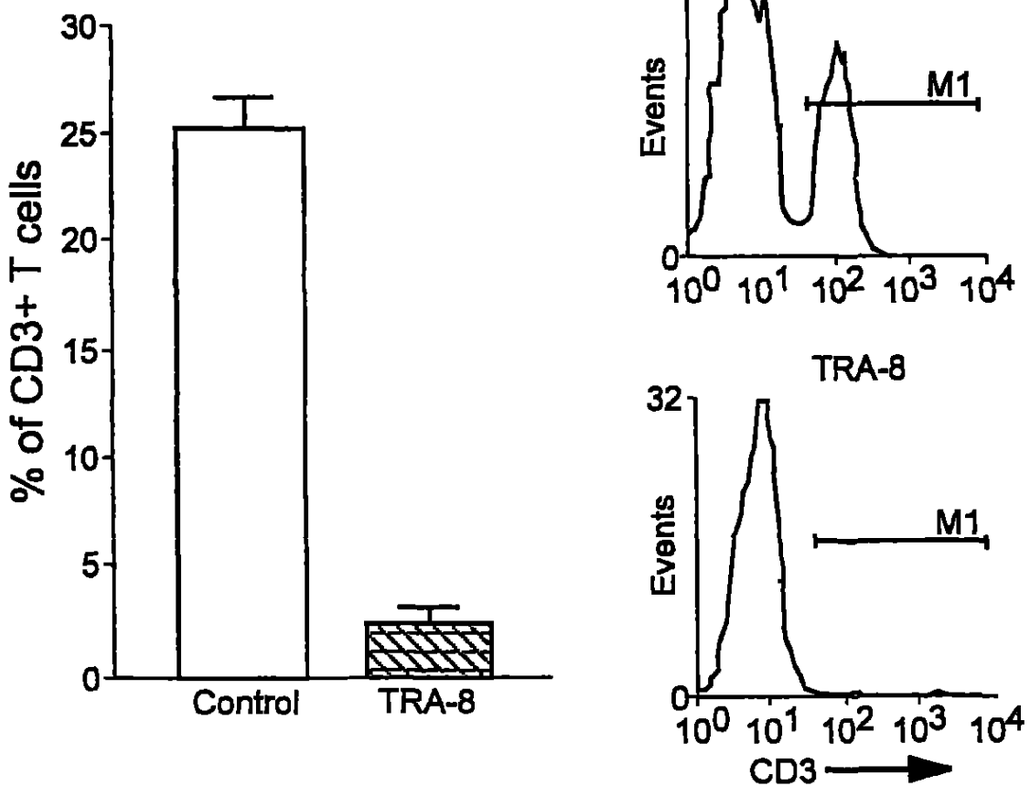

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TRA-8
Control
16
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M1
0
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TRA-8
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CD3

FIG - 17

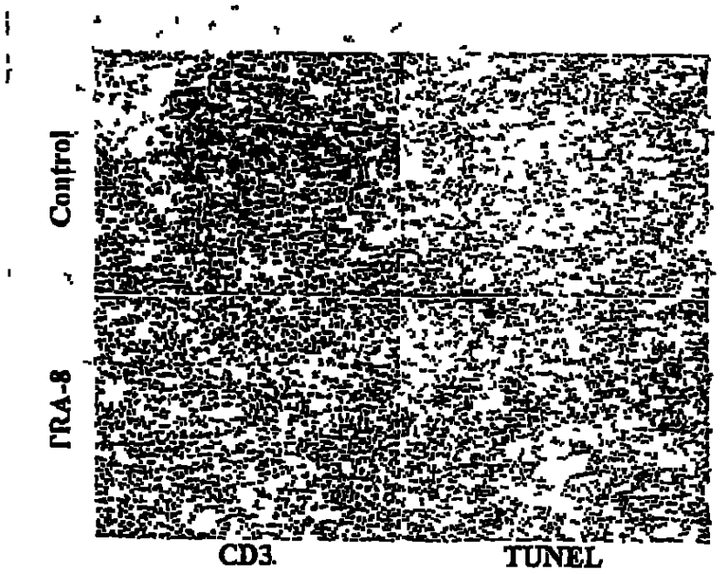

Control
TRA-8
CD3
TUNEL

FIG - 18A

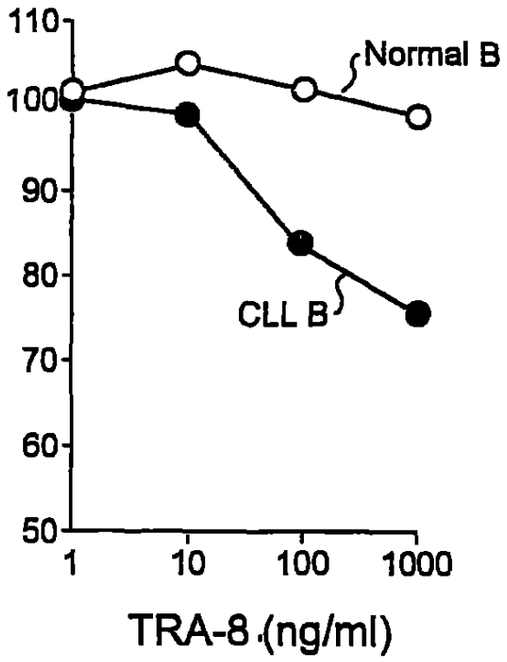


FIG - 18B

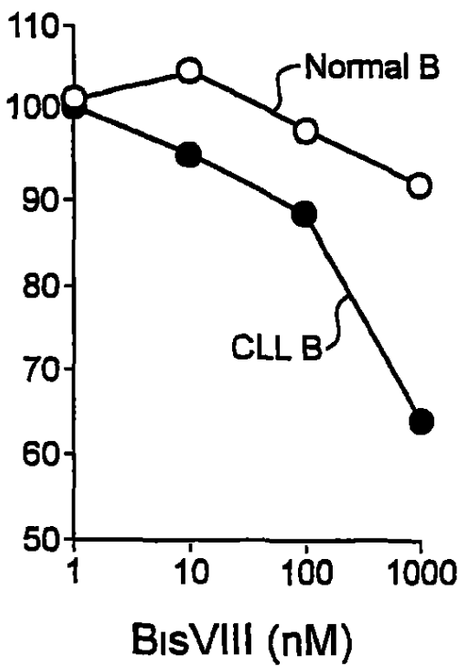
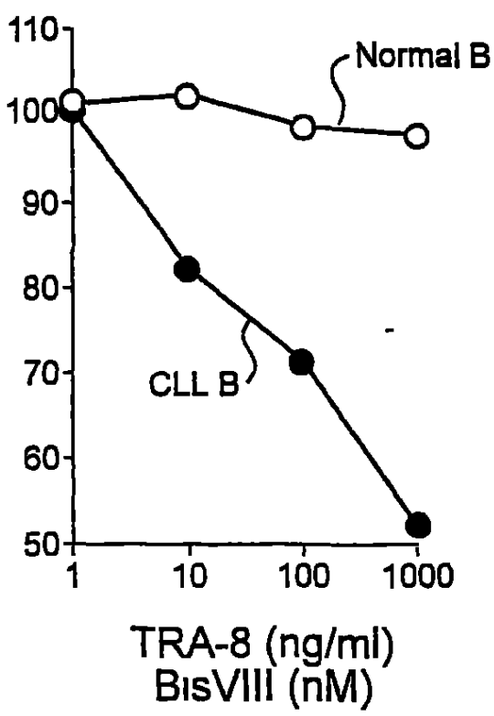


FIG - 18C



SEQUENCE LISTING

<110> The UAB Research Foundation

<120> AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

<130> UAB-17552/22

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<212> PRT
<213> Mus musculus

<400> 5

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

Asp Arg Val Ser
20

<210> 6
<211> 20
<212> DNA
<213> synthetic construct

<400> 6
cagcactgaa cacggacccc
20

<210> 7
<211> 20
<212> DNA
<213> synthetic construct

<400> 7
aaaggtaatt tattgagaag
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<210> 8
<211> 20
<212> DNA
<213> synthetic construct

<400> 8
cctcaccatg aacttcgggc
20

<210> 9
<211> 20
<212> DNA
<213> synthetic construct

<400> 9
ctgttgtatg cacatgagac
20

<210> 10
<211> 20
<212> DNA
<213> synthetic construct

<400> 10
gaagtgatgc tggtggagtc
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<210> 11
<211> 20
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<213> synthetic construct

<400> 11
agtgtgaagt gatgctggtg
20

<210> 12
<211> 21
<212> DNA
<213> synthetic construct

<400> 12
tttaccagga gagtgggaga g
21

<210> 13
<211> 20
<212> DNA
<213> synthetic construct

<400> 13
tgcagagaca gtgaccagag
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<210> 14
<211> 20
<212> DNA
<213> synthetic construct

<400> 14
tgttcaggac cagcatgggc
20

<210> 15
<211> 20
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<213> synthetic construct

<400> 15
aagacatttt ggattctaac
20

<210> 16
<211> 20
<212> DNA
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<400> 16
tatcatgaag tctttgtatg
20

<210> 17
<211> 20
<212> DNA
<213> synthetic construct

<400> 17
gatggagaca cattctcagg
20

<210> 18
<211> 20
<212> DNA
<213> synthetic construct

<400> 18
gacattgtga tgacccagtc

20

<210> 19
<211> 20
<212> DNA
<213> synthetic construct

<400> 19
ttaacactca ttcctgttga
20

<210> 20
<211> 20
<212> DNA
<213> synthetic construct

<400> 20
gactgggtca tcacaatgtc
20

<210> 21
<211> 1398
<212> DNA
<213> Mus musculus

<400> 21
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60

gtgatgctgg tggagtctgg gggaggctta gtgaagcctg gagggtccct gaaactctcc
120

tgtgcagcct ctggattcac tttcagtagc tatgtaatgt cttgggttcg ccagactccg
180

gagaagaggc tggagtgggt cgcaaccatt agtagtggtg gtagttacac ctactatcca
240

gacagtgtga aggggcgatt caccatctcc agagacaatg ccaagaacac cctgtacctg
300

caaatgagca gtctgaggtc tgaggacacg gccatgtatt actgtgcaag acgggggggac
360

tctatgatta cgacggacta ctggggccaa ggcaccactc tcacagtctc ctcagccaaa
420

acgacacccc catctgtcta tccactggcc cctggatctg ctgcccaaac taactccatg
480

gtgaccctgg gatgcctggt caagggctat ttccctgagc cagtgacagt gacctggaac
540

tctggatccc tgtccagcgg tgtgcacacc ttcccagctg tcctgcagtc tgacctctac
600

actctgagca gctcagtgac tgtcccctcc agcacctggc ccagcgagac cgtcacctgc
660

aacgttgccc acccggccag cagcaccaag gtggacaaga aaattgtgcc cagggattgt
720

ggttgtaagc cttgcatatg tacagtccca gaagtatcat ctgtcttcat cttccccca
780

aagcccaagg atgtgctcac cattactctg actcctaagg tcacgtgtgt tgtggtagac
840

atcagcaagg atgatcccga ggtccagttc agctggtttg tagatgatgt ggaggtgcac
900

acagcccaga cgcaaccccg ggaggagcag ttcaacagca ctttccgctc agtcagtgaa
960

cttcccatca tgcaccagga ctggctcaat ggcaaggagt tcaaatgcag ggtcaacagt
1020

gcagctttcc ctgcccccat cgagaaaacc atctccaaaa ccaaaggcag accgaaggct
1080

ccacaggtgt acaccattcc acctcccaag gagcagatgg ccaaggataa agtcagtctg
1140

acctgcatga taacagactt cttccctgaa gacattactg tggagtggca gtggaatggg
1200

cagccagcgg agaactacaa gaacactcag cccatcatgg acacagatgg ctcttacttc
1260

gtctacagca agctcaatgt gcagaagagc aactgggagg caggaaatac tttcacctgc
1320

tctgtgttac atgagggcct gcacaaccac catactgaga agagcctctc ccactctcct
1380

ggtaaaaatc actagtga
1398

<210> 22
<211> 705
<212> DNA
<213> Mus musculus

<400> 22
atgaagtctt tgtatgtgtt agtgtataca cattatctgt ttctgtttgc aggtgttgaa
60

ggagacattg tgatgaccca gtctcacaaa ttcatgtcca catcagtagg agacagggtc
120

agcatcacct gcaaggccag tcaggatgtg ggtactgctg tagcctggta tcaacagaaa
180

ccagggcaat ctcctaaact actgatttac tgggcatcca cccggcacac tggagtccct
240

gatcgcttca caggcagtgg atctgggaca gatttcactc tcaccattag caatgtgcag
300

tctgaagact tggcagatta tttctgtcag caatatagca gctatcggac gttcggtgga
360

ggcaccaagc tggaaatcaa acgggctgat gctgcaccaa ctgtatccat cttcccacca
420

tccagtgagc agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
480

cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa tggcgtcctg
540

aacagttgga ctgatcagga cagcaaagac agcacctaca gcatgagcag caccctcacg
600

ttgaccaagg acgagtatga acgacataac agctatacct gtgaggccac tcacaagaca
660

tcaacttcac ccattgtcaa gagcttcaac aggaatgagt gttaa
705

<210> 23
<211> 464

<212> PRT
<213> Mus musculus

<400> 23

Met Asn Phe Gly Leu Ser Leu Ile Phe Leu Val Leu Val Leu Lys Gly
1 5 10 15

Val Gln Cys Glu Val Met Leu Val Glu Ser Gly Gly Gly Leu Val Lys
20 25 30

Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45

Ser Ser Tyr Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu
50 55 60

Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
65 70 75 80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
85 90 95

Thr Leu Tyr Leu Gln Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met
100 105 110

Tyr Tyr Cys Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp
115 120 125

Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr Thr Pro Pro
130 135 140

Ser Val Tyr Pro Leu Ala Pro Gly Ser Ala Ala Gln Thr Asn Ser Met
145 150 155 160

Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr
165 170 175

Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His Thr Phe Pro
180 185 190

Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val
195 200 205

Pro Ser Ser Thr Trp Pro Ser Glu Thr Val Thr Cys Asn Val Ala His
210 215 220

Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Ile Val Pro Arg Asp Cys
225 230 235 240

Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val Ser Ser Val Phe
245 250 255

Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile Thr Leu Thr Pro
260 265 270

Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp Asp Pro Glu Val
275 280 285

Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His Thr Ala Gln Thr
290 295 300

Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Ser Val Ser Glu
305 310 315 320

Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys Glu Phe Lys Cys
325 330 335

Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu Lys Thr Ile Ser
340 345 350

Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro
355 360 365

Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu Thr Cys Met Ile
370 375 380

Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp Gln Trp Asn Gly
385 390 395 400

Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile Met Asp Thr Asp
405 410 415

Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln Lys Ser Asn Trp
420 425 430

Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu His Glu Gly Leu His
435 440 445

Asn His His Thr Glu Lys Ser Leu Ser His Ser Pro Gly Lys Asn His
450 455 460

<210> 24
<211> 234
<212> PRT
<213> Mus musculus

<400> 24

Met Lys Ser Leu Tyr Val Leu Val Tyr Thr His Tyr Leu Phe Leu Phe
1 5 10 15

Ala Gly Val Glu Gly Asp Ile Val Met Thr Gln Ser His Lys Phe Met
20 25 30

Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln
35 40 45

Asp Val Gly Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser
50 55 60

Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro
65 70 75 80

Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
85 90 95

Ser Asn Val Gln Ser Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln Tyr
100 105 110

Ser Ser Tyr Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
115 120 125

Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln
130 135 140

Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr
145 150 155 160

Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln
165 170 175

Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr
180 185 190

Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg
195 200 205

His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro
210 215 220

Ile Val Lys Ser Phe Asn Arg Asn Glu Cys
225 230

<210> 25
<211> 5
<212> PRT

<213> Mus musculus

<400> 25

Ser Tyr Val Met Ser
1 5

<210> 26
<211> 17
<212> PRT
<213> Mus musculus

<400> 26

Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val Lys
1 5 10 15

Gly

<210> 27
<211> 10
<212> PRT
<213> Mus musculus

<400> 27

Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr
1 5 10

<210> 28
<211> 11
<212> PRT
<213> Mus musculus

<400> 28

Lys Ala Ser Gln Asp Val Gly Thr Ala Val Ala
1 5 10

<210> 29
<211> 7
<212> PRT
<213> Mus musculus

<400> 29

Trp Ala Ser Thr Arg His Thr
1 5

<210> 30
<211> 8
<212> PRT
<213> Mus musculus

<400> 30

Gln Gln Tyr Ser Ser Tyr Arg Thr
1 5

<210> 31
<211> 119
<212> PRT
<213> synthetic construct

<400> 31

Glu Val Met Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> 32
<211> 80
<212> DNA
<213> synthetic construct

<400> 32
ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag

60

caacagctac aggtgtccac
80

<210> 33
<211> 80
<212> DNA
<213> synthetic construct

<400> 33
tctgaagtaa tgctggtgga gtctggggga ggcttagtac agcctggagg gtccctgaga
60

ctctcctgtg cagcctctgg
80

<210> 34
<211> 80
<212> DNA
<213> synthetic construct

<400> 34
attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga
60

gtgggttgca accattagta
80

<210> 35
<211> 80
<212> DNA
<213> synthetic construct

<400> 35
gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag
60

acaatgccaa gaacaccctg
80

<210> 36
<211> 80
<212> DNA
<213> synthetic construct

<400> 36
tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg
60

ggtgactcta tgattacgac
80

<210> 37
<211> 64
<212> DNA
<213> synthetic construct

<400> 37
ggactactgg ggccaaggga ccctggtcac agtctcctca gcctccacca agggcccatc
60

ggtc
64

<210> 38
<211> 60
<212> DNA
<213> syntnetic construct

<400> 38
ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa
60

<210> 39
<211> 80
<212> DNA
<213> synthetic construct

<400> 39
tctcagggac cctccaggct gtactaagcc tccccccagac tccaccagca ttacttcaga
60

gtggacacct gtagctgttg
80

<210> 40
<211> 80
<212> DNA
<213> synthetic construct

<400> 40
tccagaccct tccctggtgc ctgccgaacc caagacatta cataactact gaaagtgaat
60

ccagaggctg cacaggagag
80

<210> 41
<211> 80
<212> DNA
<213> synthetic construct

<400> 41
ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtaa ctaccaccac
60

tactaatggt tgcaacccac
80

<210> 42
<211> 80
<212> DNA
<213> synthetic construct

<400> 42
ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata
60

cagggtgttc ttggcattgt
80

<210> 43
<211> 84
<212> DNA
<213> synthetic construct

<400> 43
gaccgatggg cccttggtgg aggctgagga gactgtgacc agggtccctt ggccccagta
60

gtccgtcgta atcatagagt cacc
84

<210> 44

<211> 20
<212> DNA
<213> synthetic construct

<400> 44
ttggataagc ttggcttgac
20

<210> 45
<211> 21
<212> DNA
<213> synthetic construct

<400> 45
gaccgatggg cccttggtgg a
21

<210> 46
<211> 213
<212> PRT
<213> synthetic construct

<400> 46

Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

<210> 47
<211> 34
<212> DNA
<213> synthetic construct

<400> 47
cccaagctta agaagcatcc tctcatctag ttct
34

<210> 48
<211> 45
<212> DNA
<213> syntnetic construct

<400> 48
cccgaattct tactaacact ctcccctgtt gaagctcttt gtgac
45

<210> 49
<211> 60
<212> DNA
<213> synthetic construct

<400> 49
gtcccccaca gatgcagaca aagaacttgg agattgggtc atcacaatgt caccagtgga
60

<210> 50
<211> 48
<212> DNA
<213> synthetic construct

<400> 50
ccaagttctt tgtctgcatc agtaggagac agggtcacca tcacctgc
48

<210> 51
<211> 57
<212> DNA
<213> synthetic construct

<400> 51
agtgtgccgg gtggatgccc agtaaatcag tagtttagga gctttccctg gtttctg
57

<210> 52
<211> 48
<212> DNA
<213> synthetic construct

<400> 52
tgggcatcca cccggcacac tggggtccca agcaggttta gtggcagt
48

<210> 53
<211> 63
<212> DNA
<213> synthetic construct

<400> 53
ataactacta tattgctgac agtaataggt tgcaaaatcc tccggctgca gactagagat
60

ggt
63

<210> 54
<211> 63
<212> DNA
<213> synthetic construct

<400> 54

cagcaatata gcagctatcg gacgttcggt caaggcacca aggtggaaat caaacggact
60

gtg
63

<210> 55
<211> 711
<212> DNA
<213> synthetic construct

<400> 55
atggagacag acacaatcct gctatgggtg ctgctgctct gggttccagg ctccactggt
60

gacattgtga tgacccaatc tccaagttct ttgtctgcat ctgtggggga cagggtcacc
120

atcacctgca aggccagtca ggatgtgggt actgctgtag cctggtatca acagaaacca
180

gggaaagctc ctaaactact gatttactgg gcatccaccc ggcacactgg ggtcccaagc
240

aggtttagtg gcagtgggtc tgggacagac ttcaccctca ccatctctag tctgcagccg
300

gaggattttg caacctatta ctgtcagcaa tatagtagtt atcggacgtt cggtcaaggc
360

accaaggtgg aaatcaaacg gactgtggct gcaccatctg tcttcatctt cccgccatct
420

gatgagcagt tgaaatctgg aactgcctct gttgtgtgcc tgctgaataa cttctatccc
480

agagaggcca aagtacagtg gaaggtggat aacgccctcc aatcgggtaa ctcccaggag
540

agtgtcacag agcaggacag caaggacagc acctacagcc tcagcagcac cctgacgctg
600

agcaaagcag actacgagaa acacaaagtc tacgcctgcg aagtcaccca tcagggcctg
660

agctcgcccg tcacaaagag cttcaacagg ggagagtgtt agtaagaatt c
711

<210> 56
<211> 119
<212> PRT
<213> synthetic construct

<400> 56

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> 57
<211> 80
<212> DNA
<213> synthetic construct

<400> 57
tctgaagtac agctggtgga gtctggggga ggcttagtac agcctggagg gtccctgaga
60

ctctcctgtg cagcctctgg
80

<210> 58
<211> 80
<212> DNA
<213> synthetic construct

<400> 58
tctcagggac cctccaggct gtactaagcc tccccagac tccaccagct gtacttcaga
60

gtggacacct gtagctgttg
80

<210> 59
<211> 119
<212> PRT
<213> synthetic construct

<400> 59

Glu Val Met Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> 60
<211> 119
<212> PRT
<213> synthetic construct

<400> 60

Glu Val Met Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> 61
<211> 119
<212> PRT
<213> syntnetic construct

<400> 61

Glu Val Met Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly
1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Thr Leu Thr Val Ser Ser
115

<210> 62
<211> 80
<212> DNA
<213> synthetic construct

<400> 62
tatctgcaaa tgagcagtct gagagcagag gacacggctg tttattactg tgcaagaagg
60

ggtgactcta tgattacgac
80

<210> 63
<211> 80
<212> DNA
<213> synthetic construct

<400> 63
ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata
60

cagggtgttc ttggcattgt
80

<210> 64
<211> 80
<212> DNA
<213> synthetic construct

<400> 64
attcactttc agtagttatg taatgtcttg ggttcggcag actccagaga agaggctgga
60

gtgggttgca accattagta
80

<210> 65
<211> 80
<212> DNA
<213> synthetic construct

<400> 65

tccagcctct tctctggagt ctgccgaacc caagacatta cataactact gaaagtgaat
60

ccagaggctg cacaggagag
80

<210> 66
<211> 80
<212> DNA
<213> synthetic construct

<400> 66
tctgaagtaa tgctggtgga gtctgggggа ggcttagtaa agcctggagg gtccctgaaa
60

ctctcctgtg cagcctctgg
80

<210> 67
<211> 80
<212> DNA
<213> synthetic construct

<400> 67
tatctgcaaa tgagcagtct gagatctgag gacacggcta tgtattactg tgcaagaagg
60

ggtgactcta tgattacgac
80

<210> 68
<211> 64
<212> DNA
<213> synthetic construct

<400> 68
ggactactgg ggccaaggga ccactctcac agtctcctca gcctccacca agggcccatc
60

ggtc
64

<210> 69
<211> 80
<212> DNA

<213> synthetic construct

<400> 69
tttcagggac cctccaggct ttactaagcc tccccccagac tccaccagca ttacttcaga
60

gtggacacct gtagctgttg
80

<210> 70
<211> 80
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115 120 125

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Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
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Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
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Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
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Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
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Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
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Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

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165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
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Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
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Ser Val Thr Glu Gln Asp Ser Lys Asp Gly Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US01/14151

A CLASSIFICATION OF SUBJECT MATTER
IPC(7) C07K 16/00 A61K39/395 C07H 21/02 C12N 5/00 15/00 A01N 43/0-
US CL 530/387 1, 388 1 424130 1 143 1 536/23 1 435/320 1 325 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U S 530/387 1 388 1 424130.1, 143 1 536/23 1 435/320 1 325, 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable search terms used)
Please See Continuation Sheet

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document with indication where appropriate of the relevant passages	Relevant to claim No
A	SUN Q et al, Specific cytotoxicity and immuno-regulation by EBV specific CD4+ T cells. BLOOD 16 November, 2000, Vol 96 No 11 Part 2 Page. 46b, see entire document.	1-63
A	MITSIADES N et al Ewing's sarcoma family tumors are sensitive to tumor necrosis factor-related apoptosis-inducing ligand and express death receptor 4 and death receptor 5 CANCER RESEARCH, 15 March 2001 Vol 61 No 6 pages 2704-2712 see entire document.	1-63
A	KEANE M. M. et al Chemotherapy augments TRAIL-induced apoptosis in breast cell lines CANCER RESEARCH, 01 February 1999, Vol 59 No 3 pages 734-741 see entire document.	1-63
X	GRIFFITH T S Functional Analysis of TRAIL Receptors Using Monoclonal Antibodies The Journal of Immunology 1999 Vol 162 Pages 2597 2605 see pages 2597 2605 especially page 2599-2602	1 3 8

☒ Further documents are listed in the continuation of Box C ☐ See patent family annex.

* Special categories of cited documents.
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2001 (25 07 2001)	23 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No (703)305-3230	Authorized officer Sumesh Kaushal Telephone No 703 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US01/14151

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLARKE P *Reovirus-induced apoptosis is mediated by TRAIL.* JOURNAL OF VIROLOGY, September 2000, Vol 74 No 17, Pages 8135-8139 see entire document.	1-63
A	US 6 072,047 A (RAUCH et al) 06 June 2000 see col 20-21, col 26 example-4.	1-63
A	WO 99/03992 A1 (IMMNEX CORPORATION) 28 January 1999, see page 31-32	1-63
A	WO 98/35986 A1 (IMMNEX CORPORATION) 20 August 1998 see page 33-34.	1-63
X	WO 99/09165 A1 (IDUN PHARMACEUTICALS INC) 25 February 1999 see page 39-40 page 44, lines 11-16.	1-3
A	US 6,252, 050 B1 (ASHKENAZI et al) 26 June 2001, see entire document.	1-63

Form PCT/ISA/210 (second sheet) (July 1998)

229
232

INTERNATIONAL SEARCH REPORT

International application No
PCT/US01/14151

Continuation of B FIELDS SEARCHED Item 3
Medline, Agricola Caplus Biosis Medconf and STIC-SEQ search
Search terms TRAIL Receptor, antibody DrR1 DrR2, DR4, DR5 OPG, TRA-8 apoptosis programmed cell death, Vector

The demand must be filed directly wit[h] [a] competent International Preliminary Examining [Au]thority or if two or more Authorities are with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line

IPEA/ US

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated)

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference 21085 0029P1
International application No PCT/US01/14151	International filing date *(day/month/year)* 02 May 2001 (02/05/01)	(Earliest) Priority date *(day/month/year)* 02 May 2000 (02/05/00)

Title of invention
AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

Box No. II APPLICANT(S)

Name and address *(Family name followed by given name for a legal entity; full official designation. The address must include postal code and name of country.)*	Telephone No / Facsimile No / Teleprinter No
UAB RESEARCH FOUNDATION AB1120G 1530 3rd Avenue South Birmingham, Alabama 35294-0111 United States of America	

State *(that is country)* of nationality	State *(that is country)* of residence
US	US

Name and address *(Family name followed by given name for a legal entity; full official designation. The address must include postal code and name of country.)*

ZHOU, Tong
332 West Stonebrook Place
Birmingham, Alabama 35226
United States of America

State *(that is country)* of nationality	State *(that is country)* of residence
US	US

Name and address *(Family name followed by given name for a legal entity; full official designation. The address must include postal code and name of country.)*

ICHIKAWA, Kimihisa
1-3-42-205 Terayama, Tsurumi-ku Yokohama shi
Kanagawa ken 230-0015, Japan
Japan

State *(that is country)* of nationality	State *(that is country)* of residence
JP	JP

☒ Further applicants are indicated on a continuation sheet.

International application No PCT/US01/14151

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet is not to be included in the demand

Name and address *(Family name followed by given name for a legal entity full official designation. The address must include postal code and name of country)*

KIMBERLY Robert P
2045 Magnolia Ridge
Birmingham, Alabama 35243
United States of America

State *(that is country)* of nationality	State *(that is country)* of residence
US	US

Name and address *(Family name followed by given name for a legal entity full official designation. The address must include postal code and name of country)*

KOOPMAN, William J
187 Wildwood Avenue
Indian Springs, Alabama 35124
United States of America

State *(that is country)* of nationality	State *(that is country)* of residence
US	US

Name and address *(Family name followed by given name for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is country)* of nationality	State *(that is country)* of residence

Name and address *(Family name followed by given name for a legal entity full official designation. The address must include postal code and name of country)*

State *(that is country)* of nationality	State *(that is country)* of residence

☐ Further applicants are indicated on another continuation sheet.

International application No PCT/US01/14151

Box No. III AGENT OR COMMON REPRESENTATIVE, OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination

☐ is hereby appointed and any earlier appointment of (an) agent(s) /common representative is hereby revoked

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority in addition to the agent(s)/common representative appointed earlier

Name and address *(Family name followed by given name for a legal entity full official The address must include postal code and name of country.)*

NEEDLE & ROSENBERG P C , 127 Peachtree Street, N E Suite 1200 The Candler Building Atlanta Georgia 30303-1811, United States of America McKeon, Tina W Needle, William, Rosenberg Sumner Perryman David Katz Mitchell, Kirsch, Gregory Spratt Gwendolyn Setty, Nagendra, Johnson, William Weatherly, Mitchell Miller Mary Fails Charles, Ades Patricia Becker, Bruce, Corin, Shari DeCarlo Kean, DeWitt LaVonda Derrick,Wesley, Fernandez, Lizette,Maxwell, Lawrence, Murphy, Mark, Villanueva, Lawrence

Telephone No (404)688 0770

Facsimile No (404) 688 9880

Teleprinter No.

☐ Address for correspondence Mark this check box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments *

1 The applicant wishes the international preliminary examination to start on the basis of

☒ the international application as originally filed.

the description ☒ as originally filed
☐ as amended under Article 34

the claims ☒ as originally filed
☐ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34

the drawings ☒ as originally filed
☐ as amended under Article 34

2 ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3 ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examing Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69 1(d)) *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check box is marked, international preliminary examination will start on the basis of the international application as originally filed or where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report as so amended.

Language for the purposes of international preliminary examination English

☒ which is the language in which the international application was filed

☐ which is the language of a translation furnished for the purposes of international search.

☐ which is the language of publication of the international application

☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination

Box No V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect

Sheet No. 4

International application No 236
PCT/US01/14151

Box No VI CHECK LIST

The demand is accompanied by the following elements in the language referred to in Box No IV for the purposes of international preliminary examination		For International Preliminary Examining Authority use only: received	not received
1 translation of international application	sheets	☐	☐
2 amendments under Article 34	sheets	☐	☐
3 copy (or where required, translation) of amendments under Article 19	sheets	☐	☐
4. copy (or where required, translation) of statement under Article 19	sheets	☐	☐
5 letter	1 sheets	☐	☐
6. other *(specify)*	sheets	☐	☐

The demand is also accompanied by the item(s) marked below

1 ☒ fee calculation sheet
2 ☐ separate signed power of attorney
3 ☐ copy of general power of attorney reference number if any
4. ☐ statement explaining lack of signature
5 ☐ nucleotide and or amino acid sequence listing in computer readable form
6. ☒ other *(specify)* cert of exp mailing check for $827

Box No VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand)

Tina W McKeon, Agent

For International Preliminary Examining Authority use only

1 Date of actual receipt of DEMAND

2 Adjusted date of receipt of demand due to CORRECTIONS under Rule 60 1(b)

3 ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5 below does not apply ☐ The applicant has been informed accordingly

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80 5

5 ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date the delay in arrival is EXCUSED pursuant to Rule 82

For International Bureau use only

Demand received from IPEA on

237

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No	PCT/US01/14151
Applicant s or agent's file reference	21085 0029P1

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant
UAB Research Foundation, et al

Calculation of prescribed fees

1 Preliminary examination fee	490 00	P
2 Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee Where the applicant is (or all applicants are) so entitled the amount to be entered at H is 25% of the handling fee)*	137 00	H
3 Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	627 00	TOTAL

Mode of Payment

☐	authorization to charge deposit account with the IPEA (see below)	☐	cash
☒	cheque	☐	revenue stamps
☐	postal money order	☐	coupons
☐	bank draft	☐	other *(specify)*

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ US ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account

14-0629	3 December 2001	
Deposit Account Number	Date *(day/month/year)*	Signature

AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

Field of the Invention

The present invention relates to an antibody capable of specifically binding a single type of tumor necrosis factor (hereinafter referred to as "TNF")-related apoptosis-inducing ligand (hereinafter referred to as "TRAIL") receptor, more particularly, to a monoclonal antibody that induces apoptosis in *in vivo* and *vitro* cells expressing the single type receptor and therapies based thereon.

Background of the Invention

TRAIL is a member of the TNF family of proteins, which also includes TNF-α and Fas ligand (1) These proteins are potent inducers of apoptosis To date, five receptors for TRAIL have been identified, two of which, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (2-7), are capable of transducing the apoptosis signal while the other three DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG) do not transduce the apoptosis signal (8-12) All five receptors for TRAIL share significant homology in their extracellular ligand binding domains Similar to Fas and TNF receptor I (hereinafter referred to as "TNFRI"), the intracellular segments of both DR4 and DR5 contain a death domain, and transduce an apoptosis signal through a pathway that involves the Fas-associated death domain protein (hereinafter referred to as "FADD") and caspase 8 (6,7) In addition to transducing the apoptosis signal, the DR4 and DR5 receptors can also activate a pathway involving NFκb (6,7)

The biological functions of TRAIL that have been demonstrated include the capability of TRAIL to selectively induce apoptosis of transformed tumor cells, with normal cells being relatively resistant to TRAIL-mediated apoptosis (13-15) This selectivity suggests that, in contrast to Fas ligand, the administration of TRAIL is associated with very low levels of toxicity as demonstrated by systemic administration of TRAIL in an animal model without inducing significant toxicity (13) Thus, TRAIL has been proposed as a potent apoptosis inducing agent that would be a suitable therapeutic agent for the

treatment of cancer and other diseases associated with abnormal cell proliferation TRAIL also has been proposed to be a potent apoptosis inducing agent that would be suitable for the treatment of autoimmune and inflammatory diseases It has been demonstrated that TRAIL-mediated apoptosis is involved in activation-induced cell death of T cells, thereby serving as an alternative mechanism to Fas ligand (16,17) TRAIL-mediated apoptosis may also function in the induction of apoptosis of T cells and other inflammatory cells (18), and plays a role in the killing activity of NK cells (19-21), and in the immunomodulatory function of dendritic cells (22,23) Thus, TRAIL-mediated apoptosis may also function in immunoprivilege and immunosurveillance

The TRAIL receptor system is complex, and includes at least two death receptors, DR4 and DR5, and at least two non-apoptotic receptors, DcR1 and DcR2 All of these receptors not only share a high amino acid sequence homology, but also exhibit a similar binding affinity to TRAIL (2-12) The ability of the DcR1 and DcR2 receptors to compete for binding of TRAIL without inducing apoptosis suggests that they may act as decoy receptors that block or modulate the activity of the TRAIL ligand Moreover, it has been reported that untransformed cells express higher levels of decoy receptors than do transformed cells Thus, it has been proposed that the differential modulation of the expression of the death and decoy receptors may represent a key regulatory mechanism that determines the susceptibility of cells to TRAIL-mediated apoptosis, but due to the lack of receptor-specific antibodies (2) Although the expression and function of DR4 and DR5 have been studied extensively, progress has been impeded by the lack of receptor-specific monoclonal antibodies The cell surface expression of DR5 has not been documented It has been reported that a panel of anti-TRAIL receptor antibodies have been generated that are capable of inducing apoptosis of melanoma cells *in vitro* but only upon immobilization of the antibodies, to promote cross-linking, and, in some cases, the cells require culturing with actinomycin D (24) Several anti-DR5 antibodies have been generated (24) However, these previously generated anti-DR5 monoclonal antibodies have low apoptosis-inducing activity

in vitro, even under the conditions of crosslinking No *in vivo* activity has been reported These antibodies have not been used for examining cell surface expression of TRAIL receptors (24) Thus, there exists a need for a monoclonal antibody selective for each specific TRAIL receptor that is not only able to bind to cell surface receptor but also to strongly induce apoptosis of various types of abnormal cells, including tumor cells, both *in vivo* and *vitro* without the requirement for crosslinking or immobilization Such an antibody would not only provide potential therapeutic agent but also a diagnostic tool for functional analysis of TRAIL receptor There exists a particular need for an antibody specific against each of the death inducing receptors DR4 and DR5

In the development, or progression, of many diseases it is often the case that cells are not deleted In many autoimmune diseases and inflammatory conditions, the surviving activated cells attack normal tissues or cells Further, progression of tumorigenesis and the proliferative panus formation of rheumatoid arthritis are characterized by the unchecked proliferation of cells Thus, insufficient apoptosis leads to the development of disease, and the uses of apoptosis-inducing ligand or agonistic monoclonal antibody to enhance apoptosis are considered as a potential therapeutic strategy for eliminating those unwanted cells

For example, rheumatoid arthritis (hereinafter referred to as "RA") is a common human autoimmune disease The current understanding of the pathophysiology of RA is that autoimmune T cells and B cells initiate an inflammatory response in the joints, which drives hyperproliferation of the synoviocytes As a consequence of the hyperproliferation of synovial cells, metalloproteinases (hereinafter referred to as "MMPs") are over-produced, which further leads to the erosive destruction of the cartilage and bone that is characteristic of RA (25) Thus, the control of hyperproliferation of inflammatory synovial cells is a key step in the treatment of RA The molecular mechanisms leading to the hyperproliferation of synovial cells are still unknown Although the hyperproliferative synovial cells are non-malignant and non-transformed, many studies have suggested that they share some common

features with transformed cells (46) These cells, the so-called, "transformed-appearing synoviocytes", are characterized by a dense rough endoplasmic reticulum, numerous irregular nuclei, and changes in the normally spindle-shaped cell skeleton It has been proposed that the incorporation of the oncogenes and virus-derived genes might be the primary triggers for the transformed appearance of RA synovial cells (46)

At least two aspects of RA suggest that dysregulated apoptosis may contribute to the disease process and that therapeutic elicitation of apoptosis may be an effective treatment. the failure of the deletion of the activated T cells suggests that there is defective activation-induced cell death of these T cells, which is a process that involves Fas-mediated apoptosis and TRAIL-mediated apoptosis, and the hyperproliferative nature of the RA synovial cells is a contributing factor in the later stages of RA pathophysiology Indeed, it has been shown that the administration of anti-Fas antibody into the inflammatory joint inhibits the development of chronic arthritis in *tax* transgenic mice, which are an animal model for human RA (26) Moreover, localized transduction with the *fas ligand* gene by an adenoviral vector is effective in prevention of collagen-induced arthritis (27) Inhibition of the proliferation of inflammatory synovial cells by enhancement of Fas-mediated apoptosis is observed in both cases Although Fas ligand is a strong apoptosis inducer in RA synovial cells, the application of Fas ligand-mediated apoptosis as a therapy for humans has been limited by lethal liver toxicity Thus, TRAIL receptor induced apoptosis represents a safer and more effective therapeutic for the treatment of RA than Fas-ligand induced apoptosis

TRAIL receptor induced apoptosis also represents a safer and more effective therapeutic for the treatment of cancer than Fas-ligand induced apoptosis TRAIL-mediated apoptosis is known to specifically induce apoptosis of transformed tumor cells without affecting normal cells It has been shown that the systemic administration of the trimerized soluble TRAIL did not cause toxicity in experimental animals yet was able to induce regression of implanted tumors (13,28) Its potential as an adjunctive therapy for traditional treatments

was underscored by the recent finding that the expression of DR5 and susceptibility to TRAIL-induced apoptosis of breast cancer cells is enhanced by the radiation, suggesting that combined with radiation, the efficiency of TRAIL would be increased in cancer therapy (29)

In addition, the gene encoding the TRAIL receptor DR5 has been mapped to chromosome 8p21-22, a loci with a high frequency of mutation in some cancer cells (30) It has been reported that at least two kinds of tumor cells, small lung cancer (31) and head and neck cancer (32) exhibit mutations in the death domain of the DR5 gene Thus, there exists a need for an anti-DR5 antibody in cancer research to determine the effect of receptor epitope variation on the development and progression of cancers Further, the functionality of TRAIL receptor mutations would prove a useful clinical diagnostic tool when used in conjunction with other biomarkers in the early detection of cancers and as a predictor of the tumor aggressiveness

Summary of the Invention

An antibody is disclosed which recognizes a TRAIL receptor DR5 and which induces apoptosis in a DR5-expressing cell in vivo Further disclosed is an antibody that recognizes DR5 but not DR4, DcR1, nor DcR2 Specifically detailed is a monoclonal antibody produced by a hybridoma

A method provided by the invention allows inhibition of cell proliferation by exposing a cell to a therapeutic quantity of an antibody capable of binding to DR5 Also disclosed is a pharmacological composition that includes a therapeutic amount of monoclonal antibody active against a DR5, a pharmaceutically acceptable carrier and a container enclosing the antibody and the carrier Further provided by the invention is the use of an antibody recognizing DR5 for preparing a therapeutic for selective apoptosis of abnormal or dysregulated cells

An antibody of the present invention interacts with a tumor necrosis factor ligand receptor such as DR4, DR5, DcR1, DcR2 and OPG, inducing apoptosis in a cell expressing such a receptor Disclosed is an antibody of the

invention capable of selectively binding an agonistic or antagonistic tumor necrosis factor ligand receptor epitope

The present invention provides a treatment for an apoptosis related disease by a method that includes exposing a target tissue having an apoptosis related disease to a therapeutic quantity of an antibody of the invention

Further described is a fusion protein that includes an antigenic TRAIL receptor amino acid sequence having at least ten bases, coupled to an immunoglobulin protein or fragment thereof capable of eliciting an immune response within a subject

The present invention provides a method of gene therapy in which a target cell is transfected with a TRAIL receptor nucleic acid sequence in an expression vector so that the TRAIL receptor is expressed on the target cell The target cell is then exposed to an antibody that selectively binds the TRAIL receptor

Provided are nucleic acid sequences and amino acid sequences encoding the heavy and light chain immunoglobulins of an antibody selective for DR5 Also detailed are vectors that include a nucleic acid sequence of the invention and host cells transformed with a vector of the invention

The present invention provides a host cell producing a humanized TRA-8

A process for producing a humanized DR5 antibody is described in which a host is transformed with nucleic acid sequences encoding a humanized immunoglobulin light chain and a humanized immunoglobulin heavy chain after which the transformed host is incubated for a predetermined period of time

Also described is a process for inhibiting cell proliferation that includes contacting a target cell with a pharmaceutically effective amount of a humanized DR5 antibody

A commercial kit is provided for inducing cell death that includes a humanized TRA-8 antibody selective for DR5, packaged in a suitable container together with instructions for use

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Brief Description of the Drawings

Figure 1 Characterization of TRA-8 (a) Binding specificity of TRA-8 Western blot analysis (upper panel) Recombinant fusion proteins of the TNFR family probed with TRA-8 or anti-human IgG Lane 1 DR5/hIgG1 fusion protein (immunogen), Lane 2 DR4/hIgG1 (TRAIL-R1), Lane 3 DR5/hIgG1, Lane 4 TRAIL-R3 (DcR-1)/hIgG1, Lane 5 TRAIL-R4 (DcR-2)/hIgG1, Lane 6, CD95/hIgG1, Lane 7 soluble TNFRI ELISA analysis (lower panel) The well numbers match those of the Western blot except well 8 which is a murine DR5/hIgG1 fusion protein (b) Binding activity of soluble TRAIL and TRA-8 to DR5 and DR4 ELISA plates were coated with DR5/hIgG1 (left panel) or DR4/hIgG1 (middle panel) and then incubated with TRAIL or TRA-8 (c) Flow cytometry analysis of the surface expression of DR5 Cos-7 cells transfected with pcDNA3 expression vector containing the full-length DR5 cDNA (solid histogram), DR4 cDNA (open histogram, solid line) or empty vector (open histogram, dashed line) Forty-eight hours after transfection, cells were stained with TRA-8 followed by PE-conjugated anti-mouse IgG1 (d) in situ immunohistochemistry reactivity for DR5 Cytospin slides of Cos-7 cells transfected with DR5 expression or control vector were stained with TRA-8 at 48 hours after transfection, (e) Killing activity of TRA-8 Jurkat cells were incubated with the indicated concentrations of TRA-8 Cell viability was determined by ATPLite, MTT, and PI exclusion assays after overnight culture The results of ATPLite and MTT assays are presented as percent of medium control, and PI assay are presented as percent of PI negative cells (f) Western blot analysis of caspase activation Jurkat cells were incubated with 500 ng/ml TRA-8 for indicated time Cell lysates were separated by 15% SDS-PAGE, blotted, and probed with anti-caspase antibodies The arrows indicate the cleaved subunits of each caspase g Caspase inhibition assay Jurkat cells were incubated with 50 ng/ml TRA-8 overnight in the presence of various concentrations of indicated caspase inhibitors Cell viability was determined by the ATPLite assay

Figure 2 Cell surface expression of DR5 and susceptibility to DR5-mediated apoptosis Normal T and B cells, freshly isolated from peripheral blood, T cell (a and a'), glioma (b and b'), prostate cancer cell (c) and B cell (d) cell lines were incubated with TRA-8 or murine IgG1 isotype control antibody followed by PE-conjugated goat anti-mouse IgG1 The open histograms represent the isotype antibody control while the solid histograms represent TRA-8 staining Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles) as shown in a, b' and d

Figure 3a' T cell line U937 was incubated with TRA-8 or murine IgG1 isotype control antibody Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles)

Figure 3 Glioma (b) and prostate cancer (c) cell lines were incubated with TRA-8 or murine IgG1 isotype control antibody Apoptosis was determined by the ATPLite assay after overnight incubation with soluble TRAIL (open circles) or TRA-8 (closed circles)

Figure 4 is a series of graphs showing cell viability for human Jurkat cells after exposure to indicated concentrations of (A) antibody strains TRA-1, -8 and -10 and (B) TRAIL in the presence of a fixed concentration of the inventive antibody strains depicted in Figure 4A,

Figure 5 Expression of DR5 in normal and cancer tissues Normal and cancer tissue homogenates were probed with TRA-8 and developed by chemiluminescence (a) Western blot analysis of DR5 protein in normal tissues lane 1 liver, lane 2 brain, lane 3 lung, lane 4 kidney, lane 5 spleen, lane 6 testes Lane 7 ovary, lane 8 heart, lane 9 pancreas b Western blot analysis of DR5 protein in cancer tissues The cancer tissue blot containing cancers from the ovary (lane 1), lung (lane 2), liver (lane 3), rectum (lane 4), cervix (lane 5), skin (lane 6), testes (lane 7), thyroid (lane 8), uterus (lane 10), stomach (lane 11), laryngopharynx (lane 12), and pancreas (lane 13) was probed *In situ*

immunohistochemistry of normal human tissues(c) and of cancer tissues (d) Frozen sections were immunostained with TRA-8

Figure 6 Tumoricidal activity of TRA-8 SCID mice were inoculated subcutaneously with 1321N1 cells Mice were injected intravenously with a single dose of 100 μg TRA-8 on the second day after tumor inoculation (a), or with three doses of 100 μg TRA-8 beginning 7 days after tumor inoculation (b) Tumor growth was determined by the weight and examined histologically with H&E staining The photographs show viable tumor growth in control mice but not in TRA-8 treated mice (c , upper panel), and H&E staining of tumor (c , lower panel) SCID mice were injected intravenously with 10^6 Jurkat cells and treated with a single dose of TRA-8 on the second day after injection Seven days later, spleen cells were harvested, stained with anti-human CD3 antibody and analyzed by flow cytometry (d), or by immunohistochemistry (e)

Figure 7 shows expression of cell surface DR5 in RA (A) and OA (B) synovial cells 1 X 10^6 primary cultured synovial cells were stained with affinity-purified TRA-8 and followed by PE-conjugated goat anti-mouse IgG1 antibody 10,000 viable cells analyzed by FACSvantage

Figure 8 is a series of graphs showing cell viability as a function of TRAIL and TRA-8 concentration mduced apoptosis of representative strains of RA (A) and OA (B) synovial cells with various concentrations of the recombinant soluble TRAIL (the open circles) or affinity-purified TRA-8 (the closed circles) Cell viability is the percentage of the cpm of treated cells versus the cpm of untreated cells

Figure 9 is a series of graphs showing the caspase dependence of DR5-mediated apoptosis of RA synovial cells RA synovial cells (RA512) are incubated with 50 ng/ml of soluble Fas ligand (open squares), anti-Fas antibody (CH-11) (closed squares), soluble TRAIL (open circles), or anti-DR5 antibody (TRA-8) (closed circles) in the presence of variable concentrations of caspase inhibitors After overnight culture, cell viability is determined by ATPLite

Figure 10A is an electrophoretic gel-shift assav indicating NFkb activation RA1016 cells are incubated with 20 ng/ml TNF-a, 50 ng/ml soluble

TRAIL or 50 ng/ml TRA-8 for indicated time points before being subjected to electrophoresis Figures 10B and C are graphs showing the production of MMP-1 and MMP-3 1 X 10^6/ml of indicated RA synovial cells are incubated with the indicated concentrations of TNF-a (the open circles), TRAIL (the open triangles) or TRA-8 (the closed circle) After overnight culture, the culture supernatants are collected The levels of MMPs in culture supernatants are determined by ELISA

Figure 11 TRA-8 does not induce hepatocellular toxicity (a) Normal liver tissues do not express DR5 The paraffin sections of two normal liver tissues, one hepatocellular carcinoma tissue, and the cytospin preparation of HepG2 cells were prepared for H&E staining, and corresponding frozen sections were stained with TRA-8 (b) Flow cytometry analysis of cell surface expression of DR5 Hepatocytes, isolated from two normal liver tissues and from a case of hepatocellular carcinoma tissue, and HepG2 cells were stained with TRA-8, anti-Fas antibody (DX2) or an isotype control antibody The solid histograms indicate TRA-8 or DX2 staining, and the open histograms are the corresponding isotype controls

Figure 12 TRAIL but not TRA-8 induces hepatocellular toxicity Fresh normal human hepatocytes were maintained in Hepatocyte Culture Medium (a) Apoptosis of hepatocytes was induced with 1 μg/ml soluble TRAIL plus crosslinker or TRA-8 for the indicated time points Cell viability was determined by ATPLite The results are presented as percent viable cells compared to the medium control The shaded bars indicate TRAIL and the black bars indicate TRA-8 (b) The condensed nuclei of hepatocytes were stained with Hoechst 33352 and analyzed by flow cytometry (c) Effect of cycloheximide on hepatocytes apoptosis Hepatocytes were cultured in control medium or with 1 μg/ml TRAIL or TRA-8 in the presence (closed bars) or absence (open bars) of 1 μg/ml cycloheximide for 8 hours Cell viability was determined by ATPLite The results are presented as mean±SEM of triplicate cultures of two experiments d A comparison of the susceptibility of normal hepatocytes to DR5 and Fas-mediated apoptosis Freshly isolated hepatocytes were incubated

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with indicated concentrations of soluble TRAIL, TRA-8, soluble FasL or the anti-Fas mAb CH11 for 6 hours Cell viability was determined by ATPLite assay The results are presented as the percentage of viable cells compared to medium control For normal hepatocytes, mean±SEM of four normal individuals are presented The results of hepatocellular carcinoma cells from one patient and HepG2 cells are presented as the average of triplicate cultures.

Figure 13 TRAIL induces hepatitis B6 mice were intravenously inoculated with 10^9 pfu of adenoviral vector encoding the full length of human TRAIL under the control of the "Tet-on" transcriptional element TRAIL expression was induced by the indicated dose of tetracycline (a) Northern blot analysis of human TRAIL expression in the liver 24 hours after inoculation of vector and induction with tetracycline, total RNA was isolated from the livers and probed with human TRAIL cDNA or β-actin (b) Serum levels of AST 24 hours after transduction of TRAIL, serum levels of AST were determined (c) TRAIL-mediated cell death of adenoviral vector infected hepatocytes B6 mice were intravenously inoculated with tetracycline-inducible adenoviral vector 48 hours after inoculation, hepatocytes from inoculated and non-inoculated control mice were isolated and incubated with indicated concentrations of TRAIL for 8 hours (left panel) Cell viability of hepatocytes was determined by the ATPLite assay Mice, inoculated with adenoviral vector as above, were intravenously injected with 10 μg of soluble human TRAIL 48 hours later Serum levels of AST were measured at 24 hours after TRAIL injection (right panel) (d and e) Histology analysis of liver damage induced by TRAIL The livers were collected at 24 hours (d) or 7 days (e) after transduction with TRAIL The paraffin sections were H&E stained, and photographed at 100X (top panel) and 400X (lower panel)

Figure 14 is a series of graphs showing that activated T cells and B cells purified from human PBMC express increased levels of DR5 as determined by flow cytometry for resting (unfilled) and activated (shaded) cells

Figure 15 is viability graphs as a function of TRA-8 concentration for the purified T cells and B cells depicted in Figure 14 that have been stimulated for

48 hours with anti-CD3 or anti-μ, with activated and blast cells collected by different density of Ficoll-Paque Viability is determined by ATPLite assay

Figure 16 is a histogram and flow cytometry plots showing CD3 expression in a gated lymphocyte population for NK cell depleted NOD/SCID mice injected with human PBMC and TRA-8 or IgG (control)

Figure 17 shows CD3 and TUNEL stained cellular micrographs for mouse spleen tissue as detailed in Example 13

Figure 18 shows cycotoxicity plots for chronic lympholytic leukemia (CCL) and normal B cell humans in the presence of TRA-8, BISVIII, and the combination thereof

Detailed Description of the Invention

The failure to delete cells is due to defects in the apoptosis inducing system which are associated with defects illustratively including expression or function of the ligand, the receptor, or the intracellular regulatory and effector molecules The present invention affords a method to correct a deficient apoptosis inducing system as well as elucidate the specific defects inherent in a given defective apoptosis inducing system.

The present invention relates to a new class of monoclonal antibodies that have selective *in vivo* and *in vitro* apoptosis inducing activity against specific TRAIL receptors, including DR5, DR4, DcR1 and DcR2 The present invention has utility as a reagent for apoptosis signaling research, as well as a therapeutic effective against cells expressing TRAIL receptors illustratively including broad classes of cancer cells, disregulation of the apoptosis system and abnormally proliferating synovial cells of autoimmune diseases Antibodies according to the present invention are specific in binding particular types of TRAIL receptors in spite of the homology therebetween The inventive antibodies afford targeted apoptosis of only those cells expressing a target TRAIL receptor or alternatively, blocking TRAIL apoptosis of cells expressing a target receptor

An anti-DR5 monoclonal antibody of the present invention serves as a potent inducer of apoptosis in cells expressing DR5 *in vitro* and as a potent

inducer of apoptosis *in vivo* Humanized fragmentary CDR sequences engrafted on humanized antibody backbones and fusion protein anti-DR5 antibodies of the present invention exhibit similar apoptotic properties

To date, no monoclonal antibody is available which binds to cell surface DR5 and induces apoptosis of cells expressing DR5 both *in vitro* and *in vivo* in the absence of a crosslinker The present invention includes an anti-DR5 antibody operative as a therapeutic agent in animal models of disease, such as xenografted animals, or *in vivo* Although soluble TRAIL has been shown to be effective in induction of apoptosis of tumor cells *in vivo*, the killing activity appeared to be very low with the large and repeated doses often being required (13) TRA-8, one of a series of anti-DR5 antibodies according to the present invention, is pharmaceutically effective in animals carrying a human DR5 transgene and also has utility in establishing a model for the investigation of the role of DR5 and TRAIL

An antibody according to the present invention raised against a TRAIL receptor is harvested according to the present invention from an experimental animal. By humanizing the antibody according to the present invention to maintain receptor binding activity while eliciting a diminished and therapeutically tolerable immune response within a human subject, a humanized anti-TRAIL receptor antibody according to the present invention is used as therapeutic agonist or antagonist for a given TRAIL receptor The present invention being operative as an *in vivo* therapeutic since secondary crosslinking of the anti-TRAIL receptor antibody is not required

The present invention extends beyond a single anti-TRAIL receptor antibody having agonist or antagonistic apoptotic effects Rather, two or more anti-TRAIL receptor antibodies are brought into contact with a cell culture *in vitro* or a subject body tissue *in vivo* to create a synergistic treatment For example, glioma cell line U87 and hematopoietic cell lines U937 and Molt-4 are responsive to exposure to a synergistic exposure to agonistic anti-DR4 and anti-DR5 antibodies whereas exposure to agonistic anti-DR5 antibody alone shows only limited success in inducing apoptosis

Additionally, antagonistic anti-TRAIL receptor antibodies have particular utility in the present invention when an antibody is specific to binding one of the decoy receptors DcR1, DcR2 or OPG Selective blocking of a decoy receptor with an antibody according to the present invention has the effect in cell types expressing decoy receptors of shifting the TRAIL binding equilibrium towards those TRAIL receptors capable of transducing the apoptosis signal Thus, in another combined therapy according to the present invention, a decoy receptor binding antibody sensitizes an expressing cell towards agonistic apoptosis signal transducing TRAIL receptor binding

In another embodiment, the present invention affords a method of elucidating agonistic and antagonistic epitopes of a given TRAIL receptor Further, polymorphisms between individuals associated with a given TRAIL receptor are elucidated according to the present invention through the use of a panel of monoclonal antibodies each having a differing variable or CDR region A characterized panel of monoclonal antibodies provides the ability to define agonistic and antagonistic epitopes and polymorphisms Thus, a panel of monoclonal antibodies according to the present invention has utility in drug discovery and/or subject screening for disease proclivity

Still another embodiment of the present invention involves fusion proteins including an antigenic fragment of a TRAIL receptor coupled to an immunoglobulin protein, polypeptide or fragment thereof A TRAIL receptor fragment being defined as containing a sufficient number of bases to elicit an immunogenic response to a native TRAIL receptor expressed on a subject cell surface A TRAIL receptor fusion fragment including at least ten amino acids An immunoglobulin fusion protein or fragment thereof is defined herein to include a native or synthetic protein or polypeptide segment having a sufficient number of amino acid bases to activate an immunogenic cascade response within a subject An immunogen of the present invention including a fusion of a TRAIL receptor fragment coupled to an immunoglobulin fragment has utility as an *in vivo* therapeutic to elicit an anti-TRAIL receptor antibody in situ within a subject

In still a further embodiment, the present invention is operative as a gene therapy In a gene therapy aspect of the present invention, targeted cells are transfected with a vector carrying an expressible sequence corresponding to a TRAIL receptor The vector being conventional and chosen on the basis of the targeted cell susceptibility to the vector Gene therapy vectors illustratively include adenovirus, pAdCMV5 Upon the targeted cells or tissue expressing the transfected TRAIL receptor, the cells or tissue are exposed to an antibody according to the present invention specific for binding the transfected TRAIL receptor It is appreciated that the anti-TRAIL receptor antibody is either agonistic or antagonistic thereto consistent with the desired therapeutic result

The antibodies of the present invention are also operative in conjunction with a sensitizer A sensitizer as used herein is defined to include any stimulus that induces apoptosis including ultraviolet light, organic molecules specifically including the class of bisindolmaleimides, heavy metals and free radical species

In the context of a malignancy therapy, TRA-8, is able to induce apoptosis of most TRAIL-sensitive tumor cells in a caspase-dependent fashion in the absence of the secondary crosslinking TRA-8 exhibits a strong tumoricidal activity *in vivo* The ability of TRA-8 to induce apoptosis of most TRAIL-sensitive cells confirms that DR5 alone is sufficient to trigger apoptosis The majority of tumor cells detailed herein express cell surface DR5 and their susceptibility to TRA-8 induced cell death paralleled their susceptibility to TRAIL, indicating that DR5 is a primary death receptor for TRAIL-mediated apoptosis in most tumor cells Thus, differential expression of DR5 by normal and cancer cells is operative in the selectivity of TRAIL-mediated apoptosis TRA-8 bypasses the decoy receptors to induce TRAIL-mediated apoptosis Only a minority of TRAIL resistant tumor cells are sensitive to TRA-8, however, indicating that the decoy receptors do not appear to play a major role in the resistance of tumor cells to TRAIL-mediated apoptosis

Although previous studies have indicated that systemic administration of the soluble form of TRAIL in animals does induce tumor regression without causing toxicity[3,4,22], the membrane-bound form of human TRAIL induces liver

damage in mice as shown herein However, the hepatic toxicity of TRAIL is much less potent than that of Fas ligand as demonstrated by the lesser susceptibility of normal hepatocytes to TRAIL-induced injury compared to Fas ligand and by the lack of lethality of TRAIL *in vivo* Thus, titration of TRAIL has utility in cancer therapy

As detailed herein, the absence of significant levels of DR5 protein expression by normal hepatocytes is shown and is associated with hepatocyte resistance to TRA-8 induced apoptosis Crosslinking of DR5 with monoclonal antibody is insufficient to organize the homopolymeric forms of the death receptor able to trigger apoptosis Experiments in marmoset indicate no evidence of hepatic toxicity of TRA-8 administration Thus, an agonistic monoclonal anti-DR5 antibody is likely to be more selective and safer than soluble TRAIL as a therapeutic agent.

As a screening assay, the present invention is well suited for detecting small clusters of malignant cells which may still exhibit normal cell morphology *In situ* cell section staining of human cancer cells including lung, prostate and liver cancers with labeled antibodies according to the present invention readily identifies cancerous cells These cancer cells are observed to express very high levels of DR5 as compared to normal cells of the same type Thus, the present invention has utility as a sensitive screening method for early stage malignancies within tissue including at least lung, prostate and liver A therapeutic process is detailed herein for the inhibition of abnormal cell proliferation associated with diseases illustratively malignant cancers and lymphatic leukemias

The present invention is detailed herein with particularity to an anti-human DR5 monoclonal antibody designated as TRA-8, having ATCC Accession Number PTA-1428 It is appreciated that the techniques and results detailed with regard to the agonistic anti-human DR5 monoclonal antibody TRA-8 are wholly extendable and applicable to antagonistic DR5 antibodies, as well as antibodies raised against DR4, DcR1 and DcR2 acting in both agonistic and antagonistic manners

The levels of expression of an apoptosis receptor, such as Fas, do not necessarily correlate with the susceptibility of the cells to apoptosis For TRAIL-mediated apoptosis, it has been suggested that the expression of the decoy receptors for TRAIL influences the susceptibility of the cells Moreover, it has been suggested that DR5 must be associated with DR4 for effective transduction of the apoptosis signal through FADD and the caspase 8 pathway The availability of agonistic monoclonal anti-DR5 antibody allowed evaluation of the regulation of DR5 signaling and its relative role in TRAIL-mediated apoptosis Comparison of the susceptibility of the cells to TRA-8-mediated apoptosis with their susceptibility to TRAIL-mediated apoptosis offers insight into the role of DR5 in TRAIL-mediated apoptosis and the mechanisms that may affect susceptibility

This advantage generally extends to humanized anti-DR5 antibodies of the present invention. A molecular clone of an antibody to DR-5 is prepared by known techniques as detailed with respect to the following Examples Recombinant DNA methodology (33) is operative herein to construct nucleic acid sequences which encode a monoclonal antibody molecule or antigen binding region thereof

The present invention allows the construction of humanized anti-TRAIL receptor antibodies that are unlikely to induce a human anti-mouse antibody (heremafter referred to as "HAMA") response (34), while still having an effective antibody effector function As used herein, the terms "human ' and "humanized", in relation to antibodies, relate to any antibody which is expected to elicit a therapeutically tolerable weak immunogenic response in a human subject.

The present invention provides for an anti-DR5 antibody, a humanized anti-DR5 antibody, TRA-8 heavy and light chain immunoglobulins and humanized heavy and light chain immunoglobulins Certain truncations of these proteins or genes perform the regulatory or enzymatic functions of the full sequence protein or gene For example, the nucleic acid sequences coding therefor can be altered by substitutions, additions, deletions or multimeric

expression that provide for functionally equivalent proteins or genes Due to the degeneracy of nucleic acid coding sequences, other sequences which encode substantially the same amino acid sequences as those of the naturally occurring proteins may be used in the practice of the present invention These include, but are not limited to, nucleic acid sequences including all or portions of the nucleic acid sequences encoding the above polypeptides, which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change It is appreciated that the nucleotide sequence of an immunoglobulin according to the present invention tolerates sequence homology variations of up to 25% as calculated by standard methods ("Current Methods in Sequence Comparison and Analysis," *Macromolecule* Sequencing and Synthesis, Selected Methods and Applications, pp 127-149, 1998, Alan R. Liss, Inc) so long as such a variant forms an operative antibody which recognizes a TRAIL receptor DR5 For example, one or more amino acid residues within a polypeptide sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine The positively charged (basic) amino acids include arginine, lysine and histidine The negatively charged (acidic) amino acids include aspartic acid and glutamic acid Also included within the scope of the present invention are proteins or fragments or derivatives thereof which are differentially modified during or after translation, e g , by glycosolation, protolytic cleavage, linkage to an antibody molecule or other cellular ligands, etc In addition, the recombinant vector encoding nucleic acid sequences of the anti-DR5 antibodies of the present invention may be engineered so as to modify processing or expression of a vector

Additionally, an inhibitor encoding nucleic acid sequence can be mutated *in vitro* or *in vivo* to create and/or destroy translation, initiation, and/or termination sequences or to create variations in coding regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification Any technique for mutagenesis known in the art can be used, including but not limited to *in vitro* site directed mutagenesis, *J Biol Chem* 253 6551, use of Tab linkers (Pharmacia), and the like

X-ray crystallography data indicates that the antibody immunoglobulin fold generally forms a long cylindrical structure comprising two layers of antiparallel b-sheets, each consisting of three or four b-chains In a variable region, three loops from each of the V domains of H and L chains cluster together to form an antigen-binding site Each of these loops is termed a complementarity determining region (CDR) The CDRs have the highest variability in amino acid sequence with the antibody The portions of the variable region that are not part of a CDR are called "framework regions" ("FR" regions) and generally play a role in maintaining CDR structure Preferably, all the CDRs from a given antibody are grafted into an acceptor antibody, in order to preserve the binding region for the TRAIL receptor epitope region It is appreciated that grafting a portion of the total amount of CDRs into a donor is operative herein It is understood that grafting generally entails the replacement, residue for residue, of one amino acid or region, for another However, occasionally, especially with the transfer of a region, one or more residues may be added or omitted or substituted therefor, as desired, and that such deletions and insertions, as well as appropriate replacements and inversions, are within the skill of those in the art.

An antibody of the present invention is obtained by, for example, grafting each CDR of L chain and H chain subunit of an anti-TRAIL receptor monoclonal antibody into a corresponding CDR region of a human antibody, thereby humanizing a mouse monoclonal antibody effective against a TRAIL-receptor

Antibody fragments which contain the idiotype of the molecule are also generated and operative herein using known techniques For example, such fragments illustratively include the anti-TRAIL receptor (AB')2 fragment which can be produced by pepsin digestion of the antibody molecule, the TRAIL receptor antibody AB' fragments generated through reduction of the disulfide bridges of the TRAIL receptor (AB')2 fragment, and the antibody fragment which are generated by treating the antibody molecule with papain and a reducing agent.

In particular, the anti-DR5 monoclonal antibody TRA-8 may be obtained by culturing a hybridoma which, in turn, may be obtained by immunizing a mouse with human DR5 and subsequently fusing the spleen cells or lymph node cells from the mouse with mouse myeloma cells

Preparation of a monoclonal antibody illustratively involves the following steps

a) purification of a biomacromolecule for use as an antigen,

b) preparation of antibody producing cells, after first immunizing an animal using injections of the antigen, bleeding the animal and assaying the antibody titer, in order to determine when to remove the spleen,

c) preparation of myeloma cells,

d) fusing the antibody producing cells and myeloma cells,

e) selecting a hybridoma producing a desired antibody,

f) preparing a single cell clone (cloning),

g) optionally, culturing the hybridoma cells, or growing animals into which the hybridoma cells have been transplanted, for large scale preparation of the monoclonal antibody; and

h) testing the biological activities and the specificity, or assaying marker agent properties, of the monoclonal antibody thus prepared.

The procedure for the preparation of an anti-DR5 monoclonal antibody is detailed below with reference to the above described steps This method for

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preparing an antibody of the present invention is intended only to be illustrative of the methods of preparation and is not limited thereto Other known procedures may be followed, or the following method modified, for instance by using antibody producing cells other than spleen cells and myeloma

(a) Preparation of antigen

A recombinant protein (hereinafter referred to as "recombinant human DR5"), effective as the antigen, is obtained by transfecting QBI-293A cells with the expression vector pAdDR5-IgG for a fusion protein comprising the extracellular domain of human DR5 and the Fc region of human IgG1 antibody (hereinafter referred to as "IgG"), (cf PTA-1428) to express it by using the ADENO-Quest kit (Quantum Biotechnologies Inc , Canada), and collecting and partially purifying the expression product The plasmid pAdDR5-IgG is constructed by inserting DNA encoding a human DR5 and human IgG fusion protein into pAdCMV5, which is an expression vector for animal cells Other materials, such as the DNA encoding DR5, the vector, and the host, are operative herein

The human DR5 and IgG fusion protein produced in the culture supernatant of the QBI-293A cells transfected with the vector pAdDR5-IgG may be partially purified by ProteinA-Sepharose affinity chromatography or ProteinG-Sepharose affinity chromatography, or ion-exchange chromatography using a Resource Q column (trade name, Pharmacia)

Alternatively, purified DR5 obtained from the cell membranes of human cell lines is used as the antigen Further, since the primary structure of DR5 is known (cf PTA-1428), a peptide comprising the amino acid sequence of SEQ ID No 1, may be chemically synthesized by a known method such as the Sanger method, and used as the antigen

(b) Preparation of antibody producing cells

A mouse is immunized with the immunogen produced in step (a), mixed with an adjuvant, such as Freund's complete or incomplete adjuvant or alum Other suitable experimental animals illustratively include rats, guinea pigs, rabbits, dogs, chickens, horses, pigs, cows and sheep

Suitable administration routes to immunize an experimental animal include the subcutaneous, intraperitoneal, intravenous, intradermal, and intramuscular injection routes, with subcutaneous and intraperitoneal injections being preferred

Immunizations are optionally performed by a single dose or, by several repeated doses at appropriate intervals (preferably 1 to 5 weeks) Immunized animals are monitored for antibody titer in their sera, and an animal with a sufficiently high antibody titer is selected as the source of antibody producing cells Selecting an animal with a high titer makes the subsequent process more efficient Cells for the subsequent fusion are generally harvested from the animal 3 to 5 days after the final immunization

Methods for assaying antibody titer include various well known techniques such as radioimmunoassay (hereinafter, referred to as "RIA"), solid-phase enzyme immunoassay (hereinafter, referred to as "ELISA"), fluorescent antibody assay and passive hemagglutination assay, with RIA and ELISA preferred for reasons of detection sensitivity, rapidity, accuracy and potential for automation

Determination of antibody titer may be performed, for example, by ELISA, as follows First, purified or partially purified DR5 is adsorbed onto the surface of a solid phase, such as a 96-well ELISA plate, followed by blocking any remaining surface, to which DR5 has not been bound, with a protein unrelated to the antigen, such as bovine serum albumin (BSA) After washing, the well surfaces are contacted with serially diluted samples of mouse sera to enable binding of the anti-DR5 antibody in the samples to the antigen An enzyme-labeled, anti-mouse antibody, as the secondary antibody, is added to be bound to the mouse antibody After washing, the enzyme substrate is added, and antibody titer is estimated by determining absorbance change due to color development caused by the alteration of the substrate or the like

(c) Preparation of myeloma cells

Cells from established mouse cell lines serve as the source of myeloma cells, including for example 8-azaguanine resistant mouse, derived from

BALB/c myeloma strains P3X63Ag8U 1 (P3-U1) (35), P3/NSI/1-Ag4-1(NS-1) (36) Sp2/0-Ag14 (SP-2) (37), P3X63Ag8 653 (653) (38) and P3X63Ag8 (X63) (39) The cell line selected is serially transferred into an appropriate medium, such as 8-azaguanine medium 8-azaguanine medium includes Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM") or Dulbecco's, Modified Eagle Medium (hereinafter referred to as "DMEM") RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, fetal calf serum (hereinafter referred to as "FCS"), and 8-azaguanine The cells are then transferred to a normal medium, such as ASF104 medium (Ajinomoto, K. K) containing 10% FCS, 3 to 4 days prior to fusion, in order to ensure that at least 2×10^7 cells are available on the day of fusion

(d) Cell fusion

Lymphocytes and plasma cells obtained from any suitable part of the animal are precursor cells to produce the antibody Lymphocyte or plasma cell sources illustratively include spleen, lymph nodes, peripheral blood, or any appropriate combination thereof, with spleen cells being the most common source

After the last booster injection, tissue in which antibody producing cells are present is removed from a mouse having the predetermined antibody titer The currently favored technique for fusion of spleen cells with myeloma cells prepared in step c), employs polyethylene glycol

The fusion technique includes washing spleen and myeloma cells with serum-free medium (such as RPMI 1640) or phosphate buffered saline (hereinafter referred to as "PBS") so that the number ratio of spleen cells to myeloma cells is approximately between 5 1 and 10 1, and then centrifuged After the supernatant has been discarded and the pelleted cells sufficiently loosened, 1 ml of serum-free medium containing 50%(w/v) polyethylene glycol (m w 1,000 to 4,000) is added dropwise with mixing Subsequently, 10 ml of serum-free medium is slowly added and then centrifuged The supernatant is discarded again, and the pelleted cells are suspended in an appropriate amount of HAT medium containing a solution of hypoxanthine, aminopterin and thymidine

(hereinafter referred to as "HAT") and mouse interleukin-2 (hereinafter referred to as "IL-2") The suspension is then dispensed into the wells of culture plates (also referred herein simply as "plates") and incubated in the presence of 5% v/v CO_2 at 37°C for about 2 weeks, with the supplementary addition of HAT medium as appropriate

e) Selection of hybridomas

When the myeloma strain used is resistant to 8-azaguanine, i e , it is deficient in the hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme, any unfused myeloma cells and any myeloma-myeloma fusions are unable to survive in HAT medium On the other hand, fusions of antibody producing cells with each other, as well as hybridomas of antibody producing cells with myeloma cells can survive, the former only having a limited life Accordingly, continued incubation in HAT medium results in selection of only the desired hybridomas

The resulting hybridomas grow into colonies that are then transferred into HAT medium lacking aminopterin (HT medium) Thereafter, aliquots of the culture supernatant are removed to determine anti-Fas antibody titer by, for example, ELISA When the above-mentioned fusion protein is used as the ELISA antigen, it is also necessary to eliminate clones producing an antibody which is specifically bound to the Fc region of human IgG1 The presence or absence of such a clone may be verified, for example, by ELISA using Fas-IgG1or IgG1, as the antigen

(f) Cloning

Hybridomas which have been shown to produce specific antibodies, using a method similar to that described in step b) to determine antibody titer, are then transferred to another plate for cloning Suitable cloning methods include the limiting dilution method, in which hybridomas are diluted to contain one cell per well of a plate and then cultured, the soft agar method in which colonies are recovered after culturing in soft agar medium, a method of using a micromanipulator to separate a single cell for culture, and "sort-a-clone", in which single cells are separated by a cell sorter

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The cloning procedure according to, for example, the limiting dilution method is repeated 2 to 4 times for each well demonstrating an antibody titer, and clones having stable antibody titers are selected as anti-DR5 monoclonal antibody producing hybridomas Hybridomas producing an anti mouse DR5 antibody are selected by a similar method to obtain an anti-DR5 monoclonal antibody producing cell line

The mouse-mouse hybridoma TRA-8 which is a basis for antibodies of the present invention was deposited with American Type Culture Collection on March 1, 2000, and has the accession number PTA-1428 Accordingly, when preparing an antibody using the mouse-mouse hybridoma TRA-8 or any other established hybridoma, the preparation may be performed by following a procedure starting from the step (g) below, with the steps (a) to (f) omitted

(g) Culture of hybridoma to prepare monoclonal antibody

The hybridoma obtained by the cloning is then cultured in normal medium, not in HT medium Large-scale culture is performed by roller bottle culture, using large culture bottles, or by spinner culture The supernatant from the large-scale culture is then harvested and purified by a suitable method, such as gel filtration, which is well known to those skilled in the art, to obtain an anti-DR5 monoclonal antibody which is a basis for antibodies of the present invention The hybridoma may also be grown intraperitoneally in a syngeneic mouse, such as a BALB/c mouse or a nu/nu mouse, to obtain ascites containing an anti-DR5 monoclonal antibody in large quantities Commercially available monoclonal antibody purification kits (for example, MAbTrap GII Kit, Pharmacia) are conveniently used to purify the harvested antibodies

Monoclonal antibodies prepared as above have a high specificity for human DR5

(h) Assay of monoclonal antibody

Suitable identification methods of the isotype and the subclass of the monoclonal antibody include the Ouchterlony method, ELISA and RIA Preferably, a commercial kit is used for identification, such as a Mouse Typer Kit (trade name, BioRad)

Quantification of protein may be performed by the Folin-Lowry method, or by calculation based on the absorbance at 280 nm (1 4 (OD280) = Immunoglobulin 1 mg/ml)

Identification of the epitope that the monoclonal antibody recognizes are performed as follows First, various partial structures of the molecule that the monoclonal antibody recognizes are prepared The partial structures are prepared by the method wherein various partial peptides of the molecule are synthetically prepared by known oligopeptide synthesis technique, or the method wherein DNA encoding the desired partial polypeptide is incorporated in a suitable expression plasmid, and is expressed in a suitable host, such as *E coli*, to produce the peptides Generally, both methods are frequently used in combination for the above object For example, a series of polypeptides having appropriately reduced lengths, working from the C- or N-terminus of the antigen protein, can be prepared by established genetic engineering techniques By establishing which fragments react with the antibody, an approximate idea of the epitope site is obtained

The epitope is more closely identified by synthesizing a variety of smaller oligopeptides corresponding thereto or mutants of the peptide using established oligopeptide synthesis techniques to determine a binding property of the peptides to the anti-DR5 monoclonal antibody which is a basis for preparation of the antibody of the present invention and a competitive inhibition of binding of the peptide to an antigen with the monoclonal antibody Commercially available kits, such as the SPOTs Kit (Genosys Biotechnologies, Inc) and a series of multipin peptide synthesis kits based on the multipin synthesis method (Chiron Corp) may be conveniently used to obtain a large variety of oligopeptides

An antibody of the present invention has the various functional properties a) to f) described below, each of which is verified by, for example, a method described hereinbelow

a) Specific binding of TRA-8 to cells expressing human DR5

A unique feature of the present invention is the ability to bind cell surface DR5 This is demonstrated by flow cytometry analysis of cells expressing DR5 First, specific cell surface binding of DR5 is confirmed by the COS-7 cells transfected with the full-length cDNA encoding human DR5 Specifically, TRA-8 only recognizes COS-7 cells transfected with DR5 but not empty control vector or vector encoding DR4 Second, three different origins hematopoietic, glioma, and prostate cancer of human malignant tumor cells are tested The majority of these transformed tumor cells expressed significant levels of cell surface DR5, although expression levels varied largely Third, two panels of human primary synovial fibroblast cells from RA and OA patients are examined All RA synovial cells expressed significantly higher levels of DR5 compared to OA cells

b) Induction of apoptosis of human malignant tumor cells *in vitro* in the absence of crosslinking

The ability of an antibody raised according to the present invention to recognize TRAIL receptor and to directly induce apoptosis of malignant human tumor cells is determined by cell viability assay (ATPLite) during *in vitro* culture of cells with various concentrations of an antibody, specifically TRA-8 The majority of tumor cells are susceptible to TRA-8 induced apoptosis For some cells, TRA-8 exhibited a strong apoptosis-inducing activity, for example, TRA-8 is able to induce apoptosis of human Jurkat cells within the pg/ml levels Importantly, TRA-8 induced apoptosis did not require crosslinking, and in most cells, TRA-8 exhibited a stronger apoptosis-inducing activity than the recombinant soluble TRAIL in the presence of the enhancer

c) Tumoricidal activity of TRA-8 *in vivo*

Tumoricidal activity of TRA-8 is evaluated in two SCID/human tumor cell models First, SCID mice are intravenously inoculated with human leukemia Jurkat cells, and treated with a single dose (100 μg) of TRA-8 The results show that the majority of implanted Jurkat cells are eliminated from the peripheral blood and spleen by the treatment with TRA-8, as determined by flow cytometry analysis and in situ immunohistochemical staining of Jurkat cells

Second, human astrocytoma cells, 1321N1, are subcutaneously inoculated in SCID mice, and the tumor-bearing mice are treated with a single dose of TRA-8 The growth of implanted 1321N1 cells is significantly inhibited in TRA-8 treated mice as determined by the sizes of tumor and histological analysis

d) Identification of RA synovial cells by TRA-8

The primary synovial cells isolated from 8 RA and 4 OA patients are tested for cell surface expression of DR5 TRA-8 is able to positively strain all RA cells but negatively stain all OA cells Thus, RA is differentiated from OA by the surface expression of DR5 as detected by TRA-8

e) Induction of apoptosis in RA synovial fibroblast cells by TRA-8

The ability of TRA-8 to induce apoptosis of RA synovial cells is determined by cell viability assay during *in vitro* culture in the presence of various concentrations of TRA-8 All RA cells exhibited high to intermediate levels of susceptibility to 100 ng/ml of TRA-8 In contrast, all OA cells are essentially resistant to TRA-8 induced apoptosis Importantly, TRA-8 exhibited a better apoptosis-inducing activity to RA synovial cells than soluble TRAIL with the enhancer Moreover, compared to anti-Fas antibody (CH-11), TRA-8 exhibited a better selectivity to RA synovial cells

f) TRA-8 does not induce production of MMPs in RA synovial cells

Since TRA-8 is able to induce NF-kb activation in RA synovial cells as TNF-a, the effect of TRA-8 on the production of MMP1 and MMP3 of synovial cells is determined While TNF-a induced a dose-dependent increase of MMPs, TRA-8 is unable to induce any production of MMPs, and in some concentrations, TRA-8 slightly decreased the production of MMPs in RA synovial cells

g) TRA-8 induces multiple caspase activation

Since caspases play a crucial role in induction of apoptosis The ability of TRA-8 to induce caspase activation is determined in human Jurkat cells When Jurkat cells are incubated with a low dose (50 ng/ml) of TRA-8, the activation of caspase 8, caspase 9, and caspase 3 is observed as early as 15 minutes after incubation as demonstrated by Western blot analysis and caspase cleavage analysis In term of timing, number and strength of caspase activation,

antibodies of the present invention including the demonstrative antibody TRA-8 exhibited a much better activity than any other known apoptosis-inducing antibodies, such as anti-human Fas antibody (CH-11)

Thus, an antibody of the present invention is a substance having a property to selectively induce apoptosis in pathogenic cells as shown in effect (a) and (g) Accordingly, it is useful as a prophylactic and therapeutic agent for diseases associated with inappropriate survival of cells or inappropriate proliferation of cells, such as those attributable to dysregulation of apoptosis systems including the Fas/Fas ligand system

The ability of an antibody of the present invention to induce apoptosis is confirmed by culturing cells such as the human leukemia cell line Jurkat (American Type Culture No TIB-152) and astrocytoma cell line 1321N1 in medium in which the test sample has been added, and determining the survival rate by, for example, an ATPLite assay

Antibody of the present invention, especially anti-DR5 antibodies having almost the same immunogenicity to human as that of human antibodies, is used as an agent for prophylaxis or treatment of diseases associated with inappropriate survival or proliferation of cells, including those attributable to dysregulation of the apoptosis systems in autoimmune diseases illustratively including systemic lupus erythematosus, Hashimoto's disease, rheumatoid arthritis, graft-versus-host disease, Sjögren's syndrome, pernicious anemia, Addison disease, scleroderma, Goodpasture s syndrome, Crohn s disease, autoimmune hemolytic anemia, sterility, myasthenia gravis, multiple sclerosis, Basedow's disease, thrombopenia purpura, insulin-dependent diabetes mellitus, allergy, atopic disease, arteriosclerosis, myocarditis, cardiomyopathy, glomerular nephritis, hypoplastic anemia, rejection after organ transplantation and numerous malignancies of lung, prostate, liver, ovary, lymphatic and breast tissues

Such a prophylactic or therapeutic agent may be administered in various forms Suitable modes of administration include oral administration, such as by

tablets, capsules, granules, powders and syrups, or parenteral administration, such as by injection, dropping injection and suppositories

The antibody or therapeutic agent may be administered orally, rectally, intracisternally, intraventricular, intracranial, intrathecal, intravaginally, parenterally (intravenously, intramuscularly, or subcutaneously), locally (powders, ointments, or drops), by intraperitoneal injection, transdermally, by inhalation or as a buccal or nasal spray The exact amount of the antibody or therapeutic agent required will vary from subject to subject, depending on the age, weight and general condition of the subject, the severity of the disease that is being treated, the location and size of the tumor, the particular compounds used, the mode of administration, and the like An appropriate amount may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein Typical single dosages of antibody range from 0 1-10,000 micrograms, preferably between 1 and 100 micrograms Typical antibody concentrations in a carrier range from 0 2 to 2000 nanograms per delivered milliliter

Depending on the intended mode of administration, the antibody or therapeutic agent can be in pharmaceutical compositions in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, or suspensions, preferably in unit dosage form suitable for single administration of a precise dosage The compositions will include an effective amount of the selected substrate in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, or diluents By "pharmaceutically acceptable' is meant a material that is not biologically or otherwise undesirable, which can be administered to an individual along with the selected substrate without causing significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions,

variable region in terms of energy The above procedure is performed using the commercially available common molecular modeling system ABM (Oxford Molecular Limited, Inc) For the molecular model obtained, the accuracy of the structure is further evaluated using the software, PROCHECK (J Appl Cryst. (1993), 26, 283-291)

(2) Designing the amino acid sequences for humanized TRA-8

Construction of humanized TRA-8 antibodies is performed by the method generally known as CDR grafting (Proc Natl Acad Sci USA 86, 10029-10033 (1989)) The acceptor antibody is chosen based on the amino acid homology in the framework region The sequences of framework region in TRA-8 are compared with all the human framework sequences in the Kabat database of amino acid sequences of antibodies (Nuc Acid Res 29, 205-206 (2001)) As a result, mAB58'CL antibody is selected as an acceptor due to the highest sequence homology of 80% for the framework region The amino acid residues in the framework region for mAb58'CL are aligned with that for TRA-8 and the positions where different amino acids are used are identified The location of those residues are analyzed using the three dimensional model of TRA-8 constructed above and the donor residues which should be grafted on the acceptor are chosen by the criteria given by Queen et al (Proc Natl Acad Sci USA 86, 10029-10033 (1989)) Humanized TRA-8 sequences are constructed as described in the following example by transferring several donor residues into acceptor antibody, mAb58'CL

Example 18 Construction of an Expression Vector for the Heavy Chain of the Humanized Antibody

(1) Construction of plasmid carrying the heavy chain variable region DNA of Humanized TRA-8

In order to determine the activity of humanized TRA-8, the plasmid carrying the heavy chain of humanized TRA-8 is constructed as follows However, it is appreciated the humanization of TRA-8 is not limited to these examples

As shown in SEQ ID No 31 of the Sequence Listing, humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 84th amino acid (serine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, glycine, glycine, asparagine, alanine, valine, leucine, and valine, respectively

The plasmid carrying DNA encoding heavy chain variable region of humanized TRA-8 (SEQ ID No 31 of the Sequence Listing) are constructed as follows

PCR is used to construct the following DNA sequences, each of which comprised described above

The following 12 oligonucleotides are synthesized

5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32),

5'- tctgaagtaa tgctggtgga gtctgggggga ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctc cacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

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5'- tccagaccct tccctggtgc ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtaa ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42), and

5'- gaccgatggg cccttggtgg aggctgagga gactgtgacc agggtccctt ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43)

The following 2 PCR primers are synthesized as described above

5'- ttggataagc ttggcttgac -3' (P1, SEQ ID No 44), and

5'- gaccgatggg cccttggtgg a -3' (P2, SEQ ID No 45)

The synthesis of DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,

oligonucleotide B, 10 pmol,

oligonucleotide C, 10 pmol,

oligonucleotide D, 10 pmol,

oligonucleotide E, 10 pmol,

oligonucleotide F, 10 pmol,

oligonucleotide G, 10 pmol,

oligonucleotide H, 10 pmol,

oligonucleotide I, 10 pmol,

oligonucleotide J, 10 pmol,

oligonucleotide K, 10 pmol,

oligonucleotide L, 10 pmol,

oligonucleotide primer P1, 2 μM,

oligonucleotide primer P2, 2 μM,

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10 X Pyrobest buffer II, 10 µl,

dNTP mix, 8 µl,

Pyrobest DNA polymerase, 0 5 µl, and

Redistilled water to a final volume of 50 µl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

An equal volume of phenol-chloroform (50% v/v phenol saturated with water, 48% v/v chloroform, 2% v/v isoamyl alcohol) is added to 200 µl of each of the PCR products, and vigorously mixed for 1 minute After this time, the mixture is centrifuged at 10,000 X g, and the aqueous layer is recovered and mixed with an equal volume of chloroform-isoamyl alcohol (96% v/v chloroform and 4% v/v isoamyl alcohol), which is again vigorously at 10,000 X g and the aqueous layer is recovered The series of steps recited in this paragraph is referred to, hereafter, as "phenol extraction")

Ethanol precipitation is then performed on the recovered aqueous layer As used and referred to herein, "ethanol precipitation" consists of adding, with mixing, a one tenth volume of 3M sodium acetate (pH 5 2) and 2 5 volumes of 100% ethanol to the solution to be treated, and freezing the mixture using dry ice The resulting mixture is then centrifuged at 10,000 X g to recover DNA as a precipitate

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 µg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The DNA band corresponding to humanized TRA-8 DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 µl of distilled water

The resulting, each extracted DNA is cloned using pGEM-T Easy vector (Promega) as follows

The DNA fragment recovered from the PCR reaction, 5 µl,

10 X Taq polymerase buffer, 1 µl,

dNTP mixture, 1 µl

Taq polymerase (5 unit/ml), 1 µl, and

redistilled water to a final volume of 10 µl

After the above each solution is reacted at 70°C for 30 minutes, each DNA solution and pGEM-T Easy vector are ligated using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) using the manufacturer's protocol

After 4 hours incubation at 15°C, 2 µl of the incubated reaction solution is mixed with 100 µl of competent *E coli* strain JM109 at a cell density of 1-2 x 10^9 cells/ml (Takara Shuzo Co , Ltd), and the mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 1 minutes Then, 500 µl of SOC medium (2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM w/v potassium chloride, 1 mM magnesium chloride, and 20 mM glucose) is added the mixture, which is incubated for a further hour, with shaking Transformant strains are then isolated, and plasmid DNA is prepared from the strains as described in "Molecular Cloning A Laboratory Manual" The nucleotide sequences of these DNAs encoding the heavy chain of humanized TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al., (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pHB14 (the plasmid carrying cDNA encoding the heavy chain of humanized TRA-8) The transformant *E coli* strain harboring these plasmid, designated as E coli JM109/pHB14 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7556

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(2) Construction of expression plasmids carrying the heavy chain variable region DNA of Humanized TRA-8

Recombinant expression vectors for animal cells are constructed by inserting the DNA encoding the heavy chain of humanized TRA-8 (cloned in above) as follows

One μg of plasmid pSRHHH3 (European patent application EP 0-909-816-A1) carrying the heavy chain variable region of humanized anti-Fas monoclonal antibody HFE7A and human IgG1 constant region genomic DNA, an expression vector for mammalian cells, is digested with the restriction enzymes HindIII and ApaI, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The vector DNA bands containing human IgG1 constant region genomic DNA without the heavy chain variable region of humanized HFE7A are cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water and then dephosphorylated using CIP The resulting digested, dephosphorylated plasmid (100 ng) is ligated with 1 μg of the pHB14 DNA fragment containing the DNA encoding the heavy chain variable region of humanized TRA-8, which had also been digested with HindIII and ApaI, using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) The ligation mixture is then used to transform E coli JM109, which is then plated on LB agar plates containing 50 μg/ml ampicillin

The transformants obtained by this method are cultured in 2 ml of liquid LB medium containing 50 μg/ml ampicillin at 37•C overnight, and plasmid DNA is subsequently extracted from the resulting culture by the alkaline-SDS method

The extracted plasmid DNA is digested with HindIII and ApaI, and subjected to 3% w/v agarose gel electrophoresis to confirm the presence or absence of the insert of the DNA encoding the heavy chain variable region of

humanized TRA-8 The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems) The resulting expression plasmid carrying cDNA encoding the heavy chain of humanized TRA-8 is designated pHB14-1

Example 19 Construction of an Expression Vector for the Light Chain of the Humanized Antibody

(1) Construction of vectors for the light chains of humanized versions of TRA-8 antibody

As shown in SEQ ID No 46 of the Sequence Listing, in humanizing the amino acid sequence of the light chain of the mouse anti-human DR5 antibody TRA-8, 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd (serine), 60th amino acid (aspartic acid), 63rd amino acid (threonine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine),and 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, serine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine respectively The resulting sequence is designated LM2

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized TRA-8

DNA coding for the LM2 polypeptide chain (SEQ ID No 46 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig

light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- gtcccccaca gatgcagaca aagaacttgg agattgggtc atcacaatgt caccagtgga -3' (HKSPR11, SEQ ID No 49),

5'- ccaagttctt tgtctgcatc agtaggagac agggtcacca tcacctgc -3' (HKCDF11, SEQ ID No 50),

5'- agtgtgccgg gtggatgccc agtaaatcag tagtttagga gctttccctg gtttctg -3' (HKCDR12, SEQ ID No 51),

5'- tgggcatcca cccggcacac tggggtccca agcaggttta gtggcagt -3' (HKCDF22, SEQ ID No 52),

5'- ataactacta tattgctgac agtaataggt tgcaaaatcc tccggctgca gactagagat ggt -3' (HKCDR22, SEQ ID No 53), and

5'- cagcaatata gcagctatcg gacgttcggt caaggcacca aggtggaaat caaacggact gtg -3' (HKCF12, SEQ ID No 54)

2) Construction of plasmid pCR3 1/M2-1 (cloning of humanized TRA-8 light chain)

LM2-DNA fragment as defined in SEQ ID No 55 of the Sequence Listing coding for the amino acid sequence as defined in SEQ ID No 46 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM2-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA (European patent application EP 0 909 816 A1), 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR11, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase (PerkinElmer), 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F2-DNA fragment coding for a portion of FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$ and is prepared under the following conditions

Composition of the reaction solution

plasmid pL28 DNA, 25 ng

oligonucleotide primer HKCDF11, 50 pmol

oligonucleotide primer HKCDR12, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F3-DNA fragment coding for CDRL2, FRL_3, and a portion of $CDRL_3$ is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA(European patent application EP 0 909 816 A1), 25 ng

oligonucleotide primer HKCDF22, 50 pmol

oligonucleotide primer HKCDR22, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM2-F4-DNA fragment coding for $CDRL_3$, FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer HKCF12, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light. The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM2-DNA in which above described LM2-F1-DNA, LM2-F2-DNA, LM2-F3-DNA and LM2-F4-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM2-F1-DNA prepared in the first step PCR,
Gel fragment of LM2-F2-DNA prepared in the first step PCR,
Gel fragment of LM2-F3-DNA prepared in the first step PCR,
Gel fragment of LM2-F4-DNA prepared in the first step PCR
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 µl
10×PCR buffer, 5 0 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM2-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (Invitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/M2-1 (the plasmid carrying cDNA encoding the light chain variable region of humanized TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/M2-1 containing LM2-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM2-DNA fragment, that have been digested with the restriction enzymes Hind III and EcoR I, are ligated

using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM2-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M2-1-4 carrying a fusion fragment of the variable region of the humanized LM2 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M2-1-4 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7563

3) Construction of plasmid pSR/M2-1 (expression plasmid for humanized LM2 TRA-8 light chain)

The obtained plasmid pHSG/M2-1-4 carrying a fusion fragment of the variable region of the humanized LM2 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0-909-816-A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M2-1-4 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from

the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized TRA-8 is designated pSR/M2-1

Example 20 Production of Humanized Antibody

Transfection of COS-7 cells, i e , a cell line derived from a monkey kidney, with the expression plasmids for the humanized TRA-8 heavy chain and the humanized TRA-8 light chain obtained above is conducted by FUGENE6 transfection reagent methods (Boehringer Mannheim Biochemica) according to the instruction manual provided with the kit.

COS-7 cells (American Type Culture Collection No CRL-1651) are grown to semi-confluent (3×10^6 cells/dish) in a culture dish (culture area 57 cm^2, Sumitomo Bakelite) containing Dulbecco's Modified Eagle medium (hereinafter referred to as "D-MEM", Gibco BRL) supplemented with 10% fetal calf serum (heremafter abbreviated as "FCS", Moregate)

In the meantime, 10 μg/dish (total 5 dishes) of the humanized DR5 heavy chain expression plasmid DNA (pHA15-1) and 10 μg/dish of the humanized DR5 light chain expression plasmid DNA prepared by the alkaline-SDS method and cesium chloride density gradient centrifugation are mixed, and then precipitated with ethanol, followed by suspending in 5 μl/dish of dH_2O

After 15 μl/dish of FUGENE6 Transfection regent is mixed with 180 μl/dish D-MEM without FCS, this FUGENE solution (185 μl/dish) is mixed with 5 μl/dish DNA solution containing 10 μg/dish of the humanized DR5 heavy chain expression plasmid DNA and 10μg/dish of the humanized DR5 light chain expression plasmid DNA After 15 minutes incubation at room temperature, the obtained plasmid suspension (200 μl) is added to the previously prepared COS-7 plates After incubating in 5% CO_2 at 37°C for 24 hours, the culture medium is changed with D-MEM without FCS After incubating in 5% CO_2 at 37°C for 72 hours, the culture supernatant is recovered to purify the expression products in

the supernatant fluids By the method as described above, COS-7 cells are transfected with each of the following plasmid combinations

(A) no plasmid DNA

(B) cotransfection of pHB14-1 and pSR/M2-1

The culture is then centrifuged (1,000 r p m , 5 minutes) and collected the supernatant The supernatant is centrifuged again (9,800 r p m , 15 minutes) and filtrated with 0 45 μm filter (ADVANTEC TOYO DISMIC-25cs, Cat # 25CS045 AS) The purification of IgG from the filtrates are achieved using Protein G-POROS affinity chromatography (Applied Biosystems) under the following conditions

HPLC BioCAD 700E (Applied Biosystems)

column ProteinG-ID sensor cartridge (column size 2 1 mmID x 30 mm LD, bed volume 0 1 ml, Cat # 2-1002-00, Applied Biosystems)

elution buffer 0 1M Glycine-HCl (pH 2 5)

neutralization buffer 1M Tris-HCl (pH 8 5)

detection 280 nm

flow rate 1 ml/min

fraction size 0 5 ml/0 5 min

fraction tube 1 5 ml polypropylene microtube

temperature 4°C

After all the filtrates are applied to column, 30 ml of PBS (Sigma, Cat # 1000-3) is used to wash column When the elution buffer is applied, fraction collector started Each fraction microtube previously contained 55 μl of 1M NaCl, 110 μl of neutralization buffer and 74 μl of 2 mg/ml bovine serum albumin (Sigma, Cat # A-7030) in PBS The fractions from No 8 through No 10 are collected and dialyzed against 1 liter PBS (pH 7 5) at 4°C for 1 day using Slide-A lyzer (Pierce, Cat # 66450) The dialysis buffer is changed twice

Verification of the expression of the humanized antibodies and quantitative assay of the expression products in the culture supernatant fluids prepared is performed by ELISA with an antibody against anti-human IgG

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To each well of a 96-well plate (MaxiSorp, Nunc), 100 µl of goat anti-human IgG Fc specific polyclonal antibody (Kappel) dissolved at the final concentration of 0 5 µg/ml in adsorption buffer (0 05 M sodium hydrogencarbonate, 0 02% sodium azide, pH 9 6) is added and the plate is incubated at 37°C for 2 hours to cause adsorption of the antibody Then, the plate is washed with 350 µl of PBS(-) containing 0 05% Tween-20 (BioRad) (hereinafter referred to as "PBS-T") five times To the wells after washing, the culture supernatant diluted with D-MEM containing 10% FCS is added and incubated at 37°C for 2 hours After washing again with PBS-T, 100 µl of alkaline phosphatase-labeled goat anti-human IgG Fc specific polyclonal antibody (Jackson Immuno Research Lab) diluted 10,000-fold with PBS-T is added to each well and incubated at 37°C for 2 hours After washing again with PBS-T, a substrate solution of p-nitrophenyl phosphate obtained from Alkaline Phosphatase Substrate kit (Bio Rad) is added according to the instruction manual provided with the kit After incubating at 37°C for 0 5 to 1 hour, the absorbance at 405 nm is measured In the present experiments, human plasma immunoglobulin G subclass 1 (IgG1) (Biopure AG) diluted with D-MEM containing 10% FCS to certain concentrations is used as concentration reference samples of the humanized DR5 antibodies contained in the culture supernatant fluids

As a result, the expression and purified products in the culture supernatant are detected specifically with the anti-human IgG antibody The amount of human IgG antibody is 8 96 µg (800 µl)

Example 21 Apoptosis-inducing activity of Humanized Antibody

Jurkat cells (ATCC No TIB-152), are used to examine the apoptosis-inducing activity of the purified humanized TRA-8 antibody

Jurkat cells cultured in RPMI1640 medium with 10% FCS (Gibco BRL) at 37°C for 3 days in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) at 50 µl per well The humanized TRA-8 prepared in Example 20 are adjusted to have the concentration of the final product of interest of 100 ng/ml with RPMI1640 medium containing 10%

FCS by estimating their concentrations in the fluids according to the method described in Example 20 Each of the solutions of the expression products thus adjusted to 100 ng/ml is used to produce serial dilutions by repeating serial 2-fold dilution with RPMI1640 containing 10% FCS Each of the diluted humanized TRA-8 solution is added to each well at 50 µl per well After reacting at 37°C for 12 hours, 50 µl of 25 µM PMS (phenazine methosulfate, Sigma Chemical Co) containing 1 mg/ml XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt, Sigma Chemical Co) is added (final concentrations of 250 µg/ml for XTT and 5 µM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells, and "c" is the measurement of a well with no antibody added

As a result, the expression product prepared in Example 20 (humanized TRA-8) is demonstrated to induce apoptosis in cells of T lymphoma cell line expressing human DR5 antigen

Example 22 Reactivity of TRA-8 to various DR5 molecules

In order to determine the reactivity of TRA-8 to various DR5 molecules, the reactivity of TRA-8 is examined using activated lymphocytes as follows

First, peripheral blood samples are taken from a human (30 ml), marmoset (3 ml), and cynomolgus monkey (20 ml) The blood samples had 1 ml of heparin (Novoheparin, Novo) added to them and the samples are then slowly layered over an equal volume of Ficoll-Paque PLUS solution ((Amersham Pharmacia Biotech) specific gravity 1 077 for all except cynomolgus monkey, which had a specific gravity of 1 072) and centrifuged at 1,700 r p m for 30 minutes in order to obtain a fraction of peripheral blood mononuclear cells This mononuclear cell fraction is washed twice with Hanks' balanced salt solution

and then suspended in RPMI 1640 medium with 10% v/v FCS to a cell density of 1 x 10^6 cells/ml Phytohemagglutinin-P (PHA-P, Sigma Chemicals, Co) is added to the resulting suspension to a final concentration of 5 μg/ml and the sample incubated at 37°C under 5% v/v CO_2 for 24 hours After this time, the cells are recovered by centrifugation, washed and resuspended in RPMI 1640 medium containing 10% v/v FCS Then, to activate the recovered cells, interleukin-2 (Amersham Pharmacia Biotech) is added to the suspension to a final concentration of 10 units/ml, and this is incubated at 37°C under 5% v/v CO_2 for 72 hours

An amount of the activated preparation calculated to contain 1 x 10^6 activated lymphocyte cells is placed in a test tube and either suspended in 50 μl of 0 5, 1, 5, 10 μg/ml of TRA-8 in PBS or 50 μl of PBS alone The resulting suspension is allowed to stand on ice for 1 hour, after which the cells are washed 3 times with aliquots of 500 μl of PBS and then suspended in 50 μl of 20 μg/ml FITC-labeled anti-mouse IgG antibody (Bioresource) in PBS Using the cells suspended in 500 μl of PBS as controls, the fluorescence intensities are measured, using a flow cytometer (FACSCalibur; Becton Dickinson)

Distributions of cell numbers by fluorescence intensity are obtained and the proportions of the numbers of the stained cells to those of total cells are calculated Further, each Kd value is calculated using the concentration of TRA-8 and the proportions of the numbers of the stained cells to those of total cells Each frequency of reactivity to activated lymphocytes of human, marmoset, and cynomologus monkey is almost same Accordingly, TRA-8 is able to bind a wide range of primate DR5 including human against which TRA-8 is originally prepared.

Example 23 Escalating dose study of TRA-8 in marmosets

An escalating dose preliminary toxicity study of TRA-8 is performed using 1 male and 1 female marmoset Three sets of single intravenous dosing, which are separated by a 7-day withdrawal period, are carried out The dose of TRA-8 is set at 50, 250 and 1250 μg/body Forty-eight hours after each treatment, blood is collected from the femoral vein and the plasma is prepared

Plasma aspartate aminotransferase and alanine aminotransferase activities are measured using an analyzer (FUJI DRI-CHEM Fuji Film Medical Co , Ltd) All blood is taken without any anesthetization As a result, no evidences indicating hepatic injury are noted in plasma biochemical examination after each treatment

Example 24 *In vitro* and *in vivo* pharmacological studies of TRA-8 against cancer cells

In order to determine whether TRA-8 has the therapeutic efficacy in anti-cancer therapy, *in vitro* killing activity of TRA-8 using various cancer cell lines is examined as follows

Various cancer cells (2-8 x 10^3 cells/50 µl) cultured in RPMI1640 medium (for Jurkat), DMEM medium (for HCT-116), MEM-R (for WiDr), or DMEM-F12 (for COL2-Jck) obtained from Gibco BRL with 10% FCS (Gibco BRL) at 37°C in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) TRA-8 are adjusted to have the concentration of the final product of interest of 100 ng/ml with medium containing 10% FCS The TRA-8 solution (100 ng/ml) is used to produce serial dilutions by repeating serial 2-fold dilution with medium containing 10% FCS Each of the diluted TRA-8 solution is added to each well at 50 µl per well and incubated at 37°C After reacting at 37°C for 72 hours, 50 µl of 25 µM PMS (phenazine methosulfate, Sigma Chemical Co) containing 1 mg/ml XTT is added (final concentrations of 250 µg/ml for XTT and 5 µM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells, and "c" is the measurement of a well with no antibody added

The results are shown in Table 3, below

Table 3

Cells	ED50 (μg/ml)
Jurkat	0 001 - 0 01
HCT-116	0 004 - 0 02
WiDr	0 007 - 0 03
COL2-Jck	2 28

Various cancer cell lines are strongly induced apoptosis by TRA-8 under the *in vitro* conditions

Furthermore, the *in vivo* anti-tumor effect of TRA-8 in nude mice transplanted with WiDr cells is determined, because TRA-8 is not cross-reactive with murine DR5

TRA-8 anti-human DR5 antibody is administered to nude mice bearing human xenografts that express the human DR5 molecule The mice used were 6 week-old BALb/c nude/nude mice (female, from Clea Japan Inc), which were transplanted with human colon cancer cell lines WiDr (5 mm^3) At one day after tumor transplantation, these transplanted mice are daily treated with the intraarticular injection of TRA-8 (5 μg/body) to 14 times WiDr tumor growth is daily determined by the size of the tumor mass The results are shown in Table 4, below

Table 4

	8 days	11 days	15 days	18 days	22 days	25 days
Control (PBS)	196	249	469	584	833	1193
SD	±55	±77	±149	±230	±274	±419
TRA-8	158	97	155	195	365	530
SD	±78	±30	±60	±58	±91	±135

In this model, while all untreated animals exhibited visible tumor growth, tumor growth in TRA-8 treated animals is inhibited as demonstrated by the size of tumor This result indicated that TRA-8 is effective in the elimination of tumor cells *in vivo*

Example 25 Combination study of TRA-8

Human prostate cancer cell line PC-3 is obtained from American Tissue Culture Collection (ATCC) and maintained in F-12K Nutrient Mixture (21127-022, Gibco BRL) containing 10% fetal bovine serum (FBS, Hyclone), 1% L-Glutamine-200 mM (25030-149, Gibco BRL) and 0 5% Penicillin Streptomycin Solution (P-7539, Sigma) RPMI1640 medium (MED-008, IWAKI) supplemented with 10% FBS and 0 5% Penicillin Streptomycin Solution is used in the following experiment Exponentially growing PC-3 cells are collected by trypsinization and washed twice with fresh medium The cells are then counted, resuspended in fresh medium at a density of 5×10^4 cells/ml and distributed in triplicate into flat-bottomed 96 well plates (3598, Corning-Coster) in a total volume of 100 µl/well one day before the start of the experiment A representative anti-cancer drug, Paclitaxel (169-18611, Wako) dissolved in dimethylsulfoxide (10 mg/ml) is diluted in fresh medium and then added to the 96-well plates containing the cells at 50 µl/well The final concentrations of dimethylsulfoxide are less than 0 1% After incubation for 24 hr at 37°C in 5% CO_2 atmosphere, TRA-8 diluted in fresh medium is added to the wells After incubation for a further 24 hr, 50 µl of Minimum Essential Medium (11095-098, Gibco BRL) containing 1 mg/ml of XTT and 25 mM of PMS is added to the wells and the plates are incubated for 6 hr OD450 is then measured by SPECTRA MAX 250 (Molecular Devices) and the cell viability is calculated as follows

Cell viability (%) = (OD450 for the well containing cells treated with Taxol and/or TRA-8 (agent(s)) - OD450 for the well containing neither cells nor agent) x 100 / (OD450 for the well containing cells with no agent –OD450 for the well containing neither cells nor agent)

The result of the above assay for TRA-8 combined with a representative anti-cancer drug, Paclitaxel, is followed Paclitaxel reduced the cell viability of PC-3 cells but more than 40% of the signals indicating viable cancer cells still remained at concentrations of up to 200 nM Notably, the addition of 0 1 ng/ml of TRA-8 greatly decreased the cell viability of the cancer cells, up to 10%, even though no reduction in cell viability is seen after a single application of TRA-8 at

this concentration This result clearly indicates that TRA-8 exhibited anti-cancer activity synergistically when combined with other anti-cancer drugs

Example 26 Analysis of Other Type Humanized Antibodies of TRA-8

(1) Designing Humanized Antibodies

Construction of a humanized version of TRA-8 is performed by the method generally known as CDR grafting mAB58'CL antibody is used as an acceptor as described in Reference Example 2 and the CDR regions of TRA-8 antibody is grafted on the acceptor In the framework region, some amino acids are grafted on the acceptor from either TRA-8 or human consensus sequences by the criteria given by Queen et al (Proc Natl Acad Sci USA 86, 10029-10033, (1989)) and humanized TRA-8 sequences are constructed as described hereinbelow

(2) Construction of Plasmid Carrying the Heavy Chain Variable Region DNA of Other Types Humanized or Mouse TRA-8

As shown in SEQ ID No 56 of the Sequence Listing, H1 type-humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 3rd amino acid (methionine), the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 84th amino acid (serine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, glutamine, arginine, alanine, glycine, glycine, asparagine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 59 of the Sequence Listing, H3 type-humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 40th amino acid (threonine), the 42nd amino acid (glutamic acid), the 44th amino acid (arginine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, glycine, glycine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 60 of the Sequence Listing, H4 type-humanization of the amino acid sequences of the heavy chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 13th amino acid (lysine), the 19th amino acid (lysine), the 88th amino acid (serine), the 93rd amino acid (methionine), the 114th amino acid (threonine), the 115th amino acid (leucine) with glutamine, arginine, alanine, valine, leucine, and valine, respectively

As shown in SEQ ID No 61 of the Sequence Listing, the plasmid carrying the heavy chain variable region DNA of chimeric TRA-8 is designated as "M type" In addition, humanized TRA-8 described in Example 17 and 18 is designated as "H2 type"

The plasmids carrying DNA encoding heavy chain variable region of humanized or chimeric TRA-8 are constructed as follows

PCR is used to construct the following DNA sequences, each of which comprised described above

The following 24 oligonucleotide are synthesized

5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32),

5'- tctgaagtaa tgctggtgga gtctgggggg ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- tctgaagtac agctggtgga gtctgggggg ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B2, SEQ ID No 57),

5'- tctgaagtaa tgctggtgga gtctgggggg ggcttagtaa agcctggagg gtccctgaaa ctctcctgtg cagcctctgg -3' (B3, SEQ ID No 66),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag actccagaga agaggctgga gtgggttgca accattagta -3' (C2, SEQ ID No 64),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

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5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- tatctgcaaa tgagcagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E2, SEQ ID No 62),

5'- tatctgcaaa tgagcagtct gagatctgag gacacggcta tgtattactg tgcaagaagg ggtgactcta tgattacgac -3' (E3, SEQ ID No 67),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctccacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5'- ggactactgg ggccaaggga ccactctcac agtctcctca gcctccacc aagggcccat cggtc -3' (F2, SEQ ID No 68),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagct gtacttcaga gtggacacct gtagctgttg -3' (H2, SEQ ID No 58),

5'- tttcagggac cctccaggct ttactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H3, SEQ ID No 69),

5'- tccagaccct tccctggtgc ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- tccagcctct tctctggagt ctgccgaacc caagacatta cataactact gaaagtgaat ccagaggctg cacaggagag -3' (I2, SEQ ID No 65),

5'- ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtaa ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K2, SEQ ID No 63),

5'- ccttcttgca cagtaataca tagccgtgtc ctcagatctc agactgctca tttgcagata cagggtgttc ttggcattgt -3' (K3, SEQ ID No 70),

5'- gaccgatggg cccttggtgg aggctgagga gactgtgacc agggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43) and

5'- gaccgatggg cccttggtgg aggctgagga gactgtgaga gtggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L2, SEQ ID No 71)

The following 2 PCR primers are synthesized as described above

5'- ttggataagc ttggcttgac -3' (P1, SEQ ID No 44), and

5'- gaccgatggg cccttggtgg a -3' (P2, SEQ ID No 45)

The synthesis of H1 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H1 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B2, 10 pmol,
oligonucleotide C, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E, 10 pmol,
oligonucleotide F, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H2, 10 pmol,
oligonucleotide I, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K, 10 pmol,
oligonucleotide L, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The DNA bands corresponding to H1 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit (BIO 101, CA, USA) After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The synthesis of H3 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H3 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B, 10 pmol,
oligonucleotide C, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E2, 10 pmol,
oligonucleotide F, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H, 10 pmol,
oligonucleotide I, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K2, 10 pmol,
oligonucleotide L, 10 pmol,

oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The DNA bands corresponding to H3 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit. After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The synthesis of H4 type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of humanized TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The H4 type-DNA fragment is prepared as follows

Composition of the PCR reaction solution
oligonucleotide A, 10 pmol,
oligonucleotide B, 10 pmol,
oligonucleotide C2, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E2, 10 pmol,
oligonucleotide F, 10 pmol,

oligonucleotide G, 10 pmol,
oligonucleotide H, 10 pmol,
oligonucleotide I2, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K2, 10 pmol,
oligonucleotide L, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The DNA bands corresponding to H4 type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit. After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The synthesis of M type DNA encoding a polypeptide chain comprising a secretion signal sequence, a variable region of chimeric TRA-8 heavy chain and the 8 amino acid residues at the N-terminus of the IgG-CH1 region is performed using a combination of PCR respectively

The M type-DNA fragment is prepared as follows

Composition of the PCR reaction solution

oligonucleotide A, 10 pmol,
oligonucleotide B3, 10 pmol,
oligonucleotide C2, 10 pmol,
oligonucleotide D, 10 pmol,
oligonucleotide E3, 10 pmol,
oligonucleotide F2, 10 pmol,
oligonucleotide G, 10 pmol,
oligonucleotide H3, 10 pmol,
oligonucleotide I2, 10 pmol,
oligonucleotide J, 10 pmol,
oligonucleotide K3, 10 pmol,
oligonucleotide L2, 10 pmol,
oligonucleotide primer P1, 2 μM,
oligonucleotide primer P2, 2 μM,
10 X Pyrobest buffer II, 10 μl,
dNTP mix, 8 μl,
Pyrobest DNA polymerase, 0 5 μl, and
Redistilled water to a final volume of 50 μl

The PCR reaction is conducted as follows The solution is first heated at 94°C for 5 minutes, after which a cycle of heating to 98°C for 10 second, 55°C for 30 second and 72°C for 1 minute, is repeated 7 times After completion of this procedure, the reaction solution is heated at 72°C for 15 minutes

After phenol extraction and ethanol precipitation, the resulting DNA precipitate is vacuum-dried, dissolved in a minimum of redistilled water, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light. The DNA bands corresponding to M type-DNA is cut out using a razor blade and eluted from the gel using Geneclean Spin Kit After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water

The resulting, each extracted DNA (H1 type, H3 type, H4 type, and M type) is cloned using pGEM-T Easy vector (Promega) as follows

The DNA fragment recovered from the PCR reaction (H1, H3, H4 or M), 5 μl,

10 X Taq polymerase buffer, 1 μl,

dNTP mixture, 1 μl

Taq polymerase (5 unit/ml), 1 μl, and

redistilled water to a final volume of 10 μl

After the above each solution is reacted at 70°C for 30 minutes, each DNA solution and pGEM-T Easy vector are ligated using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) using the manufacturer's protocol

After 4 hours incubation at 15°C, 2 μl of the incubated reaction solution is mixed with 100 μl of competent *E coli* strain JM109 at a cell density of 1-2 x 10^9 cells/ml (Takara Shuzo Co , Ltd), and the mixture is kept on ice for 30 minutes, then at 42°C for 30 seconds, and again on ice for 1 minute Then 500 μl of SOC medium (2% v/v tryptone, 0 5% w/v yeast extract, 0 05% w/v sodium chloride, 2 5 mM w/v potassium chloride, 1 mM magnesium chloride, and 20 mM glucose) is added the mixture, which is incubated for a further hour, with shaking Transformant strains are then isolated, and plasmid DNA is prepared from the strains as described in "Molecular Cloning A Laboratory Manual" The nucleotide sequence of this DNA encoding the heavy chain of humanized or mouse TRA-8 is confirmed by the dideoxy method, respectively (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmid is designated pHA15 (the plasmid carrying cDNA encoding the H1-type heavy chain of humanized TRA-8), pHC10 (the plasmid carrying cDNA encoding the H3-type heavy chain of humanized TRA-8), pHD21 (the plasmid carrying cDNA encoding the H4-type heavy chain of humanized TRA-8), and pM11 (the plasmid carrying cDNA encoding the heavy chain of chimeric TRA-8) The transformant *E coli* strains harboring these plasmid, designated as *E coli* JM109/pHA15, *E coli* JM109/pHC10, *E coli*

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JM109/pHD21, and *E coli* JM109/pM11 were deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7555, FERM BP-7557, FERM BP-7558, and FERM BP-7559, respectively

(3) Construction of Expression Plasmids Carrying the Heavy Chain Variable Region DNA of Several Types Humanized or Mouse TRA-8

Recombinant expression vector for animal cells are constructed by inserting the DNA encoding the heavy chain of H1 type, H3 type, and H4 type humanized or M type chimeric TRA-8 (cloned in above) as follows

One μg of plasmid pSRHHH3 (European patent application EP 0 909 816 A1) carrying the heavy chain variable region of humanized anti-Fas monoclonal antibody HFE7A and human IgG1 constant region genomic DNA, an expression vector for mammalian cells, is digested with the restriction enzymes HindIII and ApaI, and separated by 3% agarose gel electrophoresis After electrophoresis, the gel is stained with a 1 μg/ml aqueous solution of ethidium bromide to allow detection of DNA under UV light The vector DNA bands containing human IgG1 constant region genomic DNA without the heavy chain variable region of humanized HFE7A are cut out using a razor blade and eluted from the gel using Geneclean Spin Kit After phenol extraction, the eluted DNA is then concentrated by centrifugation at 7,500 X g, followed by ethanol precipitation, and finally dissolved in 5 μl of distilled water and then dephosphorylated using CIP The resulting digested, dephosphorylated plasmid (100 ng) is ligated with 1 μg of the DNA fragment of pHA15, pHC10, pHD21, or pM11 containing the DNA encoding the heavy chain variable region of humanized or chimeric TRA-8, which had also been digested with HindIII and ApaI, using a DNA Ligation Kit Version 2 0 (Takara Shuzo Co , Ltd) The ligation mixture is then used to transform *E coli* JM109, which is then plated on LB agar plates containing 50 μg/ml ampicillin

The transformants obtained by this method are cultured in 2 ml of liquid LB medium containing 50 μg/ml ampicillin at 37°C overnight, and plasmid DNA is subsequently extracted from the resulting culture by the alkaline-SDS method

The extracted plasmid DNA is digested with HindIII and ApaI, and subjected to 3% w/v agarose gel electrophoresis to confirm the presence or absence of the insert of the DNA encoding the heavy chain variable region of humanized or chimeric TRA-8 The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer, Applied Biosystems) The resulting expression plasmids carrying cDNA encoding the heavy chain of humanized or chimeric TRA-8 were designated pHA15-1, pHC10-3, pHD21-1, and pM11-1, respectively

(4) Construction of Vectors for the Humanized Light Chains

(4 1) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM1 type)

As shown in SEQ ID No 72 of the Sequence Listing, other humanization (LM1 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 3rd amino acid (valine), 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd (serine), 60th amino acid (aspartic acid), 63rd amino acid (threonine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine),and 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with glutamine, proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, serine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine respectively The resulting sequence is designated LM1

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Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM1 type, SEQ ID No 72 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized TRA-8 (LM1 type)

DNA coding for the LM1 polypeptide chain (SEQ ID No 72 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain (LM1 type) and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), HKCDF11 (SEQ ID No 50), HKCDR12 (SEQ ID No 51), HKCDF22 (SEQ ID No 52), HKCDR22 (SEQ ID No 53), and HKCF12 (SEQ ID No 54)

The following oligonucleotide primers are synthesized for PCR

5'- gtcccccaca gatgcagaca aagaacttgg agattgggtc atctgaatgt caccagtgga -3' (HKSPR12, SEQ ID No 77)

2) Construction of plasmid pCR3 1/LM1-2 (cloning of humanized TRA-8 light chain type LM1)

LM1-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 72 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM1-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA, 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR12, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase (PerkinElmer), 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM1-F2-DNA fragment coding for a portion of FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$ is prepared under the following conditions

Composition of the reaction solution

plasmid pL28 DNA, 25 ng

oligonucleotide primer HKCDF11, 50 pmol

oligonucleotide primer HKCDR12, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM1-F3-DNA fragment coding for CDRL2, FRL_3, and a portion of $CDRL_3$ is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer HKCDF22, 50 pmol

oligonucleotide primer HKCDR22, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM1-F4-DNA fragment coding for $CDRL_3$, FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng
oligonucleotide primer HKCF12, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor

b) Second step PCR

LM1-DNA in which above described LM1-F1-DNA, LM1-F2-DNA, LM1-F3-DNA and LM1-F4-DNA fragments are fused is prepared under the following conditions.

Composition of the reaction solution

Gel fragment of LM1-F1-DNA prepared in the first step PCR,
Gel fragment of LM1-F2-DNA prepared in the first step PCR,

Gel fragment of LM1-F3-DNA prepared in the first step PCR,
Gel fragment of LM1-F4-DNA prepared in the first step PCR
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 µl
10×PCR buffer, 5 0 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM1-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 (LM1 type) are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM1-2 (the plasmid carrying cDNA encoding the light chain variable region of humanized TRA-8 (LM1 type) and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM1-2 containing LM1-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM1-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 µg/ml chloramphenicol (final

concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM1-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M1-2-2 carrying a fusion fragment of the variable region of the humanized LM1 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M1-2-2 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7562

3) Construction of plasmid pSR/LM1-2 (expression plasmid for humanized LM1 TRA-8 light chain)

The obtained plasmid pHSG/M1-2-2 carrying a fusion fragment of the variable region of the humanized LM1 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M1-2-2 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM1 TRA-8 is designated pSR/LM1-2

(4.2) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM3 type)

As shown in SEQ ID No 73 of the Sequence Listing, other humanization (LM3 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 87th amino acid (phenylalanine), 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, threonine, tyrosine, glutamine, valine and threonine, respectively The resulting sequence is designated LM3

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM3 type, SEQ ID No 73 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM3 TRA-8

DNA coding for the LM3 polypeptide chain (SEQ ID No 73 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- atctagttct cagagatgga gacagacaca atcctgctat gggtgctgct gctctgggtt ccagg -3' (MOD1F1, SEQ ID No 78),

5'- cagcacccat agcaggattg tgtctgtctc catctctgag aactagatga gaggatgctt cttaagctt -3' (MOD1R1, SEQ ID No 79),

5'- ctccactggt gacattgtga tgacccaatc tccaagttct ttgtctgcat ctgtgggggg cagggtc -3' (MOD1F22, SEQ ID No 80),

5'- acttggagat tgggtcatca caatgtcacc agtggagcct ggaacccaga gcag-3' (MOD1R22, SEQ ID No 81),

5'-accatcacct gcaaggccag tcaggatgtg ggtactgctg tagcctggta ccaacagaaa ccaggaa -3' (MOD1F3, SEQ ID No 82),

5'- tacagcagta cccacatcct gactggcctt gcaggtgatg gtgaccctgt cccccacaga tgcagacaaa ga -3' (MOD1R3, SEQ ID No 83),

5'- aagcacccaa actcctcatc tattgggcat ccacccggca cactggggtc ccagataggt ttacaggcag t -3' (MOD1F42, SEQ ID No 84),

5'- cccagtgtgc cgggtggatg cccaatagat gaggagtttg ggtgcttttc ctggtttctg ttggtaccag gc -3' (MOD1R4, SEQ ID No 85),

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcaacc tat -3 (MOD1F5, SEQ ID No 86),

5'- actagagatg gtgagggtga agtctgtccc agacccactg cctgtaaacc tatctgggac -3' (MOD1R52, SEQ ID No 87),

5'- tactgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3' (MOD1F6, SEQ ID No 88),

5'- cgtccgatag ctgctatatt gctgacagta ataggttgca aaatcctccg gctgcac -3' (MOD1R6, SEQ ID No 89)

5'- aaacggactg tggctgcacc atctgtcttc atcttcccgc catctgatga g -3' (MOD1F7, SEQ ID No 90),

5'- gaagatgaag acagatggtg cagccacagt ccgtttgatt tccaccttgg tgccttgacc gaa -3' (MOD1R7, SEQ ID No 91), and

5'- agatttcaac tgctcatcag atggcgggaa (LR17, SEQ ID No 101)

2) Construction of plasmid pCR3 1/LM3-3-44 (cloning of humanized TRA-8 light chain type LM3)

LM3-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 73 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM3-F31B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1 $CDRL_1$, FRL_2, and $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol
oligonucleotide primer MOD1R1, 5 pmol
oligonucleotide primer MOD1F22, 5 pmol
oligonucleotide primer MOD1R22, 5 pmol
oligonucleotide primer MOD1F3, 5 pmol
oligonucleotide primer MOD1R3, 5 pmol
oligonucleotide primer MOD1F42, 5 pmol
oligonucleotide primer MOD1R4, 5 pmol
oligonucleotide primer MOD1F5, 5 pmol
oligonucleotide primer MOD1R52, 5 pmol
oligonucleotide primer MOD1F6, 5 pmol
oligonucleotide primer MOD1R6, 5 pmol
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer MOD1R7, 5 pmol
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer LR17, 50 pmol
dNTPs cocktail, 5 µl
10× PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM3-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M3-3-22 carrying a fusion fragment of the variable region of the humanized LM3 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M3-3-22 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7564

3) Construction of plasmid pSR/LM3-3-44-10 (expression plasmid for humanized LM3 TRA-8 light chain)

The obtained plasmid pHSG/M3-3-22 carrying a fusion fragment of the variable region of the humanized LM3 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M3-3-22 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by

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DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM3 TRA-8 is designated pSR/LM3-3-44-10

(4 3) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM4 type)

As shown in SEQ ID No 74 of the Sequence Listing, other humanization (LM4 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 85th amino acid (aspartic acid), 99th amino acid (glycine) 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, threonine, glutamine, valine and threonine respectively The resulting sequence is designated LM4

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM4 type) (SEQ ID No 74 of the Sequence Listing) are constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM4 TRA-8

DNA coding for the LM4 polypeptide chain (SEQ ID No 74 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79), MOD1F22 (SEQ ID No 80),

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MOD1R22 (SEQ ID No 81), MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1F5 (SEQ ID No 86), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), and MOD1R7 (SEQ ID No 91), LR17 (SEQ ID No 101), the following oligonucleotide primers are synthesized for PCR.

5'- ttctgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3' (MOD1F62, SEQ ID No 92)

5'- cgtccgatag ctgctatatt gctgacagaa ataggttgca aaatcctccg gctgcag -3' (MOD1R62, SEQ ID No 93)

2) Construction of plasmid pCR3 1/LM4-5-3 (cloning of humanized TRA-8 light chain type LM4)

LM4-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 74 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM4-F41B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1, $CDRL_1$, FRL_2, and $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol
oligonucleotide primer MOD1R1, 5 pmol
oligonucleotide primer MOD1F22, 5 pmol
oligonucleotide primer MOD1R22, 5 pmol
oligonucleotide primer MOD1F3, 5 pmol
oligonucleotide primer MOD1R3, 5 pmol
oligonucleotide primer MOD1F42, 5 pmol
oligonucleotide primer MOD1R4, 5 pmol
oligonucleotide primer MOD1F5, 5 pmol
oligonucleotide primer MOD1R52, 5 pmol
oligonucleotide primer MOD1F62, 5 pmol

oligonucleotide primer MOD1R62, 5 pmol
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer MOD1R7, 5 pmol
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer LR17, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM4-F41C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

The template plasmids, pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 μg/ml of ethidium bromide to detect the produced DNA under UV light. The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM4-DNA in which above described LM4-F41B-DNA, and LM4-F41C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM4-F41B-DNA prepared in the first step PCR,
Gel fragment of LM4-F41C-DNA prepared in the first step PCR,
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 μl
10×PCR buffer, 5 0 μl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 μl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM4-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized LM4 TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM4-5-3 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM4 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM4-5-3 containing LM4-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM4-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM4-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure plasmid pHSG/M4-5-3-1 carrying a fusion fragment of the variable region of the humanized LM4 TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M4-5-3-1 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7565

3) Construction of plasmid pSR/LM4-5-3-3 (expression plasmid for humanized LM4 TRA-8 light chain)

The obtained plasmid pHSG/M4-5-3-1 carrying a fusion fragment of the variable region of the humanized LM4 TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III

and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M4-5-3-1 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM4 TRA-8 is designated pSR/LM4-5-3-3

(4.4) Construction of an Expression Vector for the Light Chain of the Humanized Antibody (LM5 type)

As shown in SEQ ID No 75 of the Sequence Listing, other humanization (LM5 type) of the amino acid sequences of the light chain of the mouse anti-human DR5 antibody TRA-8 entailed replacing the 8th amino acid (histidine), 9th amino acid (lysine), 10th amino acid (phenylalanine), 11th amino acid (methionine), 13th amino acid (threonine), 20th amino acid (serine), 42nd amino acid (glutamine), 43rd amino acid (serine), 77th amino acid (asparagine), 78th amino acid (valine), 80th amino acid (serine) 83rd amino acid (leucine), 103rd amino acid (leucine) and 108th amino acid (alanine) from the N-terminus of the amino acid sequence of the TRA-8 light chain are replaced with proline, serine, serine, leucine, alanine, threonine, lysine, alanine, serine, leucine, proline, phenylalanine, valine and threonine respectively The resulting sequence is designated LM5

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM5 type) (SEQ ID No 75 of the Sequence Listing) is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM5 TRA-8

DNA coding for the LM5 polypeptide chain (SEQ ID No 75 of the Sequence Listing), each of which is a fusion of the variable region of humanized anti-DR5 antibody TRA-8 light chain and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR.

Further to 7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79), MOD1F22 (SEQ ID No 80), MOD1R22 (SEQ ID No 81), MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), and LR17 (SEQ ID No 101), the following oligonucleotide primers are synthesized for PCR

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcagat tat -3' (MOD1F52, SEQ ID No 94)

5'- ttctgtcagc aatatagcag ctatcggacg ttcggtggag gcaccaaggt ggaaatc -3' (MOD1F63, SEQ ID No 95)

5'- cgtccgatag ctgctatatt gctgacagaa ataatctgca aaatcctccg gctgcag -3' (MOD1R63, SEQ ID No 96)

5'- gaagatgaag acagatggtg cagccacagt ccgtttgatt tccaccttgg tgcctccacc gaa-3' (MOD1R72, SEQ ID No 102)

2) Construction of plasmid pCR3 1/LM5-3-42 (cloning of humanized TRA-8 light chain type LM5)

LM5-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 75 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM5-F51B-DNA fragment coding for a secretion signal sequence region with a Hind III restriction enzyme cleavage site added at the 5'-end, FRL_1, $CDRL_1$, FRL_2, $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

oligonucleotide primer MOD1F1, 5 pmol

oligonucleotide primer MOD1R1, 5 pmol

oligonucleotide primer MOD1F22, 5 pmol

oligonucleotide primer MOD1R22, 5 pmol

oligonucleotide primer MOD1F3, 5 pmol

oligonucleotide primer MOD1R3, 5 pmol

oligonucleotide primer MOD1F42, 5 pmol

oligonucleotide primer MOD1R4, 5 pmol

oligonucleotide primer MOD1F52, 5 pmol

oligonucleotide primer MOD1R52, 5 pmol

oligonucleotide primer MOD1F63, 5 pmol

oligonucleotide primer MOD1R63, 5 pmol

oligonucleotide primer MOD1F7, 50 pmol

oligonucleotide primer MOD1R72, 5 pmol

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer LR17, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM5-F51C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions The template plasmids, pSRPDHH, is obtained by following the description in an European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer MOD1F7, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM5-DNA in which above described LM5-F51B-DNA, and LM5-F51C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM5-F51B-DNA prepared in the first step PCR,

Gel fragment of LM5-F51C-DNA prepared in the first step PCR,

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 0 µl

10×PCR buffer, 5 0 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM5-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized LM5 TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using DNA analyzer

The resulting plasmids are designated pCR3 1/LM5-3-42 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM5 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM5-3-42 containing LM5-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM5-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM5-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M5-3-27 carrying a fusion fragment of the variable region of the humanized LM5 TRA-8 light chain and the constant region of human Igκ chain is obtained

3) Construction of plasmid pSR/LM5-3-27-1 (expression plasmid for humanized LM5 TRA-8 light chain)

The obtained plasmid pHSG/M5-3-27 carrying a fusion fragment of the variable region of the humanized LM5 TRA-8 light chain and the constant

region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA (European patent application EP 0 909 816 A1) is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/M5-3-27 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in pSRPDHH vector is confirmed by DNA sequencing using a gene sequence analyzer (ABI Prism 3700 DNA Analyzer; Applied Biosystems)

The resulting expression plasmid carrying cDNA encoding the light chain of humanized LM5 TRA-8 is designated pSR/LM5-3-27-1

(4 5) Construction of an Expression Vector for the Light Chain of the Humanized Antibody(chimera type)

The sequence shown in SEQ ID No 76 of the Sequence Listing, the amino acid sequence of the light chain of chimera type TRA-8, is designated LM6

Expression plasmids carrying this type of humanized light chain amino acid sequences of the anti-human DR5 antibody TRA-8 (LM6 type) (SEQ ID No 75 of the Sequence Listing) is constructed as follows

1) Synthesis of primers for preparing the variable and constant regions of the light chain of humanized LM6 TRA-8

DNA coding for the LM6 polypeptide chain (SEQ ID No 75 of the Sequence Listing), each of which is a fusion of the variable region of mouse anti-DR5 antibody TRA-8 light chain (LM6 type) and the constant region of the human Ig light chain (κ chain), are respectively synthesized by using combinations of PCR

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Further to 7AL1P (SEQ ID No 47) and 7ALCN (SEQ ID No 48), the following oligonucleotide primers are synthesized for PCR

5'- tgatgtggac atgaatttgt gagactgggt catcacaatg tcaccagtgg a -3' (HKSPR13, SEQ ID No 97),

5'- tgggttccag gctccactgg tgacattgtg atgacccagt ctcacaaatt c -3' (MVF11, SEQ ID No 98),

5'- aagacagatg gtgcagccac agcccgtttg atttccagct tggtgcctc -3' (MVR11, SEQ ID No 99), and

5'- aagctggaaa tcaaacgggc tgtggctgca ccatctgtct tcatc -3'(MCF11, SEQ ID No 100)

2) Construction of plasmid pCR3 1/LM6-1-16 (cloning of humanized TRA-8 light chain type LM6)

LM6-DNA fragment coding for the amino acid sequence as defined in SEQ ID No 75 of the same is prepared by performing 2-step PCR, inserted into a plasmid vector and cloned in *E coli*

a) First step PCR

LM6-F1-DNA fragment coding for a secretion signal sequence and a portion of FRL_1 region with a Hind III restriction enzyme cleavage site added at the 5'-end is prepared under the following conditions The template plasmids, pHSGHM17 and pSRPDHH, are obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pHSGHM17 DNA, 25 ng

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer HKSPR13, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

amplitaq DNA polymerase (PerkinElmer), 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM6-F2-DNA fragment coding for a portion of FRL_1, $CDRL_1$, FRL_2, $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 and a portion of the constant region is prepared under the following conditions

Composition of the reaction solution

plasmid pL28 DNA, 25 ng

oligonucleotide primer MVF11, 50 pmol

oligonucleotide primer MVR12, 50 pmol

dNTPs cocktail, 5 µl

10× PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM6-F3-DNA fragment coding for a portion of FRL_4 and the constant region with an EcoR I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng

oligonucleotide primer MCF11, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 µl

10×PCR buffer, 5 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 μg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor blade

b) Second step PCR

LM6-DNA in which above described LM6-F1-DNA, LM6-F2-DNA, and LM6-F3-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM6-F1-DNA prepared in the first step PCR,
Gel fragment of LM6-F2-DNA prepared in the first step PCR,
Gel fragment of LM6-F3-DNA prepared in the first step PCR,
oligonucleotide primer 7AL1P, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 0 μl
10×PCR buffer, 5 0 μl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 μl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM6-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (Invitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit The nucleotide sequences of these DNAs encoding the light chain of humanized TRA-8 are confirmed by the dideoxy method using a DNA analyzer

The resulting plasmids are designated pCR3 1/LM6-1-16 (the plasmid carrying cDNA encoding the light chain variable region of mouse TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM6-1-16 containing LM6-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM6-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 μg/ml chloramphenicol (final concentrations) The white transformants obtained are cultured in liquid LB medium containing 50 μg/ml chloramphenicol, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The extracted plasmid DNA is digested with Hind III and EcoR I, and then a clone carrying LM6-DNA fragment is selected by 1% agarose gel electrophoresis

As a result of the above procedure, plasmid pHSG/M6-1-4-1 carrying a fusion fragment of the variable region of the mouse TRA-8 light chain and the constant region of human Igκ chain is obtained The transformant *E coli* strain harboring these plasmid, designated as *E coli* DH5α/pHSG/M6-1-4-1 was deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japan on April 20, 2001, in accordance with the Budapest Treaty for the Deposit of Microorganisms, and was accorded the accession number FERM BP-7566

3) Construction of plasmid pSR/LM6-1-4-6(expression plasmid for chimera type LM6 TRA-8 light chain)

The obtained plasmid pHSG/LM6-1-4-1 carrying a fusion fragment of the variable region of the mouse TRA-8 light chain and the constant region of human Igκ chain is digested with the restriction enzymes Hind III and EcoR I

One μg of cloning plasmid pSRPDHH DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pSRPDHH DNA and HindIII-EcoRI DNA fragment obtained from pHSG/LM6-1-4-1 are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar The transformants obtained are cultured in liquid LB medium containing 100 μg/ml ampicillin, and plasmid DNA is extracted from the resulting culture according to the alkaline-SDS method The insertion and orientation of the desired DNA fragment in the vector is confirmed by DNA sequencing using a gene sequence analyzer

The resulting expression plasmid carrying cDNA encoding the light chain of TRA-8 (chimera type) is designated pSR/LM6-1-4-6

(5) Production of several types-Humanized or chimeric TRA-8 Antibody

Transfection of COS-7 cells is conducted by FUGENE6 transfection reagent methods (Boehringer Mannheim Biochemica) according to the instruction manual provided with the kit

COS-7 cells (American Type Culture Collection No CRL-1651) are grown to semi-confluent (3 x 10^6 cells/dish) in a culture dish (culture area 57 cm^2, Sumitomo Bakelite) containing Dulbecco's Modified Eagle medium (heremafter referred to as "D-MEM", Gibco BRL) supplemented with 10% fetal calf serum (heremafter abbreviated as "FCS", Moregate)

In the meantime, 10 μg/dish (total 5 dishes) of the humanized DR5 heavy chain expression plasmid DNA (pHA15-1) and 10 μg/dish of the humanized DR5 light chain expression plasmid DNA prepared by the alkaline-SDS method and cesium chloride density gradient centrifugation are mixed, and then precipitated with ethanol, followed by suspending in 5 μl/dish of dH_2O

After 15 μl/dish of FUGENE6 Transfection regent is mixed with 180 μl/dish D-MEM without FCS, this FUGENE solution (185 μl/dish) is mixed with 5 μl/dish DNA solution containing 10 μg/dish of the humanized DR5 heavy chain expression plasmid DNA and 10 μg/dish of the humanized DR5 light chain expression plasmid DNA After 15 minutes incubation at room

temperature, the obtained plasmid suspension (200 µl) is added to the previously prepared COS-7 plates After incubating in 5% CO_2 at 37°C for 24 hours, the culture medium is changed with D-MEM without FCS After incubating in 5% CO_2 at 37°C for 72 hours, the culture supernatant is recovered to purify the expression products in the supernatant fluids By the method as described above, COS-7 cells are transfected with each of the following plasmid combinations

(A) cotransfection of pHA15-1 and pSR/LM1-2 (H1L1)

(B) cotransfection of pHB14-1 and pSR/M2-1 (H2L2)

(C) cotransfection of pHB14-1 and pSR/LM3-3-44-10 (H2L3)

(D) cotransfection of pHB14-1 and pSR/LM4-5-3-3 (H2L4)

(E) cotransfection of pHC10-3 and pSR/M2-1 (H3L2)

(F) cotransfection of pHC10-3 and pSR/LM3-3-44-10 (H3L3)

(G) cotransfection of pHC10-3 and pSR/LM4-5-3-3 (H3L4)

(H) cotransfection of pHD21-1 and pSR/LM5-3-27-1 (H4L5)

(I) cotransfection of pM11-1 and pSR/LM6-1-4-6 (Chimera)

The culture is then centrifuged (3,500 r p m , 15 minutes) and collected the supernatant. The supernatant is filtrated with 0 45 µm filter (ADVANTEC TOYO DISMIC-25cs, Cat # 25CS045 AS) The purification of IgG from the filtrates are achieved using Protein G-POROS affinity chromatography (Applied Biosystems) under the following conditions

HPLC system BioCAD 700E (Applied Biosystems)

column ProteinG-ID sensor cartridge

(column size 2 1 mmID x 30 mm LD, bed volume 0 1 ml, Applied Biosystems)

elution buffer 0 1 M Glycine-HCl (pH 2 5)

neutralization buffer 1 M Tris-HCl (pH 8 5)

detection 280 nm

flow rate 1 ml/min

fraction size 0 5 ml/0 5 min

fraction tube 1 5 ml polypropylene microtube

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

LM3-F31C-DNA fragment coding for a portion of the constant region with an Eco R I restriction enzyme cleavage site added at the 3'-end is prepared under the following conditions

The template plasmids, pSRPDHH, is obtained by following the description in a European patent application EP 0 909 816 A1

Composition of the reaction solution

plasmid pSRPDHH DNA, 25 ng
oligonucleotide primer MOD1F7, 50 pmol
oligonucleotide primer 7ALCN, 50 pmol
dNTPs cocktail, 5 µl
10×PCR buffer, 5 µl
ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The amplified DNA fragments after PCR are separated by 5% polyacrylamide gel electrophoresis The gel after electrophoresis is stained with 1 µg/ml of ethidium bromide to detect the produced DNA under UV light The respective DNA bands thus detected are excised with a razor

b) Second step PCR

LM3-DNA in which above described LM3-F31B-DNA, and LM3-F31C-DNA fragments are fused is prepared under the following conditions

Composition of the reaction solution

Gel fragment of LM3-F31B-DNA prepared in the first step PCR,

Gel fragment of LM3-F31C-DNA prepared in the first step PCR,

oligonucleotide primer 7AL1P, 50 pmol

oligonucleotide primer 7ALCN, 50 pmol

dNTPs cocktail, 5 0 µl

10× PCR buffer, 5 0 µl

ampliTaq DNA polymerase, 2 5 units

The reaction solution having the above composition is adjusted to a final volume of 50 µl by adding redistilled water and used in PCR.

PCR thermal conditions Heating at 94°C for 2 minutes, after which a thermal cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, repeated 30 times, followed by heating at 72°C for 10 minutes

The thus prepared LM3-DNA fragment is inserted into plasmid pCR3 1DNA using Eukaryotic TA cloning Kit (InVitrogen) following the manufacturer's protocol and introduced into the competent *E Coli* TOP10F' contained in the kit. The nucleotide sequences of these DNAs encoding the light chain of humanized LM3 TRA-8 are confirmed by the dideoxy method (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) using 3700 DNA Analyzer (ABI PRISM, Perkin Elmer Applied Biosystems, Japan)

The resulting plasmids are designated pCR3 1/LM3-3-44 (the plasmid carrying cDNA encoding the light chain variable region of humanized LM3 TRA-8 and a human Ig light chain constant region)

The obtained plasmid pCR3 1/LM3-3-44 containing LM3-DNA fragment is digested with the restriction enzymes Hind III and EcoR I

One µg of cloning plasmid pHSG399 DNA is digested with the restriction enzymes Hind III and EcoR I, and then dephosphorylated with CIP The resulting dephosphorylated pHSG399 DNA and LM3-DNA fragment, that had been digested with the restriction enzymes Hind III and EcoR I, are ligated using DNA Ligation Kit Version 2 0 (Takara Syuzo, Co Ltd) Then, *E coli* DH5α is transformed with the ligated DNA and spread onto LB agar medium containing 0 1 mM IPTG, 0 1% X-Gal and 50 µg/ml chloramphenicol (final

temperature 4°C

After all the filtrates are applied to column, 50 ml of PBS (Sigma, Cat # 1000-3) is used to wash column When the elution buffer is applied, fraction collector started Each fraction microtube previously contained 55 µl of 1 M NaCl, 110 µl of neutralization buffer and 74 µl of 2 mg/ml bovine serum albumin (Sigma, Cat # A-7030) in PBS The fractions from No 7 through No 8 are collected

Verification of the expression of the humanized antibodies and quantitative assay of the expression products in the culture supernatant fluids prepared is performed by ELISA with an antibody against anti-human IgG

To each well of a 96-well plate (MaxiSorp, Nunc), 100 µl of goat anti-human IgG Fc specific polyclonal antibody (Kappel) dissolved at the final concentration of 0 5 µg/ml in adsorption buffer (0 05 M sodium hydrogencarbonate, 0 02% sodium azide, pH 9 6) is added and the plate is incubated at 37°C for 2 hours to cause adsorption of the antibody Then, the plate is washed with 350 µl of PBS-T five times To the wells after washing the culture supernatant diluted with D-MEM containing 10% FCS is added and incubated at 37°C for 2 hours After washing again with PBS-T, 100 µl of alkaline phosphatase-labeled goat anti-human IgG Fc specific polyclonal antibody (Jackson Immuno Research Lab) diluted 10,000-fold with PBS-T is added to each well and incubated at 37°C for 2 hours After washing again with PBS-T, a substrate solution of p-nitrophenyl phosphate obtained from Alkaline Phosphatase Substrate kit (Bio Rad) is added according to the instruction manual provided with the kit After incubating at 37°C for 0 5 to 1 hour, the absorbance at 405 nm is measured In the present experiments, human plasma immunoglobulin G subclass 1 (IgG1) (Biopure AG) diluted with D-MEM containing 10% FCS to certain concentrations is used as concentration reference samples of the humanized DR5 antibodies contained in the culture supernatant fluids

As a result, the expression and purified products in the culture supernatant are detected specifically with the anti-human IgG antibody The

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final concentration of human IgG antibody is 44 03 μg/ml (H1L1), 39 8 μg/ml (H2L2), 26 7 μg/ml (H2L3), 41 0 μg/ml (H2L4), 39 3 μg/ml (H3L2), 24 7 μg/ml (H3L3), 21 5 μg/ml (H3L4), 16 7 μg/ml (H4L5) and 18 3 μg/ml (chimera), respectively

(6) Apoptosis-inducing activity of several types Humanized Antibody or Chimeric Antibody

Jurkat cells (ATCC No TIB-152), are used to examine the apoptosis-inducing activity of the purified humanized TRA-8 antibody

Jurkat cells cultured in RPMI1640 medium with 10% FCS (Gibco BRL) at 37°C for 3 days in the presence of 5% CO_2 are dispensed into each well of a 96-well microplate (Sumitomo Bakelite) at 50 μl per well The humanized TRA-8 prepared in this Example 26 are adjusted to have the concentration of the final product of interest of 100 ng/ml with RPMI1640 medium containing 10% FCS by estimating their concentrations in the fluids according to the method described in Example 26 Each of the solutions of the expression products thus adjusted to 100 ng/ml is used to produce serial dilutions by repeating serial 2-fold dilution with RPMI1640 containing 10% FCS Each of the diluted humanized TRA-8 solution (H1L1, H2L2, H2L3, H2L4, H3L3, H3L4 or H4L5) is added to each well at 50 μl per well After reacting at 37°C for 12 hours, 50 μl of 25 μM PMS containing 1 mg/ml XTT is added (final concentrations of 250 μg/ml for XTT and 5 μM for PMS) After incubating for 3 hours, the absorbance at 450 nm of each well is measured to calculate the cell viability by using the reduction ability of mitochondria as the index

The viability of the cells in each well is calculated according to the following formula

$$\text{Viability (\%)} = 100 \times (a-b) / (c-b)$$

wherein "a" is the measurement of a test well, "b" is the measurement of a well with no cells, and "c" is the measurement of a well with no antibody added

As a result, the tested humanized antibodies are demonstrated to induce apoptosis in cells of T lymphoma cell line expressing human DR5 antigen

Furthermore, the apoptosis-inducing activity of humanized TRA-8 to PC-3 is examined by adding taxol according to the method described in Example 25

Human prostate cancer cell line PC-3 (ATCC No CRL-1435) is obtained from American Tissue Culture Collection (ATCC) and maintained in F-12K Nutrient Mixture (21127-022, Gibco BRL) containing 10% fetal bovine serum (FBS, Hyclone), 1% L-Glutamine-200 mM (25030-149, Gibco BRL) and 0 5% Penicillin Streptomycin Solution (P-7539, Sigma) RPMI1640 medium (MED-008, IWAKI) supplemented with 10% FBS and 0 5% Penicillin Streptomycin Solution is used in the following experiment. Exponentially growing PC-3 cells are collected by trypsinization and washed twice with fresh medium The cells are then counted, resuspended in fresh medium at a density of 5×10^4 cells/ml and distributed in triplicate into flat-bottomed 96 well plates (3598, Corning-Coster) in a total volume of 100 µl/well one day before the start of the experiment. A representative anti-cancer drug, Paclitaxel (169-18611, Wako) dissolved in dimethylsulfoxide (10 mg/ml) is diluted in fresh medium and then added to the 96-well plates containing the cells at 50 µl/well The final concentrations of dimethylsulfoxide are less than 0 1% After incubation for 24 hr at 37°C in 5% CO_2 atmosphere, humanized TRA-8 antibody (H1L1, H2L2, H2L3, H2L4, H3L2, H3L3, H3L4 or H4L5) diluted in fresh medium is added to the wells After incubation for a further 24 hr, 50 µl of Minimum Essential Medium (11095-098, Gibco BRL) containing 1 mg/ml of XTT and 25 mM of PMS is added to the wells and the plates are incubated for 6 hr OD450 is then measured by ARVO HTS 1420 Multilabel Counter (Wallac Berthold) and the cell viability is calculated as follows

Cell viability (%) = (OD450 for the well containing cells treated with Taxol and humanized TRA-8 (agent(s)) - OD450 for the well containing neither cells nor agent) x 100 / (OD450 for the well containing cells with no agent –OD450 for the well containing neither cells nor agent)

As a result, the tested humanized antibodies are demonstrated to induce apoptosis in human prostate cancer cells expressing human DR5 antigen

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Any patents or publications mentioned in the specification are indicative of the level of those skilled in the art to which the invention pertains These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference

The present invention is not limited in scope by the above-referenced deposit or the embodiments disclosed in the examples which are intended as illustrations of a few aspects of the invention and any embodiments which are functionally equivalent are within the scope of this invention Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims

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INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY
UNITED STATES PATENT AND TRADEMARK OFFICE UNDER
PATENT COOPERATION TREATY

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UAB Research Foundation	)
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International Application No	)
PCT/US01/14151	)
	)
International Filing Date	)
02 May 2001	)
	)
For AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF	)
	)
Attorney Ref No 21085 0029P1	)

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Washington, D C 20231

April 9, 2002

AMENDMENT UNDER PCT ARTICLE 34

Sir or Madam

Pursuant to PCT Article 34, Applicants herewith submits amended claims for the above-identified PCT application in the form of substitute pages 144-156 The page number on the Abstract has been renumbered to 157 Thus, a substitute page for the Abstract is also provided herein The Abstract page contains no additional changes as compared to the originally filed Abstract

Applicants respectfully request that the United States Patent and Trademark Office (hereinafter referred to as the "PTO") accept the Amendment under PCT Article 34 and conduct an examination based on the amended claims The substitute claims are provided because new agents have assumed prosecution of this application and have modified the claims to place the claims in a better position for examination Applicants believe that the substitute claims present no new matter to the present application Support for the substitute claims can be found in the claims and specification as originally filed

It is requested that the enclosed substitute pages be incorporated into the application and taken into consideration in conducting the International Preliminary Examination Applicant requests the PTO Examiner to contact the undersigned if any further information is required

Respectfully submitted,
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Claims

1 A purified antibody which binds a TRAIL receptor DR5, wherein said antibody in its soluble form at low concentrations, has *in vivo* and *in vitro* cell death-inducing activity in target cells expressing DR5

2 The purified antibody of claim 1, wherein the antibody induces cell death *in vitro* in the absence of secondary antibody crosslinking

3 The purified antibody of claim 1, wherein the antibody does not induce significant cell death of non-transformed fibroblast cells

4 The purified antibody of claim 1, wherein the cell death-inducing activity is characterized by less than 40% target cell viability at antibody concentrations of less than 5 μg/ml

5 The purified antibody of claim 1, wherein the antibody specifically binds TRAIL receptor DR5 and does not bind TRAIL receptors DR4, DcR1, or DcR2 upon routine Western blot analysis

6 The purified antibody of claim 1, wherein said antibody is a monoclonal antibody

7 The purified antibody of claim 1, wherein the DR5 is human DR5

8 The purified antibody of claim 1, wherein said target cells are inflammatory synovial cells

9 The purified antibody of claim 1, wherein said target cells are cancer cells

10 The purified antibody of claim 1, wherein said target cell is an activated immune cell

11 The purified antibody of claim 10, wherein said immune cell is an activated lymphocyte

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12 The purified antibody of claim 10, wherein said immune cell is an activated NK cell

13 The purified antibody of claim 10, wherein said immune cell is an activated dendritic cell

14 A monoclonal antibody having the same epitope specificity as mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

15 A monoclonal antibody produced by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

16 The purified antibody of claim 1, wherein said antibody is humanized

17 A method of selectively inducing cell death in target cells expressing DR5, comprising the step of contacting the target cells with a therapeutic amount of the antibody of claim 1

18 The method of claim 17, wherein the contacting is *in vivo*

19 The method of claim 17, wherein the contacting is *in vitro*

20 The method of claim 17, wherein the target cells expressing DR5 are cancer cells

21 The method of claim 17, wherein the target cells expressing DR5 are inflammatory synovial cells

22 The method of claim 17, wherein the target cells expressing DR5 are activated immune cells

23 The method of claim 22, wherein the immune cells are activated lymphocytes

24 The method of claim 22, wherein the immune cells are activated NK cells

25 The method of claim 22, wherein the immune cells are activated dendritic cells

26 A method of selectively inducing cell death in target cells expressing DR5, comprising the step of contacting the target cells with a therapeutic quantity of the antibody of claim 16

27 A process of inhibiting proliferation of target cells expressing DR5, comprising the step of contacting the cells with a therapeutic quantity of the antibody of claim 1

28 The process of claim 27, wherein the contacting is *in vivo*

29 The process of claim 27, wherein the contacting is *in vitro*

30 The process of claim 27, wherein the target cells expressing DR5 are cancer cells

31 The process of claim 27, wherein the target cells expressing DR5 are inflammatory synovial cells

32 The process of claim 27, wherein the target cells expressing DR5 are activated immune cells

33 The process of claim 32, wherein the activated immune cells are activated lymphocytes

34 The process of claim 21, wherein the activated immune cells are activated NK cells

35 The process of claim 21, wherein the activated immune cells are activated dendritic cells

36 The process of claim 21, further comprising contacting the target cells with a therapeutic agent

37 The method of claim 36, wherein the therapeutic agent is a chemotherapeutic agent

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38 The method of claim 37, wherein the therapeutic agent is selected from the group consisting of bleomycin, carboplatin, chlorambucil, cisplatin, colchicine, cyclophosphamide, daunorubicin, actinomycin, diethylstilbestrol, doxorubicin, etoposide, 5-fluorouracil, floxuridine, melphalan, methotrexate, mitomycin, 6-mercaptopurine, paxlitaxel, teniposide, 6-thioguanine, vincristine and vinblastine

39 The method of claim 37, wherein the therapeutic agent is doxorubicin

40 The method of claim 37, wherein the therapeutic agent is methotrexate

41 The method of claim 37, wherein the therapeutic agent is paclitaxel

42 A process of inhibiting proliferation of target cells expressing DR5, comprising the step of contacting the cells with a therapeutic quantity of the antibody of claim 16

43 The process of claim 42, wherein the contacting is *in vivo*

44 The process of claim 42, wherein the contacting is *in vitro*

45 The process of claim 42, wherein the target cells expressing DR5 are cancer cells

46 The process of claim 42, wherein the target cells expressing DR5 are inflammatory synovial cells

47 The process of claim 42, wherein the target cells expressing DR5 are activated immune cells

48 The process of claim 47, wherein the immune cells are activated lymphocytes

49 The process of claim 47, wherein the immune cells are activated NK cells

50 The process of claim 47, wherein the immune cells are activated dendritic cells

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51 The process of claim 42, further comprising contacting the target cells with a therapeutic agent

52 The method of claim 51, wherein the therapeutic agent is a chemotherapeutic agent

53 The method of claim 52, wherein the therapeutic agent is selected from the group consisting of bleomycin, carboplatin, chlorambucil, cisplatin, colchicine, cyclophosphamide, daunorubicin, actinomycin, diethylstilbestrol doxorubicin, etoposide, 5-fluorouracil, floxuridine, melphalan, methotrexate, mitomycin, 6-mercaptopurine, paxlitaxel, teniposide, 6-thioguanine, vincristine and vinblastine

54 The method of claim 52, wherein the therapeutic agent is doxorubicin.

55 The method of claim 52, wherein the therapeutic agent is methotrexate

56 The method of claim 52, wherein the therapeutic agent is paclitaxel

57 A composition comprising a pharmaceutically effective amount of the antibody of claim 1 and a pharmaceutically acceptable carrier

58 Use of the antibody of claim 1 for preparing a therapeutic for selective cell death of target or dysregulated cells

59 A process of treating a subject with an apoptosis related disease comprising the step of contacting a target tissue of the subject expressing the apoptosis related disease with a therapeutic quantity of a first antibody according to claim 1

60 A process of treating a subject with an apoptosis related disease comprising the step of contacting a target tissue of the subject expressing the apoptosis related disease with a therapeutic quantity of a first antibody according to claim 16

61 A method of treating a subject with cancer, comprising administering to the subject a pharmaceutically effective amount of the antibody of claim 1, wherein the

pharmaceutically effective amount of the antibody selectively induces cell death of cancer cells

62 The method of claim 61, further comprising administering to the subject a second antibody that enhances the cell death of cancer cells

63 The method of claim 61, further comprising administering to the subject a therapeutic agent

64 The method of claim 63, wherein the therapeutic agent is a chemotherapeutic agent

65 The method of claim 64, wherein the therapeutic agent is selected from the group consisting of bleomycin, carboplatin, chlorambucil, cisplatin, colchicine, cyclophosphamide, daunorubicin, actinomycin, diethylstilbestrol doxorubicin, etoposide, 5-fluorouracil, floxuridine, melphalan, methotrexate, mitomycin, 6-mercaptopurine, paxlitaxel, teniposide, 6-thioguanine, vincristine and vinblastine

66 The method of claim 64, wherein the therapeutic agent is doxorubicin.

67 The method of claim 64, wherein the therapeutic agent is methotrexate

68 The method of claim 64, wherein the therapeutic agent is paclitaxel

69 The method of claim 64, further comprising administering to the subject radiation therapy

70 The method of claim 64, further comprising administering to the subject a therapeutic agent and radiation therapy

71 A method of treating a subject with cancer, comprising administering to the subject a pharmaceutically effective amount of the antibody of claim 16, wherein the pharmaceutically effective amount of the antibody selectively induces cell death of the cancer cells

72 A method of treating a subject having an inflammatory disease or autoimmune disease, comprising administering to the subject a pharmaceutically effective amount of the antibody of claim 1, wherein the pharmaceutically effective amount of the antibody selectively induces cell death of target cells having DR5 receptors

73 The method of claim 72, wherein the target cells are activated immune cells

74 The method of claim 73, wherein the activated immune cells are activated lymphocytes

75 The method of claim 73, wherein the activated immune cells are activated NK cells

76 The method of claim 73, wherein the activated immune cells are activated dendritic cells

77 The method of claim 72, wherein the target cells are inflammatory synovial cells

78 The method of claim 72, further comprising administering to the subject a pharmaceutically effective amount of a second antibody that enhances cell death of the target cells

79 The method of claim 72, further comprising administering to the subject a pharmaceutically effective amount of a therapeutic agent

80 The method of claim 79, wherein the therapeutic agent is a bisindolylmaleimide

81 The method of claim 79, wherein the therapeutic agent is methotrexate

82 A method of treating a subject having an inflammatory disease or autoimmune disease, comprising administering to the subject a pharmaceutically effective amount of the antibody of claim 16, wherein the pharmaceutically effective amount of the antibody selectively induces cell death of target cells

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83 A method of selectively inducing cell death in target cells comprising the steps of

(a) transfecting the target cells with a vector comprising an expressible TRAIL receptor nucleic acid sequence,

(b) expressing on said cells a TRAIL receptor encoded by said TRAIL receptor nucleic acid sequence, and

(c) contacting said cells with a cell death-inducing antibody selective for binding said TRAIL receptor

84 The method of claim 83, wherein the TRAIL receptor is a DR5 receptor

85 The method of claim 83, wherein said antibody is a monoclonal antibody having the same epitope specificity as mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

86 An isolated nucleic acid, comprising a nucleotide sequence that encodes a heavy chain immunoglobulin of an antibody capable of binding TRAIL receptor DR5 and capable of inducing cell death of a cell with DR5 receptors

87 The isolated nucleic acid of claim 86, wherein the nucleotide sequence encodes the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

88 The isolated nucleic acid of claim 86, wherein the nucleotide sequence encodes the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more conservative amino acid substitutions

89 The isolated nucleic acid of claim 86, wherein the nucleotide sequence encodes the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more amino acid deletions

90 The isolated nucleic acid of claim 86, comprising nucleotides 1-1398 of SEQ ID NO 21

91 The isolated nucleic acid of claim 86, wherein the encoded heavy chain immunoglobulin is humanized

92 The isolated nucleic acid of claim 91, wherein the encoded humanized, heavy chain immunoglobulin comprises amino acid residues 1-119 of SEQ ID NO 31

93 A vector comprising the nucleic acid of claim 86 and a regulatory element operably linked to the nucleic acid

94 A cultured cell comprising the vector of claim 93

95 A purified polypeptide comprising an amino acid sequence of a heavy chain immunoglobulin of an antibody capable of binding TRAIL receptor DR5 and capable of inducing cell death of a cell with DR5 receptors

96 The purified polypeptide of claim 95, wherein the amino acid sequence is the amino acid sequence of the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

97 The purified polypeptide of claim 95, wherein the amino acid sequence is the amino acid of the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more conservative amino acid substitutions

98 The purified polypeptide of claim 95, wherein the amino acid sequence is the amino acid of the heavy chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more amino acid deletions

99 The purified polypeptide of claim 95, wherein the amino acid sequence comprises the amino acid residues 1-464 of SEQ ID NO 23

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100 The purified peptide of claim 95, wherein the heavy chain immunoglobulin is humanized

101 The purified peptide of claim 100, wherein the amino acid sequence of the humanized heavy chain immunoglobulin comprises amino acid residues 1-119 of SEQ ID NO 31

102 The purified peptide of claim 100, wherein the amino acid sequence of the humanized heavy chain immunoglobulin comprises amino acid residues 1-119 of SEQ ID NO 31 with one or more conservative substitutions

103 The purified peptide of claim 100, wherein the amino acid sequence of the humanized heavy chain immunoglobulin comprises amino acid residues 1-119 of SEQ ID NO 31 with one or more amino acid deletions

104 An isolated nucleic acid, comprising a nucleotide sequence that encodes a light chain immunoglobulin of an antibody capable of binding TRAIL receptor DR5 and capable of inducing cell death of a cell with DR5 receptors

105 The isolated nucleic acid of claim 104, wherein the nucleotide sequence encodes the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

106 The isolated nucleic acid of claim 104, wherein the nucleotide sequence encodes the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more conservative amino acid substitutions

107 The isolated nucleic acid of claim 104, wherein the nucleotide sequence encodes the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more amino acid deletions

108 The isolated nucleic acid of claim 104, comprising nucleotides 1-705 of SEQ ID NO 22

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109 The isolated nucleic acid of claim 104, wherein the encoded light chain immunoglobulin is humanized

110 The isolated nucleic acid of claim 104, wherein the encoded humanized, light chain immunoglobulin comprises amino acid residues 1-213 of SEQ ID NO 46

111 A vector comprising the nucleic acid of claim 104 and a regulatory element operably linked to the nucleic acid

112 A cultured cell comprising the vector of claim 111

113 A purified polypeptide comprising an amino acid sequence of a light chain immunoglobulin of an antibody capable of binding TRAIL receptor DR5 and capable of inducing cell death of a cell with DR5 receptors

114 The purified polypeptide of claim 113, wherein the amino acid sequence is the amino acid sequence of the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428

115 The purified polypeptide of claim 113, wherein the amino acid sequence is the amino acid of the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more conservative amino acid substitutions

116 The purified polypeptide of claim 113, wherein the amino acid sequence is the amino acid of the light chain immunoglobulin expressed by mouse-mouse hybridoma TRA-8 having ATCC Accession Number PTA-1428 with one or more amino acid deletions

117 The purified polypeptide of claim 113, wherein the amino acid sequence comprises amino acid residues 1-234 of SEQ ID NO 24

118 The purified peptide of claim 113, wherein the light chain immunoglobulin is humanized

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119 The purified peptide of claim 118, wherein the amino acid sequence of the humanized light chain immunoglobulin comprises amino acid residues 1-213 of SEQ ID NO 46

120 The purified peptide of claim 118, wherein the amino acid sequence of the humanized light chain immunoglobulin comprises amino acid residues 1-213 of SEQ ID NO 46 with one or more conservative substitutions

121 The purified peptide of claim 118, wherein the amino acid sequence of the humanized light chain immunoglobulin comprises amino acid residues 1-213 of SEQ ID NO 46 with one or more amino acid deletions

122 A process for producing the humanized antibody of claim 16, comprising the steps of

(a) transforming a host cell with a first vector encoding a humanized immunoglobulin light chain, a humanized immunoglobulin heavy chain, or both,

(b) optionally, transforming the host cell with a second vector encoding the light chain or heavy chain of step (a) if either is absent from said first vector;

(c) incubating the transformed host cell under conditions that permit expression of the vector or vectors, and

(d) isolating the humanized antibody from the host cell or medium of the host cell

123 A commercial kit for inducing apoptosis comprising the antibody of claim 1 in a container

124 A commercial kit for inducing apoptosis comprising the antibody of claim 16 in a container

125 A process for increasing NFκB levels in a cell comprising the step of contacting the cell with an effective amount of the antibody of claim 1

126 A process for increasing NFκB levels in a cell comprising the step of contacting the cell with an effective amount of the antibody of claim 16

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Abstract of the Invention

An antibody of the invention interacts with human DR5 to produce agonistic or antagonistic effects downstream of the receptor including inhibition of cell proliferation and apoptosis Nucleic acid sequences and amino acid sequences of anti-DR5 antibodies have been elucidated and vectors and cells containing and expressing these sequences have been generated Methods and uses for the antibodies are detailed including treatment of apoptosis-related disease and treatment of dysregulated cell growth

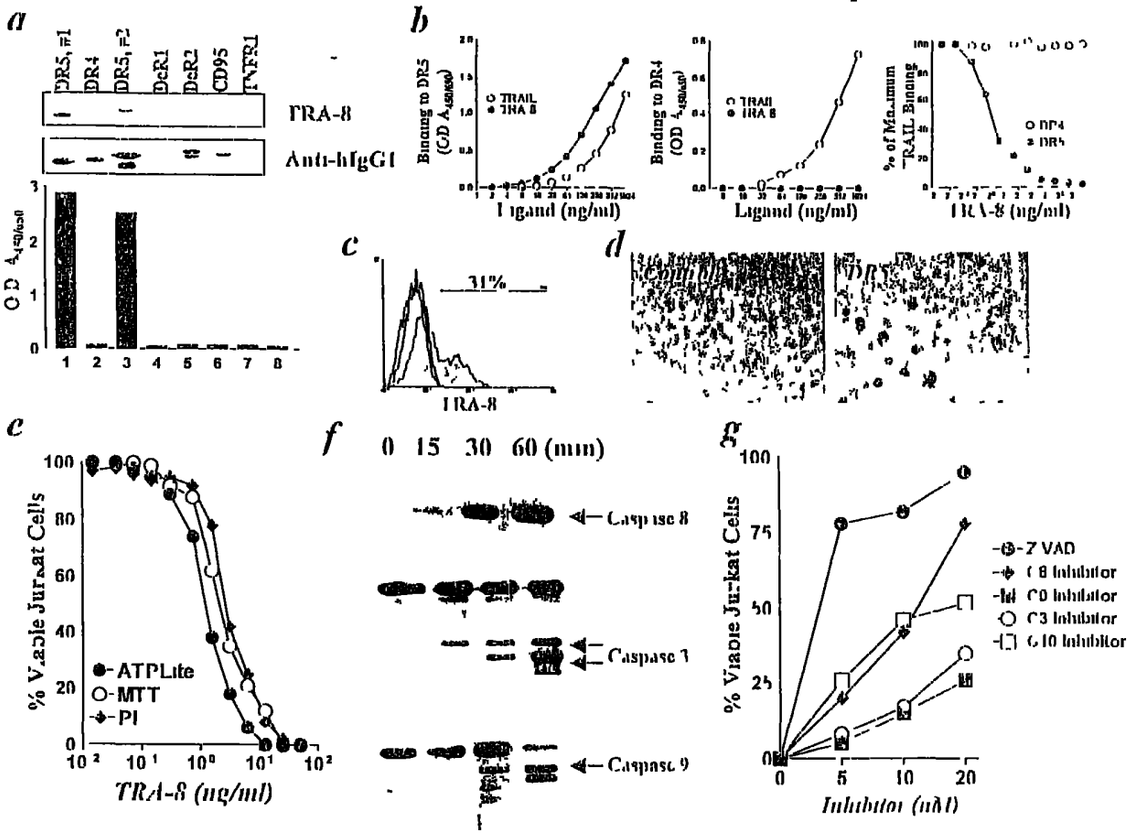

a
DR5, #1
DR4
DR5, #2
DcR1
DcR2
CD95
TNFR1
TRA-8
Anti-hIgG1
OD A450/650
1 2 3 4 5 6 7 8
b
Binding to DR5 (OD A450/650)
TRAIL
TRA-8
Ligand (ng/ml)
Binding to DR4 (OD A450/650)
TRAIL
TRA-8
Ligand (ng/ml)
% of Maximum TRAIL Binding
DR4
DR5
TRA-8 (ng/ml)
c
31%
TRA-8
d
e
% Viable Jurkat Cells
ATPLite
MTT
PI
TRA-8 (ng/ml)
f
0 15 30 60 (min)
Caspase 8
Caspase 3
Caspase 9
g
% Viable Jurkat Cells
Z-VAD
C8 Inhibitor
C9 Inhibitor
C3 Inhibitor
C10 Inhibitor
Inhibitor (nM)

Figure 1

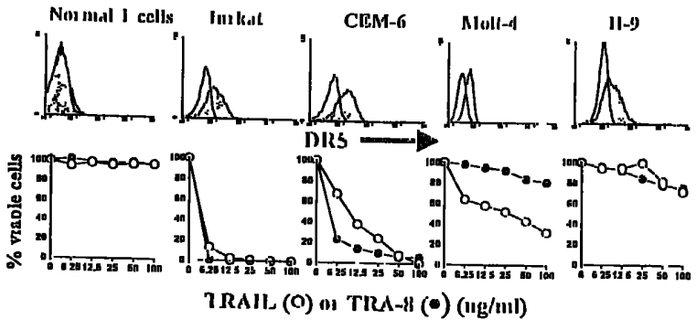

Normal T cells
Jurkat
CEM-6
Molt-4
H-9
DR5
% viable cells
TRAIL (○) or TRA-8 (●) (ng/ml)

Figure 2a

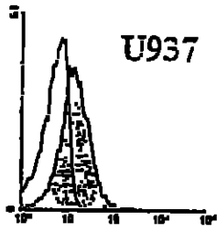


Figure 2a'

Glioma cells

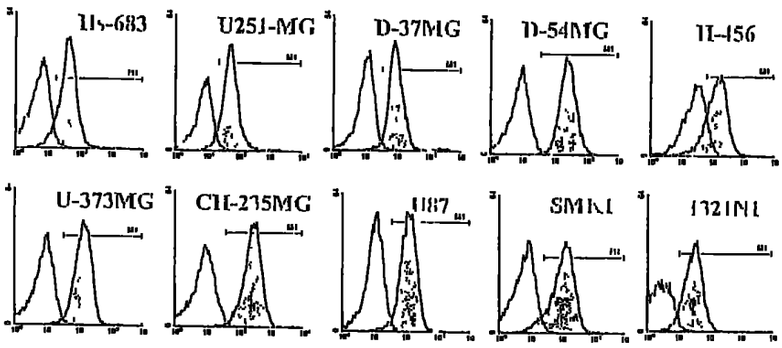


Figure 2b

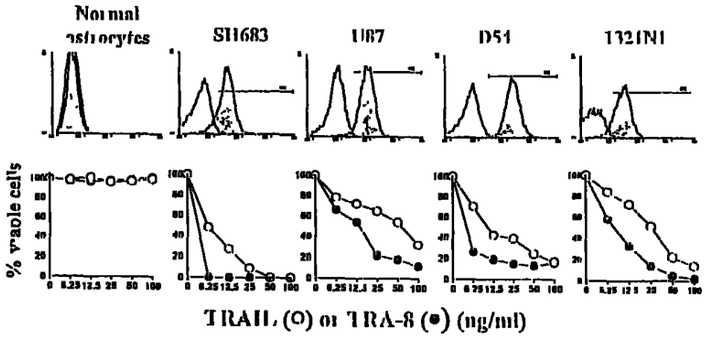

Normal astrocytes
SH683
U87
D54
T32IN1
% viable cells
TRAIL (○) or TRA-8 (●) (ng/ml)

Figure 2b'

Prostate cancer cells

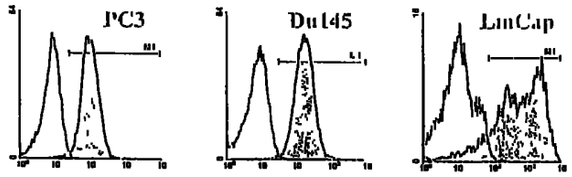


Figure 2c

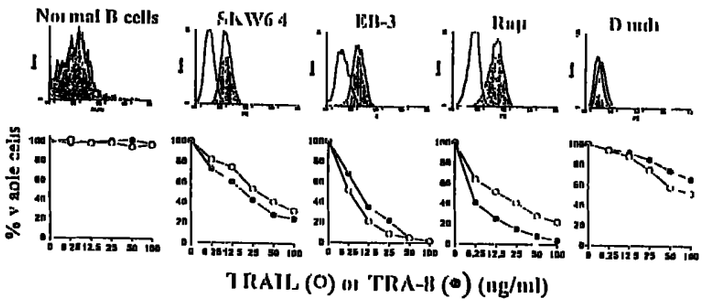

Normal B cells
SKW6.4
EB-3
Raji
Daudi
% viable cells
TRAIL (O) or TRA-8 (●) (ng/ml)

Figure 2d

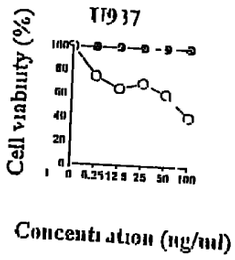
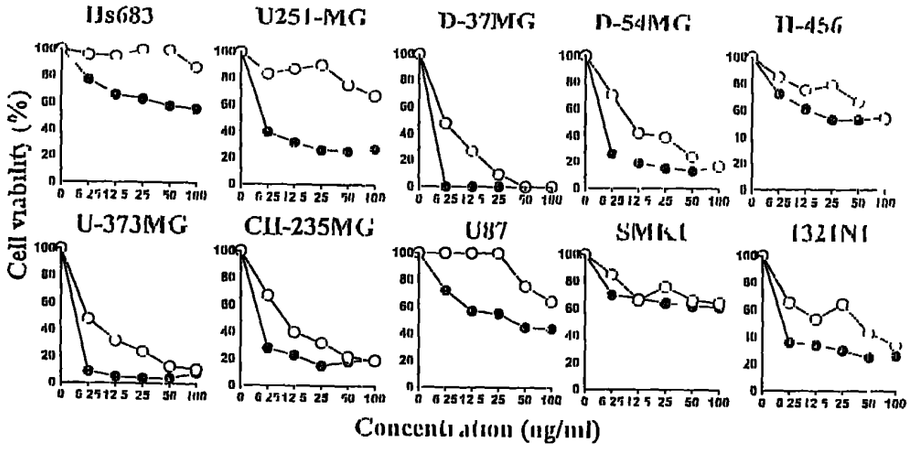

U937
Cell viability (%)
100
80
60
40
20
0
0 0.25 1 2 5 25 50 100
Concentration (ng/ml)

Figure 3a'

b. Glioma cells



c. Prostate cancer cells

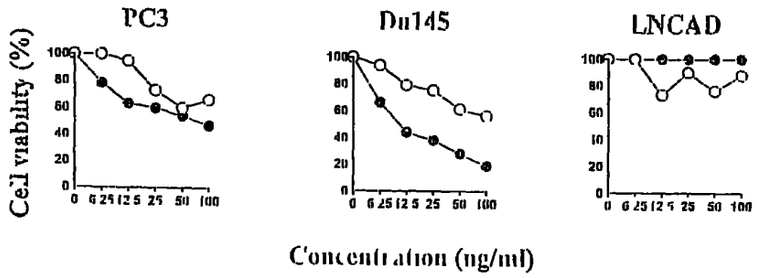
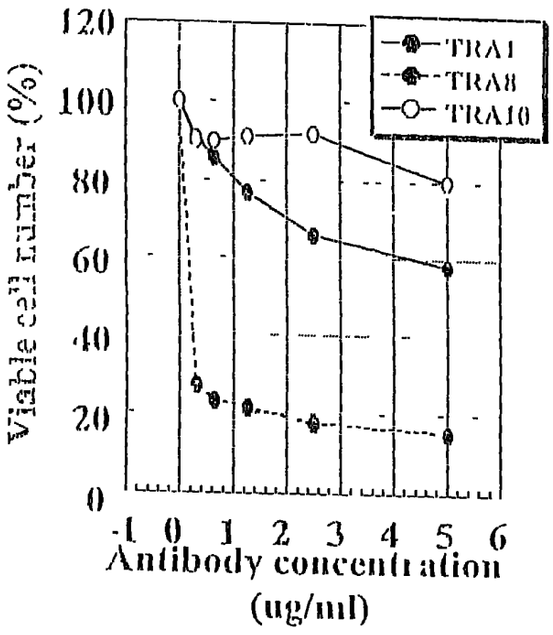


Figure 3

4A

Apoptosis-inducing activity



4B

Apoptosis-inducing activity

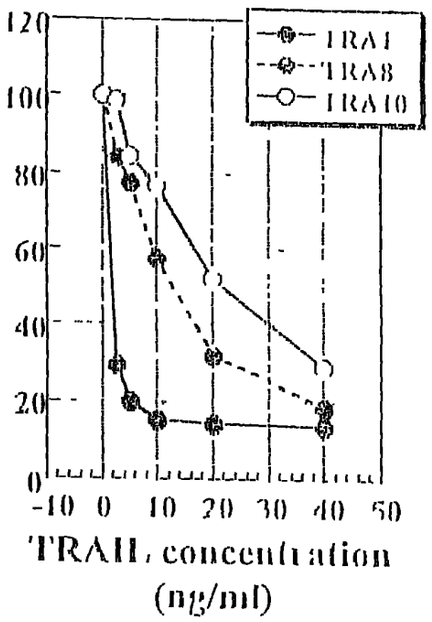


Figure 4

360

a. Normal tissue homogenates

1 2 3 4 5 6 7 8 9

b. Cancer tissue homogenates

1 2 3 4 5 6 7 8 9 10 11 12 13

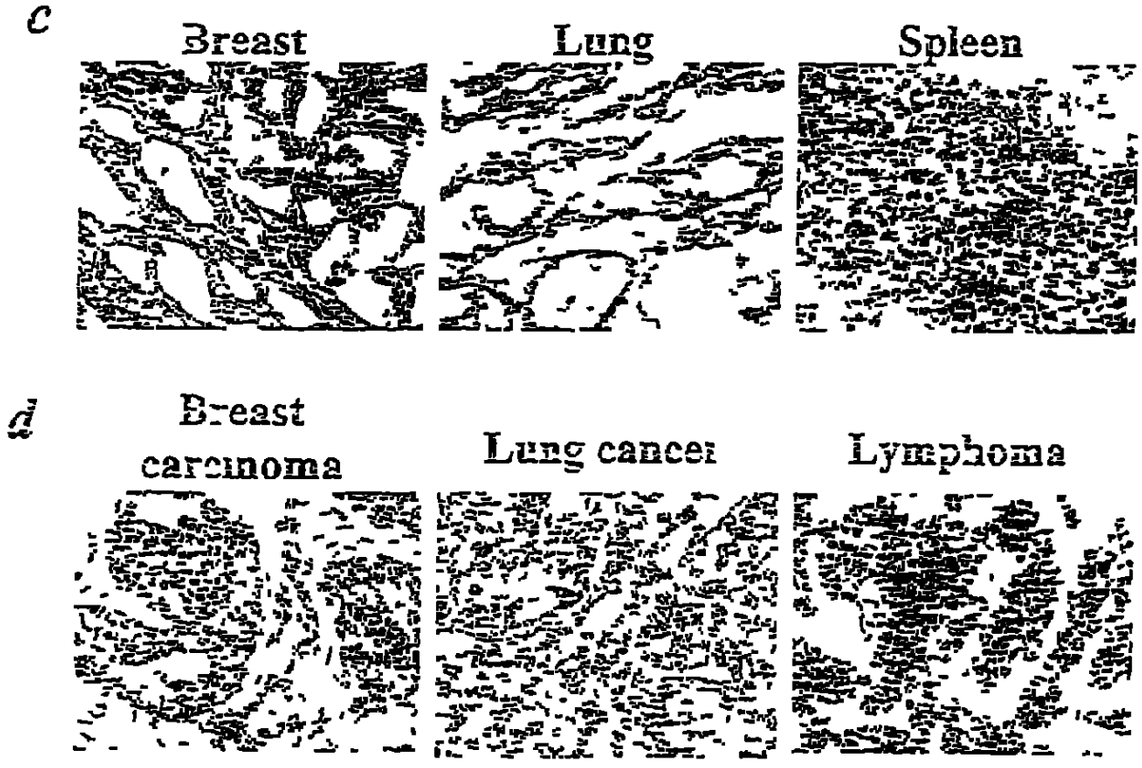


Figure 5

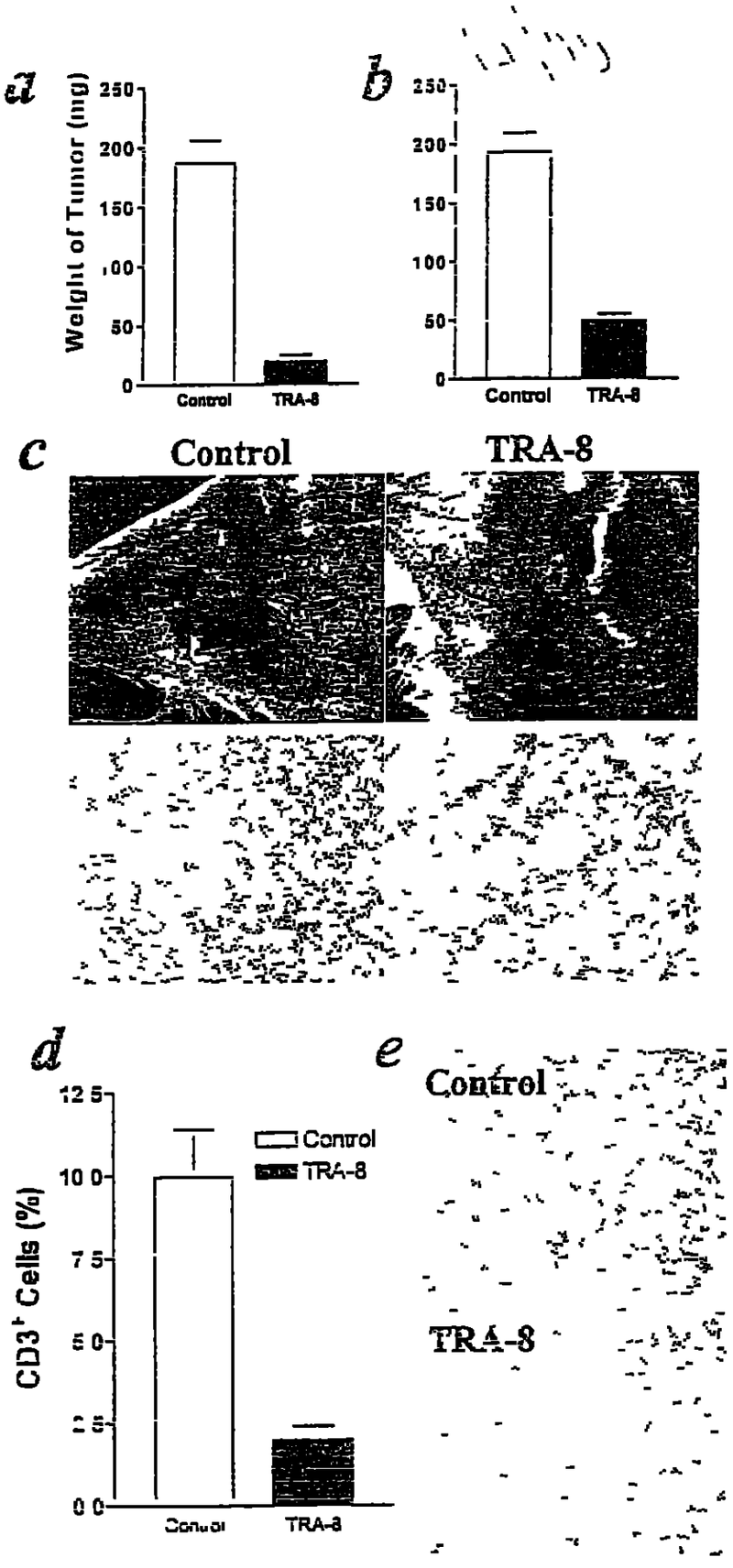
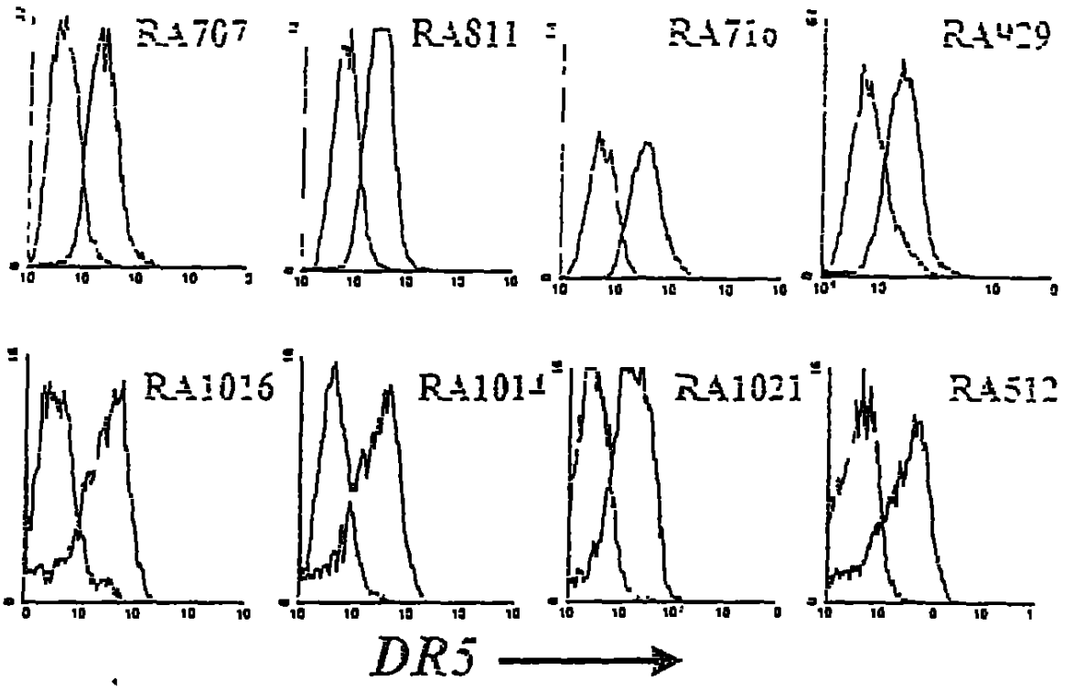

a
Weight of Tumor (mg)
250
200
150
100
50
0
Control
TRA-8
b
250
200
150
100
50
0
Control
TRA-8
c
Control
TRA-8
d
CD3+ Cells (%)
12.5
10.0
7.5
5.0
2.5
0.0
Control
TRA-8
Control
TRA-8
e
Control
TRA-8

Figure 6

c. RA synovial cells



b. OA synovial cells

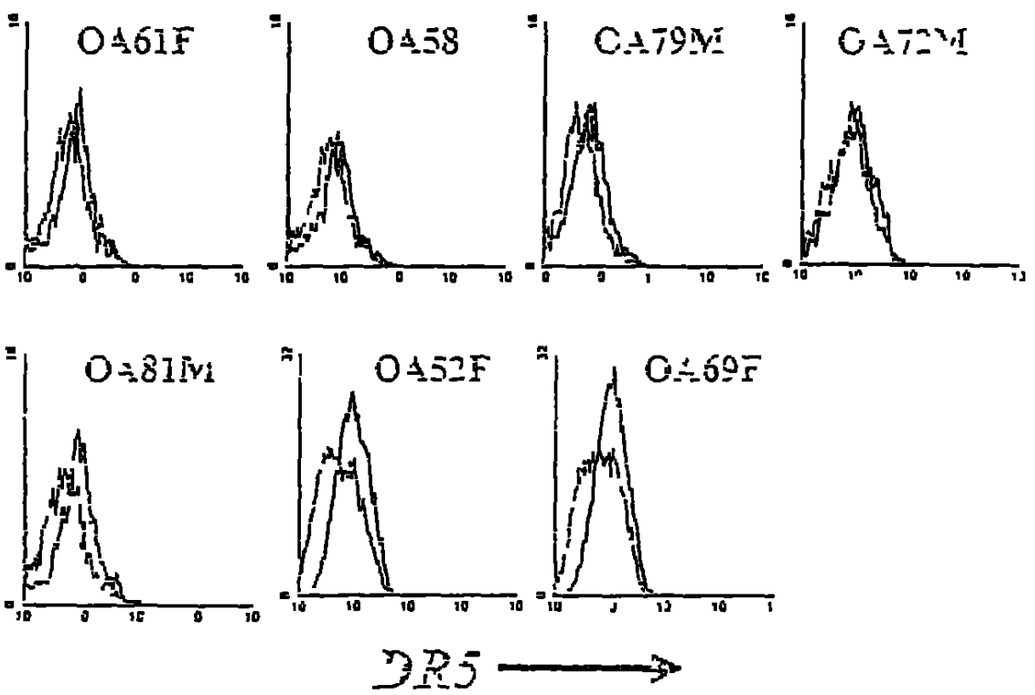


Figure 7

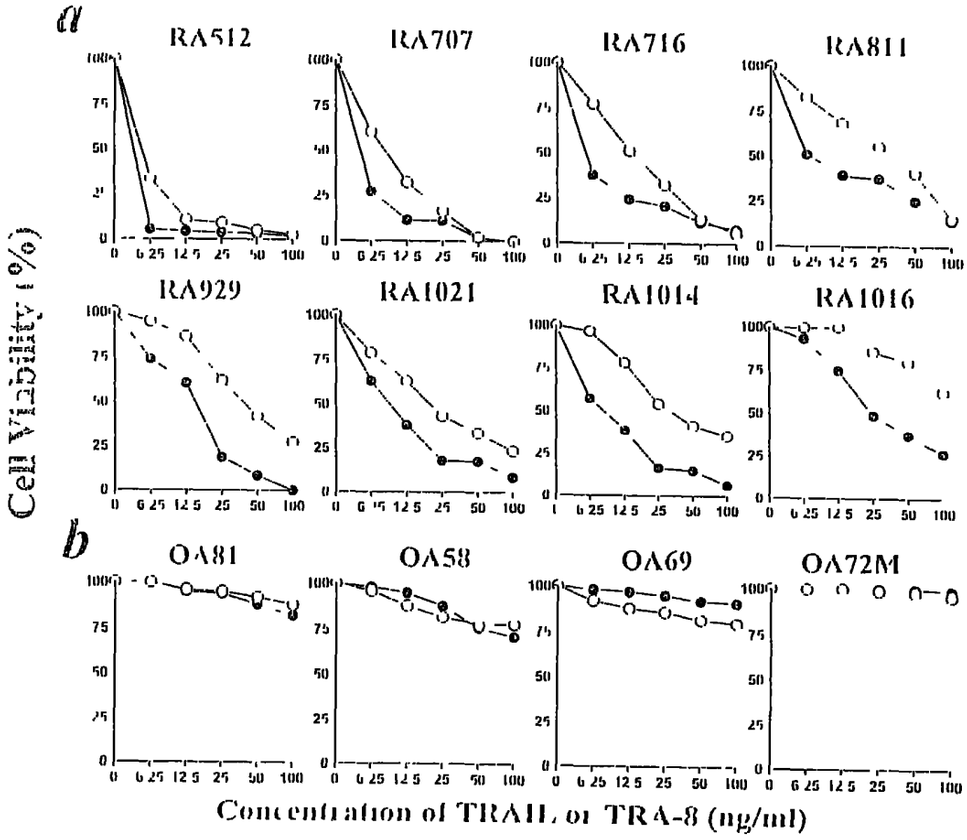

a
RA512
RA707
RA716
RA811
RA929
RA1021
RA1014
RA1016
b
OA81
OA58
OA69
OA72M
Cell Viability (%)
Concentration of TRAIL or TRA-8 (ng/ml)

Figure 8

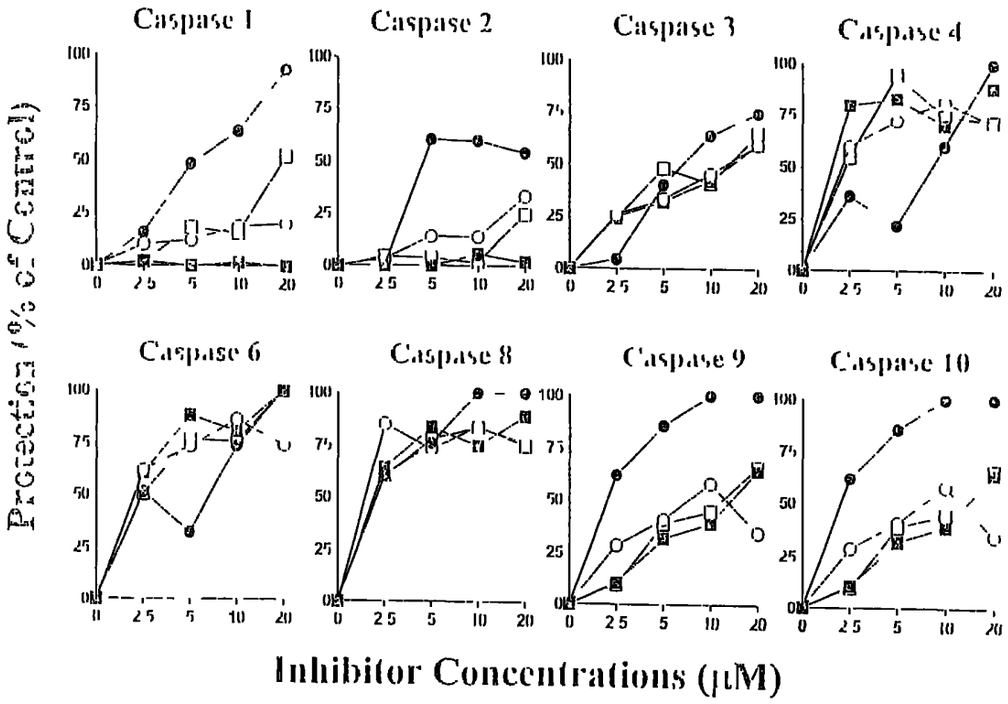
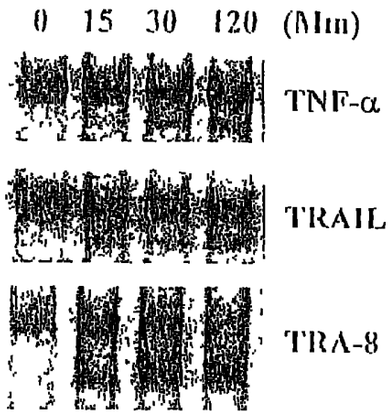
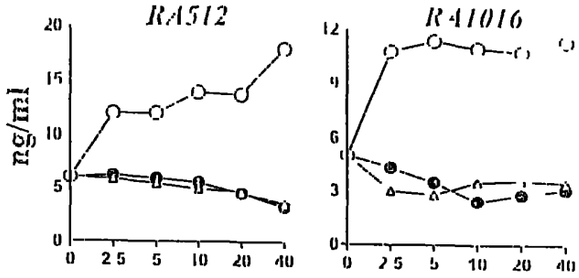

Caspase 1
Caspase 2
Caspase 3
Caspase 4
Caspase 6
Caspase 8
Caspase 9
Caspase 10
Protection (% of Control)
Inhibitor Concentrations (μM)

Figure 9

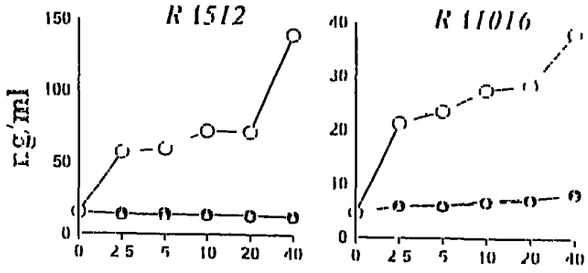
d.



b. MMP1



c. MMP3



Concentration of TNF-α (x1), TRAIL (x5), TRA-8 (x5) (ng/ml)

Figure 10

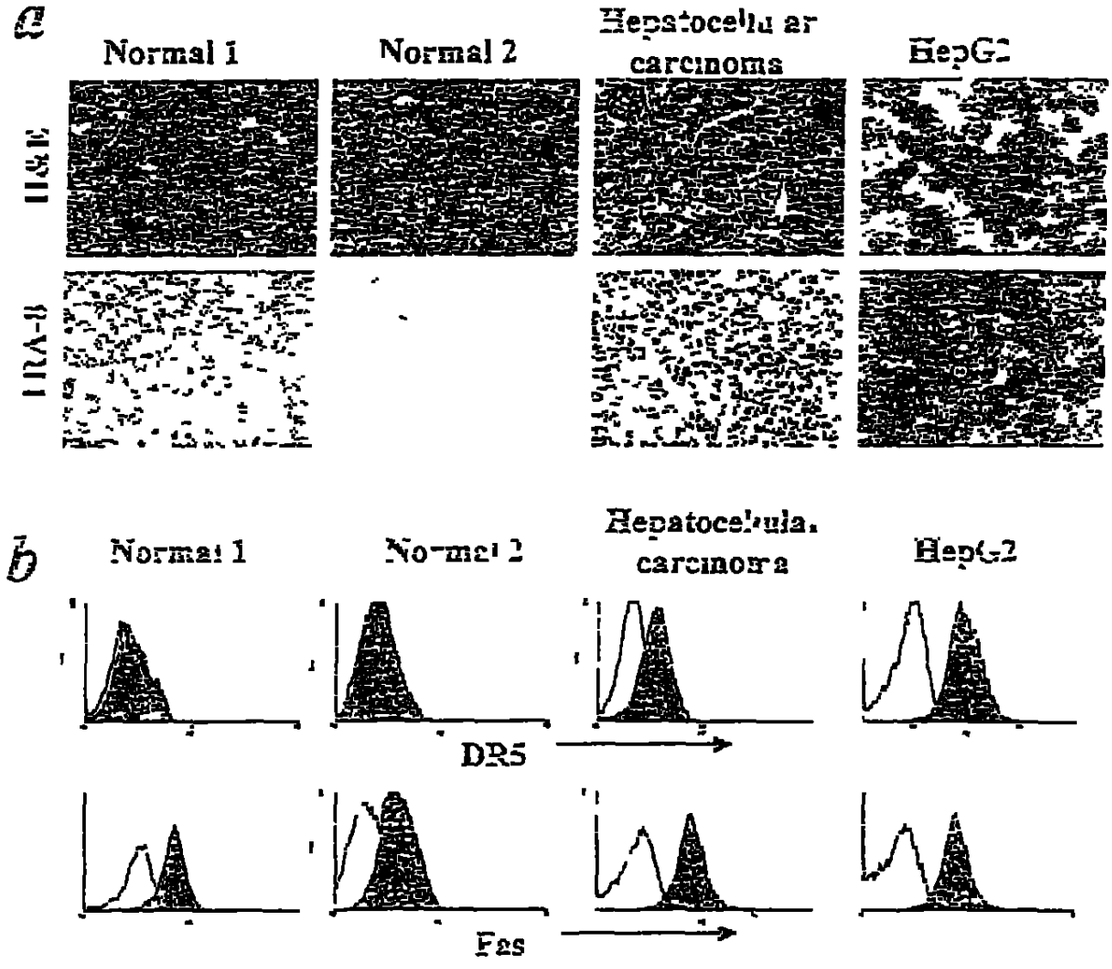

a
Normal 1
Normal 2
Hepatocellular carcinoma
HepG2
H&E
TRA-8
b
Normal 1
Normal 2
Hepatocellular carcinoma
HepG2
DR5
Fas

Figure 11

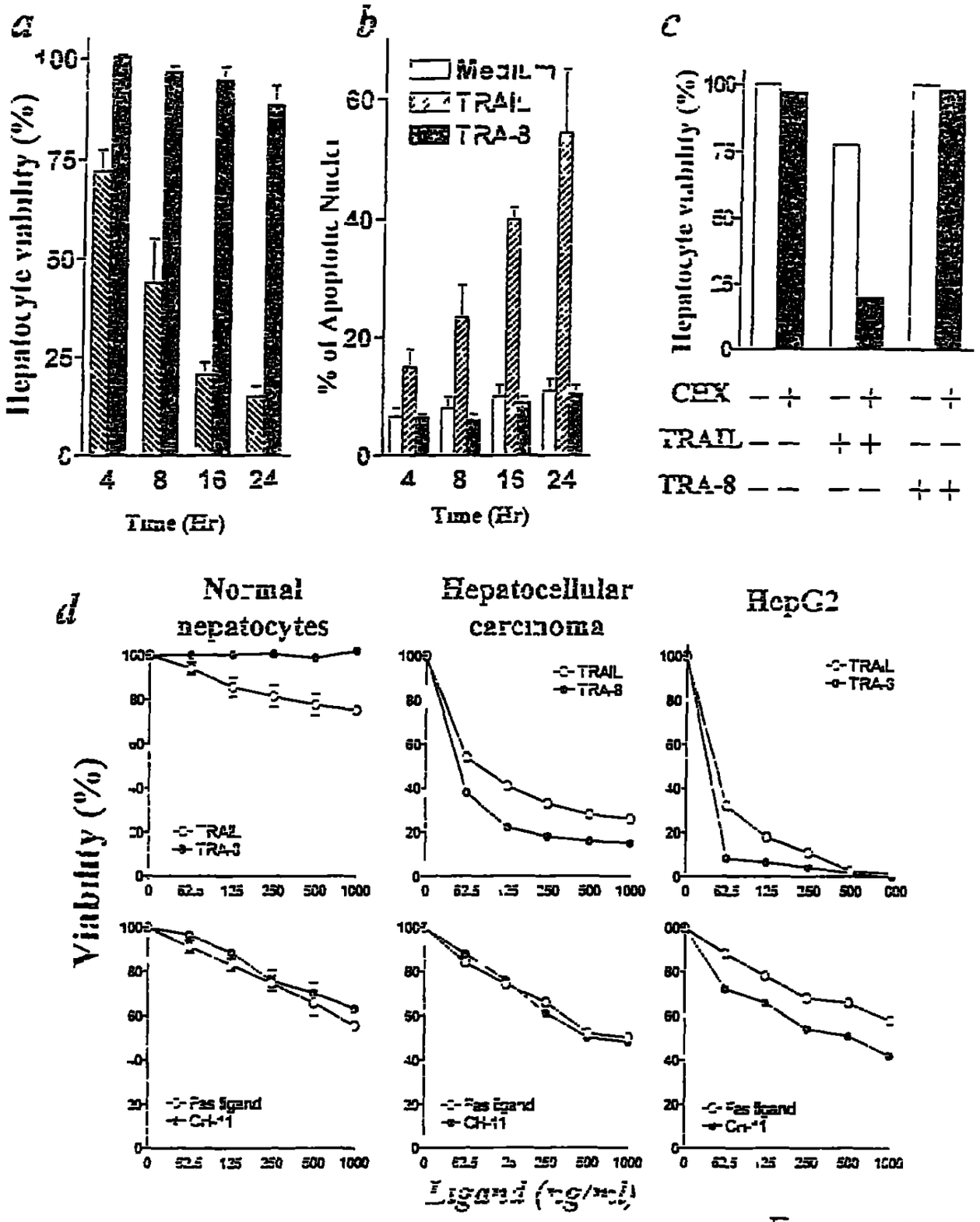

a
Hepatocyte viability (%)
100
75
50
25
0
4
8
16
24
Time (Hr)
b
% of Apoptotic Nuclei
60
40
20
0
Media
TRAIL
TRA-8
4
8
16
24
Time (Hr)
c
Hepatocyte viability (%)
100
75
50
25
0
CEX − + − + − +
TRAIL − − + + − −
TRA-8 − − − − + +
d
Normal hepatocytes
Hepatocellular carcinoma
HepG2
Viability (%)
TRAIL
TRA-8
Fas ligand
CH-11
0
62.5
125
250
500
1000
Ligand (ng/ml)

Figure 12

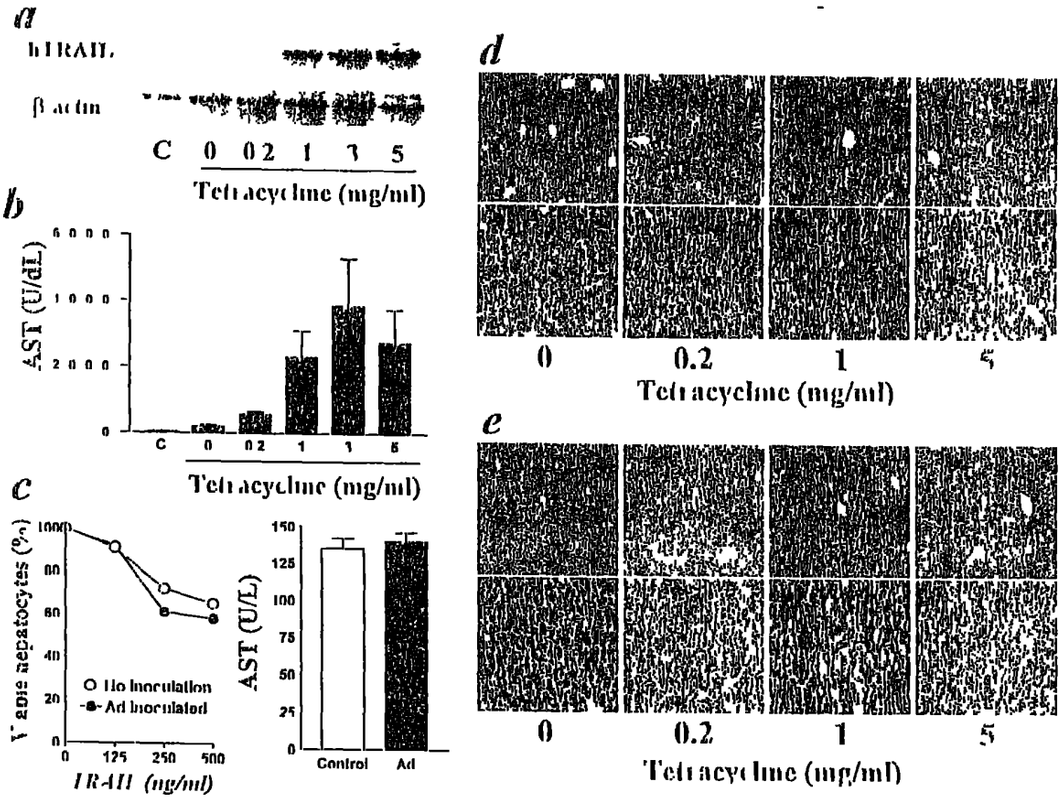

a
hTRAIL
β-actin
C 0 0.2 1 3 5
Tetracycline (mg/ml)
b
AST (U/dL)
6000
4000
2000
0
C 0 0.2 1 3 5
Tetracycline (mg/ml)
c
Viable hepatocytes (%)
100
80
60
40
20
0
No inoculation
Ad inoculated
0 125 250 500
TRAIL (ng/ml)
AST (U/L)
150
125
100
75
50
25
0
Control
Ad
d
0 0.2 1 5
Tetracycline (mg/ml)
e
0 0.2 1 5
Tetracycline (mg/ml)

Figure 13

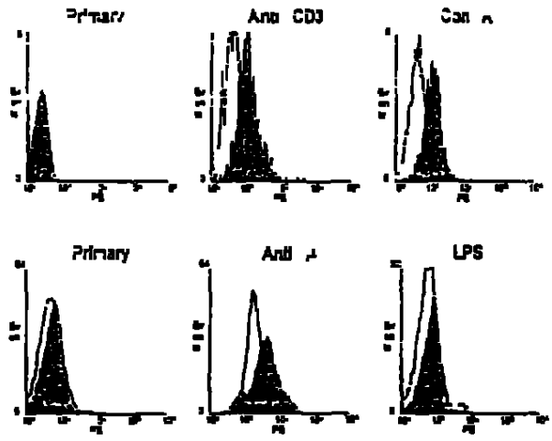


Figure 14

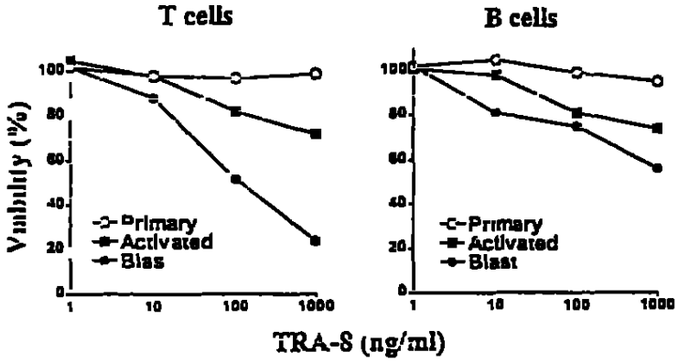


Figure 15

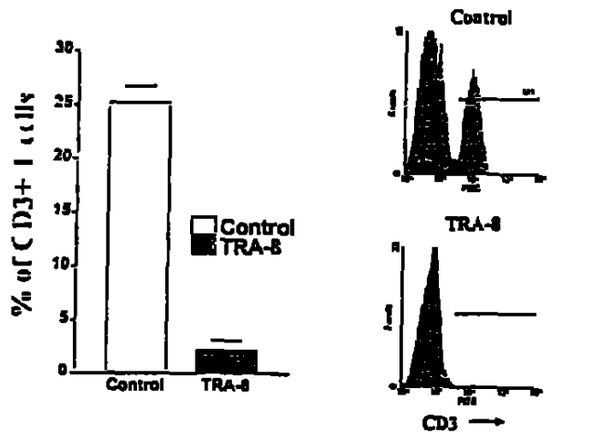


Figure 16

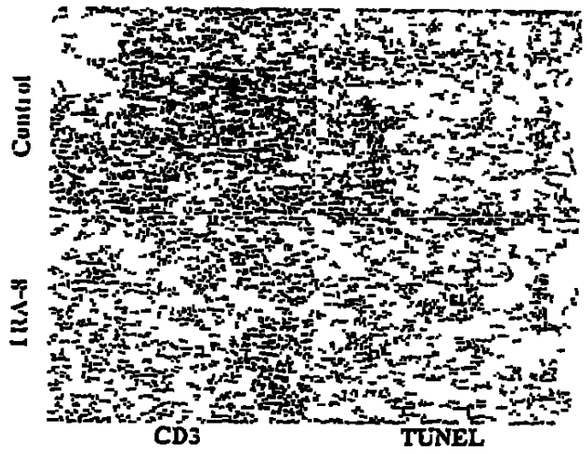


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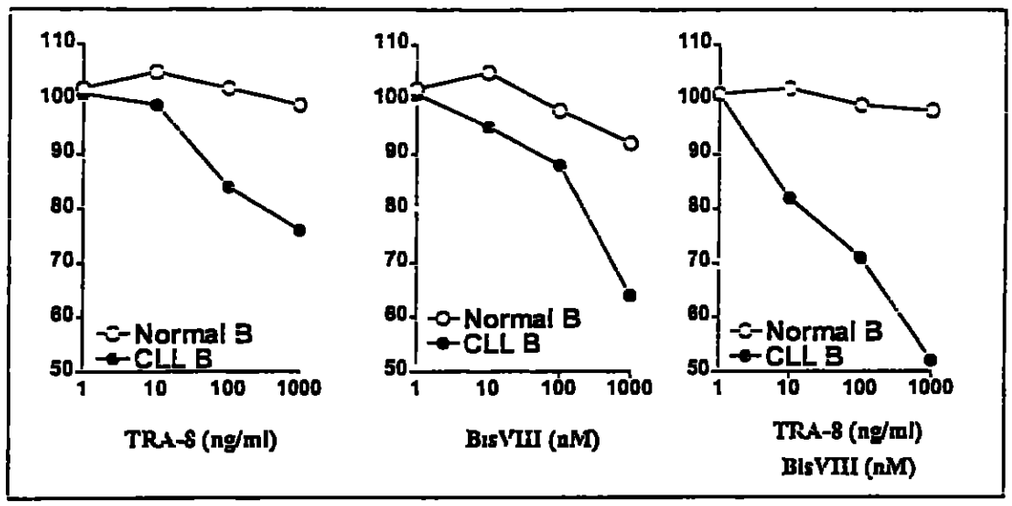

110
100
90
80
70
60
50
Normal B
CLL B
1
10
100
1000
TRA-8 (ng/ml)
BisVIII (nM)
TRA-8 (ng/ml)
BisVIII (nM)

Figure 18

~~370~~
373

SEQUENCE LISTING

<110> The UAB Research Foundation

<120> AN ANTIBODY SELECTIVE FOR A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR AND USES THEREOF

<130> UAB-17552/22

<160> 102

<170> PatentIn version 3 0

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~~923~~
376

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240

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300

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360

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420

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540

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600

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Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45

Ser Ser Tyr Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu
50 55 60

Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
65 70 75 80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
85 90 95

Thr Leu Tyr Leu Gln Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met
100 105 110

Tyr Tyr Cys Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp
115 120 125

Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr Thr Pro Pro
130 135 140

Ser Val Tyr Pro Leu Ala Pro Gly Ser Ala Ala Gln Thr Asn Ser Met
145 150 155 160

Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Pro Val Thr
165 170 175

Val Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His Thr Phe Pro
180 185 190

Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser Val Thr Val
195 200 205

Pro Ser Ser Thr Trp Pro Ser Glu Thr Val Thr Cys Asn Val Ala His
210 215 220

Pro Ala Ser Ser Thr Lys Val Asp Lys Lys Ile Val Pro Arg Asp Cys
225 230 235 240

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Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu Val Ser Ser Val Phe
                245                 250                 255

Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr Ile Thr Leu Thr Pro
            260                 265                 270

Lys Val Thr Cys Val Val Val Asp Ile Ser Lys Asp Asp Pro Glu Val
        275                 280                 285

Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val His Thr Ala Gln Thr
    290                 295                 300

Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe Arg Ser Val Ser Glu
305                 310                 315                 320

Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly Lys Glu Phe Lys Cys
                325                 330                 335

Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile Glu Lys Thr Ile Ser
            340                 345                 350

Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro
        355                 360                 365

Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser Leu Thr Cys Met Ile
    370                 375                 380

Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu Trp Gln Trp Asn Gly
385                 390                 395                 400

Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro Ile Met Asp Thr Asp
                405                 410                 415

Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val Gln Lys Ser Asn Trp
            420                 425                 430

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35 40 45

Asp Val Gly Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser
50 55 60

Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro
65 70 75 80

Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
85 90 95

Ser Asn Val Gln Ser Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln Tyr
100 105 110

Ser Ser Tyr Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
115 120 125

Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln
130 135 140

Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr
145 150 155 160

Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln
165 170 175

Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr
180 185 190

Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg
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384

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1 5

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1 5 10 15

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20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
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385

60

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35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
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Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
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Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
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145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
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Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
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480

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600

agcaaagcag actacgagaa acacaaagtc tacgcctgcg aagtcaccca tcagggcctg
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

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394

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

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<400> 61

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Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Val Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys
85 90 95

Ala Arg Arg Gly Asp Ser Met Ile Thr Thr Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Thr Leu Thr Val Ser Ser
115

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cagggtgttc ttggcattgt
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ggtgactcta tgattacgac
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394
397+

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gtccgtcgta
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Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
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Asn Arg Gly Glu
210

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<211> 213
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<400> 73

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1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
20 25 30

394
394

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

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<211> 213
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<400> 74

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1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Phe Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

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20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Asp Tyr Phe Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
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<400> 76

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1 5 10 15

Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Gly Thr Ala
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
35 40 45

Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65 70 75 80

Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln Tyr Ser Ser Tyr Arg Thr
85 90 95

Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Gly Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
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1400
403

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cttaagctt
69

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cagggtc
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<210> 82
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404

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ccaggaa
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ttggtaccag gc
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402
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60

tat
63

<210> 87
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2403
406

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gaa
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57

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60

tat
63

404
407

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57

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57

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49

~~405~~
408

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60

gaa
63

UN ANTICUERPO SELECTIVO PARA UN RECEPTOR DE LIGANDO QUE INDUCE APOPTOSIS RELACIONADA CON EL FACTOR DE NECROSIS DE TUMOR Y USOS DE ESTE

CAMPO DE LA INVENCIÓN

La presente invención se relaciona con un anticuerpo capaz de ligar específicamente a un tipo simple de receptor de ligando que induce apoptosis relacionada (en lo sucesivo denominado como "TRAIL" con el factor de necrosis de tumor (en lo sucesivo denominado como "TNF"), más particularmente, con un anticuerpo monoclonal que incluye apoptosis en células in vivo e in vitro que expresan el receptor tipo simple y las terapias basadas en este

ANTECEDENTES DE LA INVENCIÓN

El TRAIL es un miembro de la familia TNF de proteínas, que también incluye ligando (1) TNF-α y Fas Estas proteínas son potentes inductores de apoptosis A la fecha, se han identificado cinco receptores de TRAIL,

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dos de los cuales, DR4 (TRAIL-R1) y DR5 (TRAIL-R2) (2-7), son capaces de transducir la señal de apoptosis mientras que los otros tres de DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), y la osteoprotegerina (OPG) no transduce la señal de apoptosis (8-12) Todos los cinco receptores para TRAIL comparten homología significativa en sus dominios de unión de ligando extracelular Similar al receptor I Fas y TNF (en lo sucesivo denominado como "TNFRI"), los segmentos intracelulares tanto del DR4 como del DR5 contienen un dominio muerto, y transducen una señal de apoptosis a través de una senda que involucra la proteína de dominio muerta asociada con Fas (en lo sucesivo denominada como "FADD") y caspasa 8 (6, 7) Además de transducir la señal de apoptosis, los receptores DR4 y DR5 también pueden activar una senda que involucra NFkb (6, 7)

Las funciones biológicas del TRAIL que han sido demostradas incluyen la capacidad del TRAIL a inducir selectivamente apoptosis de células de tumor transformadas, con células normales que son relativamente resistentes a la apoptosis mediada por TRAIL (13-15) Esta selectividad sugiere que, en contraste con el ligando Fas, la administración del TRAIL está asociada

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con muy bajos niveles de toxicidad como se demostró mediante la administración sistémica de TRAIL en un modelo animal sin inducir toxicidad significativa (13) De esta manera, el TRAIL se ha propuesto como un agente que induce apoptosis potente que sería un agente terapéutico adecuado para el tratamiento de cáncer y otros desórdenes asociados con proliferación anormal de células El TRAIL también se ha propuesto por ser un agente potente que induce apoptosis que sería adecuado para el tratamiento de enfermedades autoinmunes e inflamatorias Se ha demostrado que la apoptosis mediada por TRAIL se involucra en la muerte celular inducida por activación de células T, sirviendo de esta manera como un mecanismo alternativo al ligado Fas (16, 17) La apoptosis mediada por TRAIL también puede funcionar en la inducción de apoptosis de células T y otras células inflamatorias (18), y juega un papel en la actividad de muerte de las células NK (19-21), y en la función inmunomodulatoria de células dendríticas (22, 23) De esta manera, la apoptosis mediada por TRAIL también puede funcionar en inmunoprivilegio e inmunosupervisión

El sistema receptor TRAIL es complejo, e incluye al menos dos receptores de muerte, DR4 y DR5, y al menos dos

receptores no apoptósicios DcR1 y DcR2 Todos estos receptores no solo comparten una homología alta de secuencia aminoácido, sino también exhiben una afinidad de unión similar al TRAIL (2-12) La capacidad de los receptores DcR1 y DcR2 para competir por la unión del TRAIL sin inducir apoptosis sugiere que ellos pueden actuar como receptores señuelo que bloquean o modulan la actividad del ligando TRAIL Más aún, se ha reportado que célula no transformadas expresan niveles más altos de receptores señuelo que lo que lo hacen las células transformadas Así, se ha propuesto que la modulación diferencial de la expresión de la muerte y los receptores señuelo pueden representar un mecanismo regulatorio clave que determina la susceptibilidad de las células a la apoptosis mediada por TRAIL, pero debido a la falta de los anticuerpos específicos a los receptores (2) Aunque la expresión y función del DR4 y DR5 ha sido estudiada intensamente, se ha impedido el progreso por la falta de anticuerpos monoclonales específicos de los receptores La expresión de superficie celular del DR5 no se ha documentado Se ha reportado que un panel de anticuerpos de receptor anti-TRAIL se han generado que son capaces de inducir apoptosis de células de melanoma in vitro pero solo a la inmovilización de los anticuerpos, para

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promover reticulación, y, en algunos casos, las células requieren cultivo con actinomicina D (24) Varios anticuerpos anti-DR5 se han generado (24) Sin embargo, estos anticuerpos monoclonales anti-DR5 previamente generados tienen una actividad que induce baja apoptosis in vitro, aún bajo las condiciones de reticulación No se ha reportado actividad in vivo Estos anticuerpos no han sido utilizados para examinar la expresión de la superficie celular de los receptores TRAIL (24) De esta manera, existe necesidad de un anticuerpo monoclonal selectivo para cada receptor TRAIL específico que no es solo capaz de unirse al receptor en la superficie celular sino también de inducir fuertemente apoptosis de varios tipos de célula anormales, que incluyen células de tumor, tanto in vivo como in vitro sin el requisito de reticulación o inmovilización Tal anticuerpo no solo suministraría un agente terapéutico potencial sino también una herramienta de diagnóstico para análisis funcional del receptor TRAIL Existe necesidad particular de un anticuerpo específico contra cada uno de los receptores que inducen muerte DR4 y DR5

En el desarrollo, o progresión de muchas enfermedades es a menudo el caso que las células no sean

suprimidas En muchas enfermedades autoinmunes y condiciones inflamatorias, las células sobrevivientes activadas atacan los tejidos o células normales Además, la progresión y tumorigénesis y la formación de panus proliferativo de la artritis reumatoide son características de la proliferación no revisada de células Así, la apoptosis insuficiente conduce al desarrollo de la enfermedad, y el uso de un ligando que induce apoptosis o anticuerpo monoclonal agonístico para mejora la apoptosis se considera como una estrategia potencial terapéutica para eliminar aquellas células no requeridas

Por ejemplo, la artritis reumatoide (en lo sucesivo denominada como "RA") es una enfermedad autoinmune común en los humanos El entendimiento habitual de la patofisiología del RA es que las células T autoinmunes y las células B inician una respuesta inflamatoria en las articulaciones que conduce a la hiperproliferación de sinoviocitos Como consecuencia de la hiperproliferación de células sinoviales, las metanoproteinasas (en lo sucesivo denominadas como "MMPS") son sobreproducidas lo cual además conduce a la destrucción erusiva del cartílago y el hueso que es característica de RA (25)

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Así, el control de hiperproliferación de las células sinoviales inflamatorias es una etapa clave en el tratamiento de RA El mecanismo molecular que conduce a la hiperproliferación de células sinoviales es aún desconocido Aunque las células sinoviales hiperproliferativas son no malignas y no transformadas, muchos estudios han sugerido que ellas comparten algunas características comunes con las células transformadas (46) Estas células, las así llamadas "sinoviocitos que parecen transformados", se caracterizan por un retículo endoplasmático aproximadamente tenso, numerosos núcleos irregulares, y cambios en el esqueleto normal de las células en forma de huso Se ha propuesto que la incorporación de los oncogenes y los genes derivados de virus podrían ser los disparadores primarios para la apariencia transformada de la célula sinovial RA (46)

Al menos dos aspectos de RA sugieren que la apoptosis desregulada podía contribuir al proceso de la enfermedad y que la elicitación terapéutica de la apoptosis podría ser un tratamiento efectivo La falla de la supresión de las células T activadas sugiere que hay una muerte defectuosa de las células inducidas por activación de estas células T, lo cual es un proceso que

involucra apoptosis mediada por Fas y apoptosis mediada por TRAIL, y la naturaleza hiperproliferativa de la célula sinovial RA es un factor que contribuye a la etapa posterior de la patofisiología RA De hecho, se ha mostrado que la administración de anticuerpo antifaz en las articulaciones inflamatorias inhibe el desarrollo de artritis crónica en ratones transgénicos tax, que son modelos animales para RA humano (26) Más aún, la transducción localizada del gen de ligando Fas mediante un vector adenoviral es efectiva en la prevención de artritis inducida por colágeno (27) La inhibición de la proliferación de células sinoviales inflamatorias mediante el mejoramiento de apoptosis mediada por Fas se observa en ambos casos Aunque el ligando Fas es un inductor de apoptosis fuerte en las células sinoviales RA, la aplicación de una apoptosis mediada por ligando Fas como una terapia para humanos se ha limitado por su toxicidad letal al hígado De esta manera, la apoptosis inducida por el receptor TRAIL representa una terapia más segura y más efectiva para el tratamiento de RA que la apoptosis inducida por ligando Fas

La apoptosis inducida por receptor TRAIL también representa una terapia más segura y más efectiva para el

tratamiento de cáncer que la apoptosis inducida por ligando Fas La apoptosis mediada por TRAIL es conocida por inducir específicamente apoptosis de células de tumor transformadas sin afectar las células normales Se ha mostrado que la administración sistémica de TRAIL soluble trimerizado no causa toxicidad en animales experimentales siendo aún capaces de inducir regresión de tumores implantados (13, 28) Su potencial como una terapia adjunta para tratamientos tradicionales fue infracalificada por el hallazgo reciente de que la expresión del DR5 y la susceptibilidad de la apoptosis inducida por TRAIL de las células de cáncer de mama se mejora mediante la radiación sugiriendo que combinada con la radiación, la eficiencia del TRAIL se incrementaría en terapia de cáncer (29)

Además, el gen que codifica el receptor TRAIL DR5 se ha mapeado en el cromosoma 8p21-22, un loci con una alta frecuencia de mutación en algunas células cancerosas (30) Se ha reportado que al menos dos clases de células tumorales, cáncer pequeño de pulmón (31) y cáncer de cabeza y cuello (32) exhiben mutaciones en el dominio de muerte del gen DR5 Así, existe necesidad de un anticuerpo anti-DR5 en la investigación de cáncer para

determinar el efecto de la variación del epítopo receptor sobre el desarrollo de la progresión de los cánceres Además, la funcionalidad de las mutaciones del receptor TRAIL probarían una herramienta de diagnóstico clínica útil cuando se utiliza en conjunto con otros biomarcadores en la detección temprana de cánceres y como un predictor de agresividad de tumor

RESUMEN DE LA INVENCIÓN

Se describe un anticuerpo que reconoce un receptor TRAIL DR5 y que induce apoptosis en las células que expresan DR5 in vivo Además describe un anticuerpo que reconoce DR5 pero no DR4, DcR1, no DcR2 Se detalla específicamente un anticuerpo monoclonal producido por un hibridoma

El método suministrado por la invención permite la inhibición de proliferación de células al exponer una célula o una cantidad terapéutica de un anticuerpo capaz de unirse al DR5 También se describe una composición farmacológica que incluya una cantidad terapéutica de anticuerpo monoclonal activo contra un DR5, un portador farmacéuticamente aceptable y un recipiente que incluye

el anticuerpo y el portador Además se suministra por la invención el uso de un anticuerpo que reconoce el DR5 para preparar una terapia para apoptosis selectiva de células anormales o desreguladas

Un anticuerpo de la presente invención interactúa con un receptor de ligando de factor de necrosis tumoral tal como DR4, DR5, DcR1, DcR2 y OPG, que induce apoptosis en una célula que expresa tal receptor Se describe un anticuerpo de la invención capaz de unir selectivamente un epítopo receptor de ligando de factor de necrosis tumoral agonista o antagonista

La presente invención suministra un tratamiento para una enfermedad relacionada con apoptosis mediante un método que incluye exponer un tejido objetivo que tiene una enfermedad relacionada con apoptosis a una cantidad terapéutica de un anticuerpo de la invención

Además describe una proteína de fusión que incluye una secuencia de aminoácido receptora de TRAIL antigénico que tiene al menos 10 bases, acopladas a una proteína de inmunoglobulina o un fragmento de esta capaz de elicitar una respuesta inmune en un sujeto

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La presente invención suministra un método de terapia génica en la cual una célula objetivo se transfecta con una secuencia de ácido nucleico de receptor TRAIL en un vector de expresión de tal forma que el receptor TRAIL se expresa sobre la célula objetivo La célula objetivo se expone entonces a un anticuerpo que se une selectivamente al receptor TRAIL

Se suministran secuencias de ácido nucleico y secuencias de aminoácido que codifican las cadenas de inmunoglobulinas pesada y ligera del anticuerpo selectivo para DR5 También se detallan vectores que incluyen una secuencia de ácido nucleico de la invención y células hospederas transformadas con un vector de la invención

La presente invención suministra una célula hospedera que produce un TRA-8 humanizado

Se describe un proceso para la producción de un anticuerpo DR5 humanizado en el cual el huésped se transforma con secuencias de ácido nucleico que codifican una cadena liviana de inmunoglobulina humanizada y una cadena pesada de inmunoglobulina humanizada después de lo

cual el huésped transformado se incuba durante un periodo predeterminado de tiempo

También se describe un proceso para inhibir la proliferación de células que incluye poner en contacto una célula objetivo con una cantidad farmacéuticamente efectiva de un anticuerpo DR5 humanizado

Se suministra un kit comercial para inducir la muerte de células que incluye un anticuerpo TRA-8 humanizado selectivo para DR5, empacado en un recipiente adecuado junto con instrucciones de uso.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La figura 1, caracterización de TRA-8 (a) Uniendo especificidad de un análisis Western blot TRA-8 (panel superior) proteínas de fusión recombinante de la familia TNFR probada con TRA-8 o IgG antihumano Carril 1 proteína de fusión DR5/hIgG1 (inmunógeno), carril 2 DR4/ hIgG1 (TRAIL-R1), carril 3 DR5/hIgG1, carril 4 TRAIL-R3 (DcR1)/hIgG1, carril 5 TRAIL-R4 (DcR2)/hIgG1, carril 6, CD95/hIgG1, carril 7 TNFRI soluble Análisis ELISA (panel inferior) los números de los pozos hacen pareja

con aquellos del Western blot exceptuando el pozo 8 que es una proteína de fusión DR5/hIgG1 de ratón (b) Actividad de unión del TRAIL soluble y TRA-8 a DR5 y DR4 los platos ELISA fueron recubiertos DR5/ hIgG1 (panel izquierdo) o DR4/ hIgG1 (panel medio) y luego incubado con TRAIL o TRA-8 (c) El análisis de citometría de flujo de la expresión de superficie del DR5 Las células Cos-7 transfectadas con vector de expresión pcDNA 3 que contienen el cADN del DR5 de longitud total (histograma sólido), cADN de DR4 (histograma abierto, línea sólida) o vector vacío (histograma abierto, línea punteada) 48 horas después de la transfección, las células fueron teñidas con TRA-8 seguido por hIgG1 anti ratón conjugada con PE (d) La reactividad inmunohistoquímica in situ para DR5 placas de citospina de células Cos-7 transfectadas con expresión DR5 o vector de control fueron teñidas con TRA-8 48 horas después de la transfección, (e) Actividad de muerte del TRA-8 las células Jurkat fueron incubadas con las concentraciones indicadas de TRA-8 La viabilidad de las células determinó mediante ensayos de exclusión del ATPLite, MTT, y PI después de cultivo durante toda la noche Los resultados de los ensayos ATPLite y MTT se presentan como porcentaje del control medio, y el ensayo PI se presenta

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como porcentaje de las células negativas PI (f) El análisis de Western blot de activación de caspasa las células jurkat se incubaron con 500 ng/ml de TRA-8 durante el tiempo indicado Los lisados de células se separaron con 15% de SDS-PAGE, manchada y probada con anticuerpos anticaspasa Las flechas indican las subunidades divididas de cada caspasa(g) Ensayo de inhibición de caspasa las células Jurkat fueron incubadas con 50 ng/ml de TRA-8 durante toda la noche en la presencia de varias concentraciones de inhibidores de caspasa indicados La viabilidad de las células se determinó mediante el ensayo ATPLite

Figura 2 Expresión de superficie celular de DR5 y susceptibilidad a la apoptosis mediada por DR5 Las células normales T y B, aisladas recientemente de sangre periférica, célula T (a y a'), glioma (b y b') célula de cáncer de próstata (c) y célula B (d) las líneas celulares fueron incubadas con TRA-8 o anticuerpo de control isotipo IgG1 de ratón seguido por IgG1 anti ratón cabra conjugado con PE Los histogramas abiertos representan el control de anticuerpo isotipo mientras que los histogramas sólidos representan manchado TRA-8 La apoptosis se determinó mediante el ensayo ATPLite después

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de incubación durante toda la noche con TRAIL soluble (círculos abiertos) o TRA-8 (círculo cerrado) como se muestra en a, b' y d

La figura 3a' la línea de células T U937 se incubó con TRA-8 o anticuerpo de control de isotipo IgG1 de ratón La apoptosis se determinó mediante el ensayo ATPLite después de incubación durante toda la noche contra el soluble (círculos abiertos) o TRA-8 (círculo cerrado)

La figura 3 filas de células de glioma (b) y cáncer de próstata (c) se incubaron con TRA-8 o anticuerpo de control de isotipo IgG1 de ratón La apoptosis se determinó mediante el ensayo ATPLite después de incubación durante toda la noche con un TRAIL soluble (círculos abiertos) o TRA-8 (círculos cerrados)

La figura 4 es una serie de gráficos que muestran la viabilidad de las células para células Jurkat humanas después de exposición a las concentraciones indicadas de (a) sepas de anticuerpo TRA-1, -8 y -10 y (b) TRAIL en la presencia de concentración fija de las sepas de anticuerpo de la invención descritas en la figura 4A,

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La figura 5 La expresión de DR5 en tejidos normales y de cáncer los homogenados de tejido normal y de cáncer se probaron con TR-8 y se desarrollaron mediante quicio luminiscencia (a) El análisis Western blot de la proteína DR5 en tejidos normales carril 1 hígado, carril 2 cerebro, carril 3 pulmón, carril 4 riñón, carril 5 vaso, carril 6 testículos Carril 7 ovario, carril 8 corazón, carril 9 páncreas b El análisis Western blot de la proteína DR5 en tejidos de cáncer La mancha del tejido de cáncer que contiene cánceres del ovarios (carril 1), pulmón (carril 2), hígado (carril 3), recto (carril 4), cervix (carril 5), piel (carril 6), testículos (carril 7), tiroide (carril 8), útero (carril 10), estómago (carril 11), laringofaringe (carril 12), y páncreas (carril 13) se probaron La inmuno histoquímica in situ de tejidos humanos normales (C) y tejidos de cáncer (d) Secciones congeladas se inmunomancharon con TRA-8

Figura 6 Actividad tumoricida de TRA-8 el ratón SCID se inoculó subcutáneamente con células 1321N1 Los ratones fueron inyectados intravenosamente con una dosis

simple de 100 µg de TRA-8 en el segundo día después de la inoculación del tumor (a), o con tres dosis de 100 µg de TA-8 iniciando siete días después de la inoculación del tumor (b) el crecimiento del tumor se determinó mediante el peso y examinado histológicamente con manchado H&E Las fotografías muestran el crecimiento viable del tumor en ratones de control pero no en ratones tratados con TRA-8 (c , panel superior), y manchado H&E de tumor (c , panel inferior) Los ratones SCID fueron inyectados intravenosamente con 10^6 células Jurkat y tratados con una dosis simple de TA-8 en el segundo día después de la inyección Siete días más tarde, las células de vaso fueron cosechadas, manchadas con anticuerpo CD3 antitumor y analizadas mediante citometría de flujo (d), o mediante inmunohistoquímica (e)

La figura 7 muestra la expresión de la superficie celular DR5 en RA (A) y OA (B) células sinoviales 1 x 10^6 células sinoviales cultivadas primarias se mancharon con TRA-8 purificado con afinidad y seguida por anticuerpo IgG1 antiratón cabra conjugada con PE 10 000 células viables se analizaron mediante FACSventaja

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La figura 8 es una serie de gráficos que muestran la viabilidad de las células como una función de la apoptosis inducida por concentración TRAIL y TRA-8 de sepas representativas RA (A) y OA (B) células sinoviales con varias concentraciones del TRAIL soluble recombinante (el círculo abierto) o TRA-8 purificado de afinidad (los círculos cerrados) La viabilidad de las células es el porcentaje del cpm de células tratadas contra el cpm de células no tratadas

La figura 9 es una serie de gráficos que muestran la independencia de caspasa de la apoptosis mediada con DR5 de célular sinoviales RA las células sinoviales RA (RA 512) se incuban con 50 ng/ml de ligando de Fas soluble (cuadrados abiertos), anticuerpo anti-Fas (CH-11) (cuadrado cerrado), TRAIl soluble (círculos abiertos), o anticuerpo anti DR-5 (TRA-8) (círculo cerrado) en la presencia de concentraciones variables de inhibidores de caspasa Después de cultivo durante toda la noche, la viabilidad de las células se determinó mediante ATPLite

La figura 10A es un ensayo de cambio de gel electroforético que indica la activación NFkb Las células RA1016 se incubaron con 20 ng/ml de TRAIL soluble

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o 50 ng/mg de TRA-8 durante los puntos de tiempo indicados antes de ser sometidos a electroforesis La figura 10B y C son gráficos que muestran la producción de MMP-1 y MMP-3 1 x 10^6/ml de células sinoviales RA indicadas se incubaron con las concentraciones indicadas de TNF-a (los círculos abiertos), TRAIL (los triángulos abiertos) o TRA-8 (el círculo cerrado) Después de cultivo durante toda la noche, los supernadantes del cultivo se recolectaron Los niveles de MMP en supernadantes de cultivo se determinan mediante ELISA

Figura 11 E TRA-8 no induce toxicidad hepatocelular (a) tejidos de hígado normal no expresan DR5 Las secciones de parafina de dos tejidos de hígado normales, un tejido de carcinoma hepatocelular, y la preparación de citospina de las células HepG2 se prepararon para teñido H&E, y las correspondientes secciones congeladas se tiñeron con TRA-8 (b) El análisis de citometría de flujo de la expresión de al superficie celular de DR5 Hepatocitos, aislados de dos tejidos de hígado normal y de un caso de tejido de carcinoma hepatocelular, y células HepG2 se tiñeron con TRA-8, anticuerpo anti Fas (DX2) o un anticuerpo de control isotipo Los histogramas sólidos indican manchado

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con TRA-8 o DX2, y los histogramas abiertos son los controles de isotipo correspondiente

Figura 12 TRAIL pero no el TRA-8 induce toxicidad hepatocelular Los hepatocitos humanos normales frescos se mantuvieron en un medio de cultivo de hepatocito (a) La apoptosis de los hepatocitos se indujo con 1 µg/ml de TRAIL soluble más reticulador o TRA-8 para los puntos de tiempo indicados La viabilidad celular se determinó mediante ATPLite Los resultados se presentaron como porcentaje de células viables comparadas con el medio de control Las barras sombreadas indicaron TRAIL y las barras negras indicaron TRA-8 (b) Los núcleos condensados de hepatocitos se tiñeron con Hoechst 33352 y se analizaron mediante citometría de flujo (c) Efecto de cicloheximida sobre apoptosis de hepatocitos Los hepatocitos fueron cultivados en un medio de control o con 1 µg/ml de TRAIL o TRA-8 en la presencia (barra cerrada) o ausencia (barras abiertas) de 1 µg/ml de cicloheximida durante 8 horas La viabilidad de las células se determinó mediante ATPLite Los resultados se presentan como media±SEM de cultivos triplicados de dos experimentos d Una comparación de susceptibilidad de

hepatocitos normales a apoptosis mediada por DR5 y Fas Los hepatocitos aislados frescos se incubaron con las concentraciones indicadas de TRAIL soluble, TRA-8, Fas soluble, o el anti-Fas mAb CH11 durante 6 horas La viabilidad de las células se determinó mediante el ensayo de ATPLite Los resultados se presentaron como porcentaje de las células viables comparadas con el medio de control Para los hepatocitos normales, se presenta la media±SEM de 4 individuos normales Los resultados de las células de carcinoma hepatocelular provenientes de un paciente y de las células HepG2 se presentan como el promedio de los cultivos triplicados

Figura 13 TRAIL induce hepatitis Los ratones B6 fueron inoculados intravenosamente con 10^9 pfu de un vector adenoviral que codifica la longitud completa del TRAIL humano bajo el control del elemento transcripcional "tet-on" La expresión del TRAIL se indujo mediante la dosis indicada de tetraciclina (a) el análisis Northern blot de la expresión TRAIL de humano en le hígado 24 horas después de la inoculación del vector y la inducción con tetraciclina, en ARN total se aisló de los hígados se sondeó con cADN TRAIL o β-actina (b) Los niveles de

suero de AST 24 después de las transducción del TRAIL, los niveles de suero de AST se determinaron (c) la muerte celular mediada por TRAIL del vector adenoviral infectado con hepatocitos los ratones B6 se inocularon intravenosamente con un vector adenoviral inducible con tetraciclina 48 horas después de la inoculación, los hepatocitos de ratones control inoculados y no inoculados se aislaron y se incubaron con las concentraciones indicadas de TRAIL durante 8 horas (panel izquierdo) La viabilidad de las células de los hepatocitos se determinó mediante el ensayo ATPLite Los ratones, inoculados con vector adenoviral como se hizo anteriormente, fueron inyectados intravenosamente con 10 µg de TRAIL humano soluble 48 horas después los niveles de suero de AST se midieron 24 horas después de la inyección TRAIL (panel derecho) (d) y (e)el análisis histológico del daño en el hígado inducido por TRAIL Los hígados fueron recolectados a las 24 horas (d) o a los 7 días (e) después de las transducción con TRAIL Las secciones de parafina fueron teñidas con H&E, y fotografiadas a 100 x (panel superior) y 400X (panel inferior)

La figura 14 es una serie de gráficos que muestran unas células T activadas y unas células T purificadas de PBMC humano que expresan niveles crecientes de DR5 según se determinó mediante la densitometría de flujo para células en descanso (no llenadas) y activadas (sombreadas)

La figura 15 es una gráfica de viabilidad como una función de la concentración de TRA-8 para las células T y B purificadas descritas en la figura 14 que se han estimulado durante 48 horas con anti-CD3 o anti-μ, con células activadas y apagadas recolectadas con diferente densidad de Ficoll-Paque La viabilidad se determina mediante el ensayo ATPLite

La figura 16 es un histograma y una gráfica de densitometría de flujo que muestra la expresión CD3 una población de linfocitos de puerta para ratones NOD/SCID vaciados de células NK inyectados con PBMC humano y TRA-8 o IgG (control)

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La figura 17 muestra microfotografías celulares teñidas CD3 y TUNEL para tejido de vaso de ratón según se detalla en el ejemplo 13

La figura 18 muestra gráficas de citotoxicidad para leucemia linfolítica crónica (CCL) y para células humanas B en la presencia de TRA-8, BISVIII, y combinación de estas

DESCRIPCIÓN DETALLA DE LA INVENCIÓN

La falla para suprimir células se debe a los efectos en el sistema de inducción de apoptosis que está asociado con defectos que ilustrativamente incluyen la expresión o la función del ligando, el receptor, o las moléculas regulatorias o efectoras intracelulares La presente invención logra un método para corregir un sistema de inducción de apoptosis deficiente así como también elucidar los defectos específicos inherentes en un sistema de inducción de apoptosis deficiente dado

La presente invención se relaciona con una nueva clase de anticuerpos monoclonales que tienen actividad de inducción de apoptosis in vivo e in vitro contra

receptores TRAIL específicos que incluyen DR5, DR4, DcR1, y DcR2 La presente invención tiene utilidad como un reactivo para la investigación que señala la apoptosis así como también una terapia efectiva contra la expresión de células Los receptores TRAIL incluyen de manera ilustrativa clases amplias de células cancerosas, desregulación del sistema de apoptosis y células sinoviales que proliferan anormalmente de enfermedades autoinmunes Los anticuerpos de acuerdo con la presente invención son específicos en los tipos particulares de unión de los receptores TRAIL a pesar de la homología entre ellos Los anticuerpos de la invención logran la apoptosis objetivo de solo aquellas células que expresan un receptor TRAIL objetivo o alternativamente, bloquean la apoptosis TRAIL de las células que expresan un receptor objetivo

Un anticuerpo monoclonal DR5 de la presente invención sirve como un inductor potente de apoptosis en células que expresan DR5 in vitro y como un potente inductor de apoptosis in vivo La secuencia CDR fragmentarias humanizadas implantadas sobre esqueletos de anticuerpos humanizados y anticuerpos anti-DR5 de

proteína de fusión de la presente invención exhiben similares propiedades apoptósicas

A la fecha, ningún anticuerpo monoclonal se encuentra disponible que se una al DR5 de la superficie célular e induzca apoptosis de las células que expresan DR5 tanto in vitro como in vivo en la ausencia de un reticulador La presente invención incluye un anticuerpo anti-DR5 operativo como un agente terapéutico en modelos animales de enfermedades, tal como animales semi-injertados, o in vivo Aunque el TRAIL soluble ha mostrado ser efectivo en la inducción de apoptosis de células tumorales in vivo, la actividad de muerte parece ser muy baja con dosis grandes y repetidas que a menudo se requieren (13) El TRA-8, uno de una serie de anticuerpos anti-DR5 de acuerdo con la presente invención, es farmacéuticamente efectivo en animales que llevan un transgen DR5 humano y también tiene la utilidad de establecer un modelo para la investigación del papel DR5 y TRAIL

Un anticuerpo de acuerdo con la presente invención levantado contra un receptor TRAIL se cosecha de acuerdo con la presente invención de un animal experimental Al

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humanizar el anticuerpo de acuerdo con la presente invención para mantener la actividad de unión del receptor el cual elicita una respuesta inmune disminuida y terapéuticamente tolerable dentro de un sujeto humano, un anticuerpo receptor anti-TRAIL humanizado o antagonistas para un receptor TRAIL dado La presente invención es operativa como una terapia in vivo en razón de que no se requiere la segunda reticulación del anticuerpo receptor anti-TRAIL

La presente invención se extiende más allá de un simple anticuerpo receptor anti-TRAIL que tiene efectos apoptosis agonistas o antagonistas En lugar de esto, dos o más anticuerpos receptores anti-TRAIL se ponen en contacto con el cultivo de células in vitro o un tejido de cuerpo de sujeto in vivo para crear un tratamiento sinérgico Por ejemplo, la línea celular glioma U87 y las líneas celulares hematopoyéticas U937 y Molt-4 responden la exposición de una exposición sinérgica a un anti-DR4 agonista y anticuerpos anti-DR5 mientras que la exposición a anticuerpos anticuerpo-DR5 agonistas solos muestran solo un éxito limitado en la inducción de apoptosis

Adicionalmente, los anticuerpos o receptores anti-TRAIL antagonistas tienen utilidad particular en la presente invención cuando un anticuerpo es específico para ligar uno de los receptores señuelo DcR1, DcR2 o OPG El bloqueo selectivo de un receptor señuelo con un anticuerpo de acuerdo con la presente invención tiene el efecto en los tipos de células que expresan receptores señuelo de cambio del equilibrio de unión del TRAIL hacia aquellos receptores TRAIL capaces de transducir la señal de apoptosis De esta manera, en otra terapia combinada de acuerdo con la presente invención, un receptor señuelo que se une a un anticuerpo sintetiza una célula de expresión hacia la señal de apoptosis agonista que transduce la unión del receptor TRAIL

En otra modalidad, la presente invención logra un método de elusitar epítopos agonistas y antagosistas de un receptor TRAIL dado Además, los polimorfismos entre individuos asociados con un receptor TRAIL dado son elucidados de acuerdo con la presente invención a través del uso de un panel de anticuerpos monoclonales que tiene cada uno una variable diferente o región CDR Un panel caracterizado de anticuerpos monoclonales suministra la capacidad de definir epítopos y polimorfismos agonistas y

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antagonistas De esta manera, un panel de anticuerpos monoclonales de acuerdo con la presente invención tiene utilidad en el descubrimiento de drogas y/o determinar sujetos con proclinidad a enfermedades

Aún otra modalidad de la presente invención involucra proteínas de fusión que incluyen un fragmento antigénico de un receptor TRAIL acoplado a una proteína de inmunoglobulina, polipéptido o fragmento de este Un fragmento receptor TRAIL se define por contener un número suficiente de bases para elicitar una respuesta inmunogénica a un receptor TRAIL nativo expresado en la superficie de la célula de un sujeto Un fragmento de fusión receptor TRAIL que incluye al menos 10 aminoácidos Una proteína de fusión de inmunoglobulina o fragmento de esta se define aquí por incluir una proteína nativa o sintética o segmento de polipéptido que tiene un número suficiente de bases de aminoácido para activar una respuesta encascada inmunogénica en un sujeto Un inmunógeno de la presente invención que incluye una fusión de un fragmento receptor TRAIL acoplado a un fragmento de inmunoglobulina tiene utilidad como una terapia in vivo para elicitar un anticuerpo receptor anti-TRAIL in situ en un sujeto

En aún una modalidad adicional, la presente invención es operativa como terapia génica En un aspecto de la terapia génica de la presente invención, las células objetivo se transfectan con un vector que lleva una secuencia expresable que corresponde a un receptor TRAIL El vector es convencional y escogido sobre la base de la susceptibilidad de la célula objetivo al vector Los vectores de terapia génica incluyen de manera ilustrativa adenovirus, pAdCMV5 Sobre las células o tejido objetivo que expresan el receptor TRAIL transfectado, las células o tejidos se exponen a un anticuerpo de acuerdo con la presente invención específico para unir el receptor TRAIL transfectado Se aprecia que el anticuerpo receptor anti-TRAIL es agonista o antagonista a este consistente con el resutlado terapéutico deseado

Los anticuerpos de la presente invención también son operativos en conjunto con un sensibilizador Un sensibilizador como se utiliza aquí se define por incluir cualquier estímulo que induce apoptosis que incluye luz ultravioleta, moléculas orgánicas incluyen

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específicamente la clase de bisindolmaleimidas, metales pesados y especies radicales libres

En el contexto de la terapia de malignidad, TRA-8, es capaz de inducir apoptosis en la mayoría de las células tumorales sensibles a TRAIL en una forma dependiente de caspasa en la ausencia de una reticulación secundaria El TRA-8 exhibe una actividad tumoral fuerte in vivo La capacidad del TRA-8 de inducir apoptosis de la mayoría de células sensibles TRAIL confirma que el DR5 es suficiente para dispara la apoptosis La mayoría de las células tumorales detalladas aquí expresan DR5 en la superficie celular y su susceptibilidad al TRA-8 indujeron muerte celular paralela a susceptibilidad al TRAIL lo que indica que el DR5 es un receptor de muerte primario para la apoptosis mediada por TRAIL en la mayoría de las células tumorales De esta manera, la expresión diferencial del DR5 mediante células normales y cancerosas es operativa en la selectividad de la apoptosis mediada por TRAIL Los puentes TRA-8 de los receptores señuelo para inducir la apoptosis mediada por TRAIL Solo una minoría de las células tumorales resistentes a TRAIL son sensibles al TRA-8, sin embargo, indicando que los receptores señuelo no parecen jugar un

papel importante en la resistencia de las células tumorales de la apoptosis mediada por TRAIL

Aunque estudios previos han indicado que la administración sistémica de la forma soluble de TRAIL en animales induce regresión tumoral sin causar toxicidad [3,4 22], la forma de unión de membrana de TRAIL humano induce daño en el hígado en ratones como se muestra aquí Sin embargo, la toxicidad hepática del TRAIL es mucho menos potente que aquella del ligado Fas como se demostró por la menor susceptibilidad de los hepatocitos normales al daño inducido por TRAIL comparado con el ligando Fas y mediante la falta de letalidad del TRAIL in vivo De esta manera, la titulación del TRAIL tiene utilidad en terapia de cáncer

Como se detalló aquí, se muestra la ausencia de niveles significativos de expresión de proteína DR5 mediante hepatocitos normales y se asocia con resistencia de hepatocitos a apoptosis inducida con TRA-8 La reticualción de DR5 con anticuerpo monoclonal es suficiente para organizar las formas homopoliméricas del receptor de muerte capaz de disparar la apoptosis Los experimentos en titis no indican evidencia de toxicidad

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hepática de la administración de TRA-8 De esta forma, un anticuerpo anti-DR5 monoclonal agonista es probablemente más selectivo y más seguro que el TRAIL soluble como agente terapéutico

Como un ensayo de tamizado, la presente invención es adecuada para detectar clústers pequeños de células malignas que pueden aún exhibir morfologías de células normales La sección de células in situ que se tiñe de células cancerosas humanas incluyen pulmón, próstata y cánceres de hígado con anticuerpos etiquetados de acuerdo con la presente invención fácilmente identifican células cancerosas Estas células cancerosas se observan por expresar muy altos niveles de DR5 comparados con las células normales del mismo tipo De esta forma, la presente invención tiene utilidad como método de tamizado sensible para malignidades en etapa temprana dentro del tejido que incluye al menos pulmón, próstata e hígado Un proceso terapéutico se detalla aquí para la inhibición de proliferación de células anormales asociadas con enfermedades de manera ilustrativa cánceres malignos y leucemias linfáticas

La presente invención se detalla aquí con particularidad a un anticuerpo monoclonal DR5 antihumano designado como TRA-8, que tiene un número de acceso ATCC PTA-1428 Se aprecia que las técnicas y resultados detallados con relación al anticuerpo monoclonal DR5 antihumano agonistra TRA-8 son completamente extendibles y aplicables a anticuerpos DR5 antagonistas, así como también anticuerpos elevados contra DR4, DcR1, y DcR2, que actúan tanto de manera agonista como antagonista

Los niveles de expresión de un receptor de apoptosis, tal como el Fas no necesariamente se correlacionan con la susceptibilidad de las células a la apoptosis Para la apoptosis mediada por TRAIL, se ha sugerido que la expresión de los receptores señuelo para TRAIL influencia la susceptibilidad de las células Más aún, se ha sugerido que el DR5 se debe asociar con el DR4 para transducción efectiva de la señal de apoptosis a través del FADD y la senda de la caspasa 8 La disponibilidad del anticuerpo anti-DR5 monoclonal agonista permitió la evaluación de la regulación de la señalización DR5 y su papel relativo en la apoptosis mediada por TRAIL La comparación de la susceptibilidad de las células a la apoptosis mediada por TRA-8 con su

susceptibilidad a la apoptosis mediada por TRAIL ofrece penetración en el papel del DR5 en la apoptosis mediada por TRAIL y el mecanismo puede afectar la susceptibilidad

Esta ventaja se extiende generalmente a los anticuerpos anti DR5 humanizados de la presente invención Un clón molecular de un anticuerpo a dr-5 se prepara mediante técnicas conocidas como se detalla con respecto a los siguientes ejemplos La metodología del ADN recombinante (33) es operativa aquí para construir secuencias de ácidos nucleicos que codifican una molécula de un anticuerpo monoclonal o una región que se une a una antígeno de este

La presente invención permite la construcción de anticuerpos receptores anti-TRAIL humanizados que es poco probablemente inducirán una respuesta de anticuerpo antiratón humano (en lo sucesivo denominado como "HAMA") (34), mientras que aún tenga un función efectora anticuerpo efectiva Como se utiliza aquí, en los términos "humano" y "humanizado", en relación con los anticuerpos, relacionan a cualquier anticuerpo que se

espera para elicitar una respuesta inmunogénica débil terapéuticamente tolerable en un sujeto humano

La presente invención proporciona un anticuerpo anti-DR5, un anticuerpo anti-DR5 humanizado, inmunoglobulinas de cadena pesada y ligera TRA-8 e inmunoglobulinas de cadena pesada ligera y humanizada Ciertos truncamientos de estas proteínas o genes desarrollan las funciones regulatorias o enzimáticas de la proteína o el gen de secuencia completa Por ejemplo, la codificación de la secuencia de ácido nucleico pueden por lo tanto ser alterados mediante sustitución, adiciones, supresiones o expresión multimérica que proporcionan las proteínas o genes de funcionalidad equivalente Debido a la degeneración de las secuencias que codifican los ácidos nucleicos, otras secuencias que codifican sustancialmente las mismas secuencias de aminoácidos que aquellas proteínas de ocurrencia natural se pueden utilizar en la práctica de la presente invención Estas incluyen, pero no están limitadas a, secuencias de ácido nucleico que incluyen todas o porciones de las secuencias de ácido nucleico que codifican los polipéptidos anteriores, que se alteran mediante la sustitución de diferentes codones que

codifican un residuo de aminoácido funcionalmente equivalente dentro de la secuencia produciendo así el cambio silente Se aprecia que la secuencia de nucleótidos de una inmunoglobulina de acuerdo con la presente invención tolera las variaciones de homología de la secuencia de hasta el 25% calculada mediante métodos estándar ("Current Methods in Sequence Comparison and Analysis", Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp 127-149, 198, Alan R Liss, Inc) en tanto que tal variante forma un anticuerpo operativo que reconoce un receptor TRAIL DR5 Por ejemplo, uno o más residuos de aminoácido con una secuencia de polipéptido se pueden sustituir mediante otro aminoácido de polaridad similar que actúa como una función equivalente, dando como resultado una alteración silente Los sustitutos para un aminoácido dentro de una secuencia se pueden seleccionar de otros miembros de la clase a los cuales pertenece al aminoácido Por ejemplo, los aminoácidos no polares (hidrófobos) incluyen alanina, leucina, isoleucina, valina, prolina, fenilalanina, triftano y metionina Los aminoácidos neutros polares incluyen glicina, cerina, treonina, cisteína, tirosina, asparagina, y glutamina Los aminoácidos cargados positivoamente (básico) incluyen arginina, licina e

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histidina Los aminoácidos cargados negativamenmte (ácido) incluyen ácido aspártico y ácido glutámico También se incluyen dentro del alcance de la presente invención proteínas o fragmentos o derivados de estos que son diferencialmente modificados durante o después de la traducción, por ejemplo mediante glicosolación, división protolítica, unión a una molécula de anticuerpo u otros ligadnos celulares, etc además, el vector recombinante que codifica secuencias de ácidos nucleicos anti-DR5 de la presente invención podrían ser trabajos por ingeniería con el fin de modificar el procesamiento o la expresión de un vector

De acuerdo con esto, un inhibidor que codifica la secuencia de ácido nucleico se puede mutar in vitro o in vivo para crear y/o destruir las secuencias de traducción, iniciación, y/o determinación para crear variaciones en las regiones codificantes y/o formar nuevos sitios donde nucleasas de restricción o destruir la preexistentes, para facilitar adicionalmente la modificación in vitro Cualquier técnica conocida para mutagénesis en el arte se puede utilizar incluyendo pero no estando limitados a mutagénesis dirigida a sitio in

vitro, J Biol Chem 253 6651, el uso de ligadores Tab (Pharmacia), y similares

Los datos de cristalografía de rayos X indican que el doblés de la inmunoglobulina del anticuerpo forma generalmente una estructura cilíndrica larga que comprende dos capas de láminas V antiparalelas, que consisten cada una de tres o cuatro cadenas V En una región variable, tres aros de cada uno de los dominios V de los clúster de las cadenas H y L forman juntos un sitio de unión de antígeno Cada uno de los aros es denominado como región de determinación de complementariedad (DCR) Los CDR tienen la más alta variabilidad en las secuencias de aminoácidos con el anticuerpo Las porciones de la región variable que no son parte de un CDR son llamadas "regiones marco" (regiones "FR") y generalmente juegan un papel en el mantenimiento de la estructura CDR Preferiblemente, todos los CDR de un anticuerpo dado son injertados en un anticuerpo aceptador, con el fin de preservar la región de unión para la región epítopo del receptor TRAIL Se aprecia que el injerto de una porción de la cantidad total de CDR en un donador es operativa aquí Se entiende que el injerto vincula el reemplazo, residuo por residuo,

de un aminoácido o región, por otro Sin embargo, ocasionalmente, especialmente con la transferencia de una región, uno o más residuos se pueden agregar u omitir o sustituir para este, según se desee, y que tales supresiones o inserciones, así como también los reemplazos adecuados e inversiones, están dentro del conocimiento de aquellos de la técnica

Un anticuerpo de la presente invención se obtiene, por ejemplo, injertar cada CDR de la subunidad de la cadena H de un anticuerpo monoclonal receptor anti-TRAIL en una región correspondiente CDR de un anticuerpo humano, humanizando de esta manera, un anticuerpo monoclonal de ratón efectivo contra un receptor TRAIL

Los fragmentos de anticuerpo que contienen un idiotipo de las moléculas también son generados y operativos aquí utilizando dos técnicas Por ejemplo, tales fragmentos incluyen ilustrativamente el fragmento (AB')2 del receptor anti-TRAIL que se puede producir mediante digestión de pepsina de la molécula del anticuerpo, los fragmentos AB' del anticuerpo receptor TRAIL geenrados a través de la reducción de los puentes de disulfuro del fragmento (AB')2 del receptor TRAIL y el

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fragmento del anticuerpo que es generado al tratar la molécula de anticuerpo con papaína y un agente reductor

En particular, el anticuerpo monoclonal anti-DR5 DRA-8 se puede obtener al cultivar un hibridoma que, a su vez, se pude obtener al inmunizar un ratón con DR5 humano y subsecuentemente fusionar las células de base o las células de nodo de linfa de un ratón con células de mieloma de ratón

Preparación de un anticuerpo monoclonal involucra ilustrativamente las siguientes etapas

a) purificación de biomacromoléculas para uso como un antígeno,
b) preparación de células que producen anticuerpos, después de inmunizar primero un animal utilizando inyecciones del angtígeo, sangrando el animal y ensayando el título del anticuerpo con el fin de determinar cuando remover el vaso,
c) preparación de células de mieloma,
d) fusionar el anticuerpo que produce células y células de mieloma,

e) seleccionar un hibridoma que produce un anticuerpo deseado,

f) preparar un clon de células simple (clonación),

g) opcionalmente cultivar las células hibridoma, o hacer crecer animales en los cuales las células hibridoma han sido transplantadas, para preparación a gran escala del anticuerpo monoclonal, y

h) ensayar las actividades biológicas y la especificidad, o ensayar las propiedades del agente marcador, del anticuerpo monoclonal así preparado

El procedimiento para la preparación del anticuerpo monoclonal anti-DR5 se detalla adelante con referencia a las etapas anteriormente descritas Este método para la preparación de un anticuerpo de la presente invención se pretende solo como ilustración de los métodos de preparación y no está limitado a este Otros procedimientos conocidos pueden ser seguidos, o el método siguiente modificado, por ejemplo mediante el uso de células que producen anticuerpos diferentes de las célula de vaso y mieloma

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(a) preparción de antígeno

Una proteína recombinante (en los sucesivo denominada como una "DR5 humano recombinante") efectiva como el antígeno, se obtiene mediante la transfección de células QBI-293A con el vector de expresión pAdDR5-IgI para una proteína de fusión que comprende el dominio extracelular del DR5 humano y la región Fc del anticuerpo IgG1 humano (en lo sucesivo denominado como "IgG") (cf PTA-1428) para expresarlo al utilizar el kit ADENO-Quest (Quantum Biotechnologies Inc , Canadá), y recolectar y purificar parcialmente el producto de expresión El plásmido pAdDR5-IgG se construye al insertar ADN que codifica un DR5 humano y una proteína de fusión IgG humana en pAdCMV5, que es un vector de expresión para células animales Otros materiales, tales como el ADN que codifica DR5, el vector, y el hospedero son operativos aquí

La proteína de fusión DR5 e IgG humanas producidas en el cultivo supernadante de las células QBI-293A transfectadas con el vector pAdDR5-IgG pueden ser parcialmente purificadas mediante cromatografía de afinidad de proteína A-sefarosa o cromatografía de

afinidad de proteína G-sefarosa, o cromatografía de intercambio de iones utilizando una columna Resource Q (nombre comercial Pharmacia)

Alternativamente, el DR5 purificado obtenido de las membranas de líneas de células humanas se utilizan como el antígeno Además, en razón a que la estructura primaria del DR5 es conocida (cf PTA-1428), un péptido que comprende las secuencias de aminoácidos del SEC ID No 1, pueden ser químicamente sintetizadas mediante un método conocido tal como el método Sanger, y utilizadas como el antígeno

(b) Preparción de las células productoras de anticuerpo

Se inmuniza un ratón con el inmunógeno producido en la etapa (a), mezclada con un adyuvante, tales como el adyuvante o alumbre completo o iincompleto Freund Otros animales experimentales adecuados ilustrativamente incluyen ratas, cerdos de guinea, conejos, perros, pollos, caballos, cerdos, y ovejas

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Las rutas de administración adecuadas para inmunizar un animal experimental incluyen las rutas de inyección subcutánea, intraperitoneal, intravenosa, intradermal, e intramuscular, cuyas inyecciones subcutáneas e intraperitoneal son preferidas

Las inmunizaciones son opcionalmente desarrolladas mediante una dosis simple o, mediante varias dosis repetidas a intervalos apropiados (preferiblemente 1 a 5 semanas) Los animales inmunizados se monitorean para titular el anticuerpo en su suero, y un animal con un título de anticuerpo suficientemente alto se selecciona como la fuente de células que producen anticuerpos Seleccionar un animal con un título alto hace el subsecuente proceso más eficiente Las células para la fusión subsecuente son generalmente cosechadas del animal tres a cinco días después de la inmunización final

Los métodos para ensayar el título del anticuerpo incluyen varias técnicas bien conocidas tales como radioinmunoensayo (en lo sucesivo, denominado como "RIA"), inmunoensayo de enzima de fase sólida (en lo sucesivo denominado como "ELISA"), ensayo de anticuerpo fluorescente y ensayo de hemaglutinación, siendo el RIA y

el ELISA preferidos por razones de sensibilidad, rapidez, precisión y potencial de detección de manera automática

La determinación del título del anticuerpo se puede desarrollar, por ejemplo, mediante ELISA como sigue Primero, el DR5 purificado o parcialmente purificado se adsorbe sobre la superficie de la fase sólida, tal como un plato ELISA de 96 pozos, seguido por el bloqueo de cualquier superficie restante, a la cual el DR5 no se ha unido, con un proteína no relacionada con el antígeno, tal como seroalbúmina de bovino (BSA) Después de lavado, la superficie de los pozos son puestas en contacto con muestras serialmente diluidas de suero de ratón para posibilitar la unión del anticuerpo anti-DR5 en las muestras al antígeno Un anticuerpo anti-ratón etiquetado con enzima, como el anticuerpo secundario, se agrega por estar unido al anticuerpo del ratón Después de lavado, el sustrato de enzima se agrega y en el título el anticuerpo se estima al determina el cambio de absorbancia debido al desarrollo del color causado por la alteración del sustrato o similar

(c) Preparación de células de mieloma

Las células de líneas de célula de ratón establecido sirven como la fuente de células de mieloma que incluyen por ejemplo ratón resistente 8-azaguanina, derivado de las sepas de mieloma BALB/c P3X62Ag8U 1 (P3-U1)(35), P3/NSI/1-Ag4-1(NS-1)(36) Sp2/0-Ag14(SP-2)(37), P3X63Ag8 653(653)(38) y P3X63 Ag8(X63)(39) La línea de células seleccionada es transferida serialmente a un medio apropiado, tal como un medio 8-azaguanina El medio 8-azaguanina incluye un medio de Dulbeco modificado de Iscove (en lo sucesivo denominado como "IMDM") o medio Eagle modificado de Dulbeco (en lo sucesivo denominado como "DMEM") El medio RPMI-1640 suplementado con glutamina 2-mercaptoetano, gentamicina, suero de ternero fetal (en lo sucesivo denominado como "FCS"), y 8-azaguanina Las células son entonces transferidas a un medio normal, tal como un medio ASF 104 (Ajinomoto, K K) que contiene 10% de FCS, tres a cuatro días antes de la fusión, con el fin de asegurar que al menos $2x10^7$ células estén disponibles en el día de la fusión

(d) fusión de células

Los linfocitos y las células de plasma obtenidsa de cualquier parte adecuada del animal son células

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precurosras para producir el anticuerpo Las fuentes de células de linfocitos o plasma incluyen de manera ilustrativa el vaso, los nodos linfáticos, la sangreperiférica o cualquier combinación apropiada de estos, siendo las células del vaso la fuente más común

Después de la ultima inyección propulsora, el tejido en el cual las células que producen anticuerpo están presentes se remueve de un ratón que tienen un título de anticuerpo predeterminado La técnica habitualmente favorecida por la fusión de células de vaso con células de mieloma preparadas en la etapa (c), emplea polietilenglicol

La técnica de fusión incluye lavado de células de vaso y mieloma con 0 libre (tal como RPMI 1640) o solución salina amortiguada con fosfato (en lo sucesivo denominada como "PBS") de tal forma que la proporción de número de células de vaso a células de mieloma está aproximadamente entre 5 1 a 10 1, y luego centrifugada Después el supernadante se ha descartado y las células peletizadas suficientemente sueltas, se agrega 1 ml de medio libre de suero que contiene 50% (p/v) de polietilneglicol (p m 1 000 a 4 000) gota a gota con

mezclador Subsecuentemente, 10 ml de medio libre de suero se agrega lentamente y luego se centrifuga El supernadante se descarta de nuevo, y las células peletizadas se suspenden en una cantidad apropiada de medio HAT que contiene una solución de hipoxantina, aminopterina y timidina (en lo sucesivo denominada como "HAT") e interleucina-2 de ratón en lo sucesivo denominada como "IL-2") La suspensión es entonces dispensada dentro de los pozos de los platos de cultivo (aquí denominados simplemente como "plato") e incubados en la presencia de 5% v/v de CO_2 a 37° C durante dos semanas con una adición suplementaria de medio HAT según sea apropiado

(e) Selección de hibridomas

Cuando la sepa mieloma utilizada es resistente a la 8-azaguanina, es decir es deficiente en la enzima hipoxantina y guanina fosforibosil transferasa (HGPRT), cualquier célula de mieloma no fusionada y cualquier fusión mieloma-mieloma es incapaz de sobrevivir en un medio HAT De otra parte las fusiones de las células que producen anticuerpo una con la otra, así como también los hibridomas de células que producen anticuerpo con células

de mieloma pueden sobrevivir, la última solo teniendo una vida limitada De acuerdo con esto, la incubación continuada en un medio HAT da como resultado la selección de solo los hibridomas deseados

Los hibridomas resultantes crecen en colonias que son luego transferidas en un medio HAT al que le falta aminopterina (medio HAT) Luego de esto, alícuotas del cultivo supernadante se remueven para determinar el título del anticuerpo anti-Fas mediante, por ejemplo, ELISA Cuando la proteína difusión anteriormente mencionada se utiliza como el antígeno ELISA, es también necesario eliminar clones que producen un anticuerpo que se une específicamente a la región Fc del IgG1 humano La presencia o ausencia de tales clones puede ser verificada, por ejemplo, mediante ELISA utilizando Fas-IgG1 o IgG1, como el antígeno

(f) Clonación

Los hibridomas que han mostrado que producen anticuerpos específicos, utilizando un método similar al de la etapa b) para determinar el título del anticuerpo, son entonces transferidos a otro plato para clonación

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Los métodos de clonación adecuados incluyen el método de dilusión limitada, en el cual los hibridomas son diluidos por contener una célula por pozo de un plato y luego cultivados, el método de agar suave en el cual las colonias son recubiertas después de cultivar el medio agar suave, un método de usar un micro manipulador para separar una célula simple para el cultivo, y "clasificar un clón", en el cual las células simples se separan mediante un clasificador de células

El procedimiento de clonación de acuerdo con, por ejemplo, el método de dilusión limitada se repite de dos a cuatro veces por cada pozo que demuestra un título de anticuerpo, y los clones que tienen títulos de anticuerpo estables se recolectan como hibridomas que producen anticuerpos monoclonales anti-DR5 Los hibridomas que producen un anticuerpo DR5 anti-ratón se seleccionan mediante un método similar para obtener un anticuerpo monoclonal anti-DR5 que produce la línea de células

El hibridoma ratón-ratón TRA-8 que es una base para los anticuerpos de la presente invención se depositó con la American Type Culture Collection en marzo 1, 2000, y tiene el número de acceso PTA-1428 De acuerdo con esto,

cuando se prepara un anticuerpo que utiliza el hibridoma ratón-ratón TRA-8 o cualquier otro hibridoma establecido, la preparación se puede desarrollar al seguir un procedimiento de partida de la etapa (g) adelante, con las etapas (a) a (f) omitidas

(g) Cultivo de hibridoma para prepara anticuerpo monoclonal

El hibridoma obtenido mediante la clonación es luego cultivado en un medio normal, no en un medio HT El cultivo a gran escala se desarrolla mediante un cultivo en botella rodillo, utilizando grandes botellas de cultivo o mediante un cultivo hilador El supernadante proveniente del cultivo a gran escala es entonces cosechado y purificado mediante un método adecuado, tal como filtración de gel, el cual es bien conocido por aquellos expertos en la técnica, para obtener un anticuerpo monoclonal anti-DR5 que es una base para anticuerpos de la presente invención El hibridoma también puede crecer intraperitonealmente en un ratón singeneico, tal como el ratón BALB/c o un ra ton un/un, para obtener acitos que contienen un anticuerpo monoclonal anti-DR5 en grandes cantidades Los kits de

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purificación de anticuerpos monoclonales comercialmente disponibles (por ejemplo, el kit MabTrap GII, de Pharmacia) se utiliza convencionalmente para purificar los anticuerpos cosechados

Los anticuerpos monoclonales preparados como anteriormente tienen una alta especificidad para el DR5 humano

(h) Ensayo de anticuerpo monoclonal

Los métodos de identificación adecuados del isotipo y las subclases del anticuerpo no clonal incluyen el método Ouchterlony, ELISA y RIA Preferiblemente, se utiliza un kit comercial para la identificación, tal como un kit Mouse Typer (nombre comercial, BioRad)

La cunatificación de la proteína se puede desarrollar mediante el método Folin-Lowry, o mediante el cálculo basado en la absorbancia a 280 nm (1,4 (OD280) = inmonuglobulina 1 mg/ml)

La identificación del epítopo que el anticuerpo monoclonal reconoce se desarrolla como sigue Primero,

varias estructuras parciales de la molécula que reconoce el anticuerpo monoclonal se preparan Las estructuras parciales se preparan mediante el método en donde varios péptidos parciales de la molécula se preparan sintéticamente mediante técnica de síntesis de oligopéptidos conocida, o el método en donde el ADN que codifica el polipéptido parcial deseado se incorpora en un plásmido de expresión adecuada y se expresa en un hospedero adecuado, tal como E Coli, para producir los péptidos Generalmente, ambos métodos son frecuentemente utilizados en combinación para el objeto anterior Por ejemplo, una serie de polipéptido que tienen longitudes apropiadamente reducidas, trabajan desde el terminal C- o N- de la proteína de antígeno, se pueden preparar mediante técnicas de ingeniería genética establecidas Al estableces cuales fragmentos reaccionan con el anticuerpo, se obtiene una idea aproximada del epítopo

El epítopo está más cercanamente identificado al sintetizar una variedad de oligopéptidos más pequeños que corresponden a este mutantes del péptido que utilizan las técnicas de síntesis de oligopéptidos establecidas para determinar una propiedad de unión de los péptidos al anticuerpo monoclonal anti-DR5 que es una base para la

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preparación del anticuerpo de la presente invención y una inhibición competitiva de la unión del péptido a un antígeno con el anticuerpo monoclonal Los kits comercialmente disponibles, tales como el kit SPOTs (Genosys Biotechnologies, Inc) y una serie de kits de síntesis de péptido multipasador basados en el método de síntesis multipasador (Chiron Corp) pueden ser convenientemente utilizados para obtener una gran variedad de péptidos

Un anticuerpo de la presente invención tiene las varias propiedades funcionales a) a f) descritas adelante, cada una de las cuales se verifica mediante, por ejemplo, un método descrito en lo sucesivo

a) Unión específica del TRA-8 a las células que expresan en DR5 humano

Una característica única de la presente invención es la capacidad de unir superficies de célula DR5 Esto se demuestra mediante análisis de citometría de flujo de las células que expresan DR5 Primero, la superficie de células específicas que unen el DR5 se confirman mediante las células transfectadas COS-7 con el DR5 humano que

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codifica el cADN de longitud completa Específicamente, el TRA-8 reconoce solo las células COS-7 transfectadas con DR5 pero no el vector de control vacío o el vector que codifica DR4 Segundo, tres orígenes diferentes se ensayan en hematopoyético, el glioma, y las células de tumor maligno de cáncer de próstata de humano La mayoría de las células de tumor transformadas expresaron niveles significativos de superficie celular DR5, aunque los niveles de expresión varían grandemente Tercero, se examinaron dos paneles de células de fibroblasto sinovial primario de humano provenientes de paciente RA y OA Todas las células sinoviales RA expresaron niveles significativamente altos de DR5 comparados con las células OA

b) Inducción de apoptosis de células de tumor maligno de humano in vitro en la ausencia de reticulación

La capacidad de un anticuerpo levantado de acuerdo con la presente invención para reconocer el receptor TRAIL y para inducir directamente apoptosis de las células de tumor humano maligno se determinaron mediante ensayo de viabilidad (ATPLite) durante el cultivo in

vitro de células con varias concentraciones de un anticuerpo, específicamente TRA-8 La mayoría de células de tumor con susceptibles de apoptosis inducida por TRA-8 Para algunas células, el TRA-8 exhibió una actividad que induce fuerte apoptosis, por ejemplo, el TRA-8 es capaz de inducir apoptosis de células Jurkat de humano con niveles pg/ml De manera importante, la apoptosis inducida por TRA-8 no requirió reticulación, y en la mayoría de las células, el TRA-8 exhibió una actividad que induce apoptosis más fuerte que el TRAIL soluble recombinante en la presencia del mejorador

c) Actividad tumoricida del TRA-8 in vivo

La actividad tumoricida del TRA-8 se evalúa en dos modelos de células de tumor SCID/humano Primero, los ratones SCID son intravenosamente inoculados con células Jurkat de leucemia de humanos, y tratadas con una dosis simple (100 µg) de TRA-8 Los resultados muestran que la mayoría de las células Jurkat implantadas se eliminan de la sangre periférica del vaso mediante el tratamiento con TRA-8, como se determinó mediante el análisis de

densitometría de flujo y tamizado inmunohistoquímico in situ de células Jurkat

Segundo, las células astrocitoma de humano, 1321N1, son inoculadas subcutáneamente en ratones SCID, y los ratones que llevan el tumor son tratados con una dosis simple de TRA-8 El crecimiento de las células 1321N1 implantadas es significativamente inhibido en los ratones tratados con TRA-8 como se determinó mediante los tamaños de tumor y el análisis histológico

d) Identificación de células sinoviales RA mediante TRA-8

Las células sinoviales primarias aisladas provenientes de ocho pacientes RA y cuatro pacientes OA son ensayadas para expresión de superficie de célula de DR5 El TRA-8 es capaz de tamizar positivamente todas las células RA pero teñir negativamente todas las células OA De esta forma, el RA es diferenciado del OA mediante la expresión de superficie del DR5 como se detecto mediante TRA-8

e) Inducción de apoptosis en células de fibroblastos sinovial RA mediante TRA-8

La capacidad del TRA-8 de inducir apoptosis de las células sinoviales RA se determina mediante el ensayo de viabilidad celular durante el cultivo in vitro en la presencia de varias concentraciones de TRA-8 Todas las células RA exhibieron niveles alto a intermedio de susceptibilidad a 100 mg/ml de TRA-8 En contraste, todas las células OA son esencialmente resistentes a la apoptosis inducida TRA-8 De manera importante, las TRA-8 exhibieron una mejor actividad inductora de apoptosis a las células sinoviales RA que el TRAIL soluble con el mejorador Más aún, comparado con el anticuerpo anti-Fas (CH-11), el TRA-8 exhibió una mejor selectividad a las células sinoviales RA

f) El TRA-8 no indujo la producción de MMP en células sinoviales RA en razón a que el TRA-8 es capaz de inducir la activación NF-kb en células sinoviales RA como TNF-a, el efecto del TRA-8 sobre la producción de MMP1 y MMP3 de células sinoviales se determinó Mientras que el TNF-a indujo un incremento dependiente de dosis de MMP, el TRA-8 es incapaz de inducir cualquier producción de

MMP, y en algunas concentraciones, el TRA-8 disminuye ligeramente la producción de MMP en las células sinoviales RA

g) El TRA-8 induce activación de caspasa múltiple

En razón a que las capasas juegan un papel crucial en la inducción de apoptosis La capacidad del TRA-8 para inducir activación de caspasa se determina en célula Jurkat de humano Cuando las células Jurkat se incuban con una dosis baja (50 mg/ml) de TRA-8, la actividad de caspasa 8, caspasa 9, y caspasa 3 se observa tan tempranamente como 15 minutos después de la incubación como se demostró mediante el análisis Western blot y el análisis de separación de caspasa En términos de tiempo, número y resistencia de activación de la caspasa, los anticuerpos de la presente invención incluyen el anticuerpo demostrativo TRA-8 exhiben una mucho mejor actividad que cualquier otros anticuerpos que inducen apoptosis conocidos tales como el anticuerpo Fas anti-humano (CH-11)

De esta forma, el anticuerpo de la presente invención es una sustancia que tiene una propiedad para

inducir selectivamente apoptosis en células patogénicas como se meustra en efecto (a) y (g) De acuerdo con esto, es útil como un agente profiláctico y terapéutico para enfermedades asociadas con supervivencia inadecuada de células o proliferación inadecuada de células, tales como aquellas atribuíbles a la desregulación de sistemas de apoptosis que incluyen el sistema ligando Fas/Fas

La capacidad de un anticuerpo de la presente invención a inducir apoptosis se confirma medainte el cultivo de células tales como el Jurkat de línea de células de leucemia de humano (American Type Culture No TIB-152) y la línea celular de astrocitoma 1321N1 en un medio en el cual la muestra de ensayo se ha agregado, y determinar la proporción de supervivencia mediante, por ejemplo, un ensayo ATPLite

El anticuerpo de la presente invención, especialmente los anticuerpos anti-DR5 que tienen casi la misma inmunogenicidad a un humano como auqel de los anticuerpos humnaos, se utiliza como un agente para profilaxis o tratamietno de enfermedades asociadas con sobrevivencia inadecuada o proliferación de células que incluye aquellos atribuibles a la desregulación de

sistemas de apoptosis en enfermedades autoinmunes que incluyen de manera ilustrativa lupus eritematoso sistémico, enfermedad de Hashimoto, artritis reumatoide, enfermedad de injerto versus huésped, síndrome de Sjögren, anemia perniciosa, enfermedad de Addison, escleroderma, síndrome de Goodpasture, enfermedad de Crohn, anemia hemolítica autoinmune, esterilidad, miastenia gravis, esclerosis múltiple, enfermedad de Basedow, trombopenia púrpura, diabetes mellitus insulinodependiente, alergia, enfermedad atópica, arterioesclerosis, miocarditis, cardiomiopatía, nefritis glomerular, anemia hipoplástica, rechazo después del transplante de órganos y numerosas malignidades de pulmón, próstata, hígado, ovario, tejido linfático y pecho

Tal agente profiláctico y terapéutico se puede administrar en varias formas Los modelos adecuados de administración incluyen administración oral, tal como mediante tabletas, cápsulas, gránulos, polvos y melazas, o administración parenteral, tal como mediante inyección, inyección por goteo y supositorios

El anticuerpo o el agente terapéutico se puede administrar oralmente, por el recto, intrasisternalmente, intraventricular, intracraneal, intratecal, intravaginal, parenteralmente, (intravenosamente, intramuscularmente, o subcutáneamente), localmente (polvos, ungüentos, o gotas), mediante inyección intraperitoneal, transdérmicamente, mediante inhalación o como un rociado bucal o nasal La cantidad exacta del anticuerpo o agente terapéutico requerida variará de sujeto a sujeto, dependiendo de la edad, peso y condición general del sujeto, la severidad de la enfermedad que está siendo tratada, el sitio y el tamaño del tumor, los compuestos particulares utilizados, el modo de administración, y similar Una cantidad apropiada se puede determinar mediante un experto en la técnica utilizando experimentación de rutina dada las enseñanzas de aquí Las dosis típicas simples de rango de anticuerpo de 0,1-10 000 µg, preferiblemente entre 1 a 100 µg las concentraciones de anticuerpo típicas en un portador varían desde 0,2 a 2 000 nanogramos por mililitro suministrado

Dependiendo del modo pretendido de administración, el anticuerpo o agente terapéutico puede estar en composiciones farmacéuticas en la forma de formas semisólidas o formas de dosis líquidas, tales como, por ejemplo, tabletas, supositorios, píldoras, cápsulas, polvos, líquidos, o suspensiones, preferiblemente en forma de dosis unitaria adecuada para la administración simple de una dosis precisa La composición incluirá una cantidad efectiva del sustrato seleccionado en combinación con un portador farmacéuticamente aceptable y, en adición, puede incluir otro agentes medicinales, agentes farmacéuticos, portadores, o diluyentes Por "farmacéuticamente aceptables" se entiende un material que no es biológicamente o de otra manera indeseable, el cual se puede administrar a un individuo junto con el sustrato seleccionado sin causar efectos biológicos significativamente indeseables o interactuar de una manera deletérea con cualquiera de los otros componentes de la composición farmacéutica en la cual está contenida

Las composiciones adecuadas para inyección parenteral pueden comprender soluciones acuosas estériles o soluciones no acuosas fisiológicamente aceptables, dispersiones,

Región variable en términos de energía El procedimiento anterior se lleva a cabo usando un sistema de modelado molecular común disponible comercialmente ABM (Oxford Molecular Limited, Inc) Para el modelo molecular obtenido, la precisión de la estructura es evaluada adicionalmente usando el programa PROCHECK (J Appl Cryst 1993), 26, 283-291)

(2) diseñando las secuencias de aminoácido para el TRA-8 humanizado

La construcción de anticuerpos TRA-8 humanizado se lleva a cabo por el método generalmente conocido como injertación CDR (Proc Natl Acad Sci USA 86, 10029-10033 (1989)) El anticuerpo aceptor es escogido con base en la homología del aminoácido en la región de la estructura Las secuencias de la región de estructura en el TRA-8 son comparadas con todas las secuencias de estructura humana en la base de datos Kabat de secuencias de aminoácido de anticuerpos (Nuc Acid Res 29, 205-206 (2001)) Como resultado, el anticuerpo mAB58'CL es seleccionado como un aceptor debido a la homología de

secuencia más alta del 80% para la región de trabajo Los residuos de aminoácido en la región de estructura para el mAB58' CL son alineados con aquella para el TRA-8 y las porciones en donde diferentes aminoácidos son usados se identifican La localización de aquellos residuos son analizados usando el modelo tridimensional del TRA-8 construido anteriormente y los residuos donor que deben ser injertados en el aceptor son escogidos por los criterios dados por Queen et al (Proc Natl Acad Sci USA 86, 10029-10022 (1989)) Las secuencias TRA-8 humanizadas son construidas como se describió en el siguiente ejemplo por medio de transferir diversos residuos donores en los anticuerpos aceptores, mAB58'CL

Ejemplo 18 Construcción de un vector de expresión para la cadena pesada del anticuerpo humanizado

(1) construcción del plásmido que lleva la variable de la región de ADN de cadena pesada del TRA-8 humanizado

Para poder determinar la actividad del TRA-8 humanizado, el plásmido que lleva la cadena pesada del TRA-8 humanizado es construido como sigue Sin embargo,

podrá apreciarse que la humanización del TRA-8 no está limitada a estos ejemplos

Como se muestra en la SEC ID No 31 de la lista de secuencias, la humanización de la secuencia de aminoácidos de la cadena pesada del anticuerpo DR5 antihumano de ratón TRA-8 reemplazo ocasionado al 13^{avo} aminoácido (glicina), el 19^{avo} aminoácido (glicina), el 40^{avo} aminoácido (treonina), el 42^{avo} aminoácido (ácido glutámico), el 44^{avo} aminoácido (arginina), el 84^{avo} aminoácido (cerina), el 88^{avo} aminoácido (cerina), el 93^{avo} aminoácido (metionina), el 114^{avo} aminoácido (treonina), el 115^{avo} aminoácido (leucina), con glutamina, arginina, alanina, glicina, asparagina, alanina, valina, leucina y valina, respectivamente

El plásmido lleva ADN que codifica la región variable de la cadena pesada del TRA-8 humanizado (SEC ID No 31 de la lista de secuencias) y construido como sigue

El PCR se utiliza para construir las siguientes secuencias de ADN, cada una de las cuales comprende los descritos anteriormente

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Los siguientes 12 oligonucleótidos se sintetizan

5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32)

5'- tctgaagtaa tgctggtgga gtctgggggg ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- attcacttttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaagcg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg ttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctc cacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

5 - tccagaccct tccctggtgc ctgccgaacc caagacatta catnactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- ctctggagat ggtgaatcgg cccttcacac tgtctggata gtaggtgtna ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42), and

5'- gaccgatggg ccctggtgg aggctgagga gactgtgacc agggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43)

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Los siguientes dos iniciadores PCR se sintetizaron como se describió anteriormente

5'- ttggataagc ttggcttgac -3' (P1, SEQ ID No 44), y
5'- gaccgatggg cccttggtgg a -3' (P2, SEQ ID No 45)

La síntesis de ADN que codifica una cadena de polipéptido que comprende una secuencia de señal de secreción, una región variable de la cadena pesada de TRA-8 humanizada y los ocho residuos de aminoácido en el N-terminal de la región IgG-CH1 se desarrolla utilizando una combinación de PCR respectivamente

El fragmento de ADN se prepara como sigue

Composición de la solución de reacción PCR

Oligonucleótido A, 10 pmol,
Oligonucleótido B, 10 pmol,
Oligonucleótido C, 10 pmol,
Oligonucleótido D, 10 pmol,
Oligonucleótido E, 10 pmol,
Oligonucleótido F, 10 pmol,
Oligonucleótido G, 10 pmol,

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Oligonucleótido H, 10 pmol,

Oligonucleótido I, 10 pmol,

Oligonucleótido J, 10 pmol,

Oligonucleótido K, 10 pmol,

Oligonucleótido L, 10 pmol,

Iniciador de Oligonucleótido P1, 2µM,

Iniciador de Oligonucleótido P2, 2µM,

Solución pirobest II, 10 µl,

Mezcla dNTP, 8 µl,

Polimerasa de ADN pirobeeet, 0,5 µl, y

Agua redestilada hasta un volumen final de 50 µl

La reacción PCR se conduce como sigue Las soluciones se calientan primero a 94° C durante cinco minutos, después de lo cual se repite siete veces un ciclo de calentar a 98° durante 10 segundos, 55° durante 30 segundos y 72° durante un minuto Después de completar este procedimiento, la solución de reacción se calienta a 72° C durante 15 minutos

Un volumen igual que fenol-cloroformo (50% v/v de fenol saturado con agua, 48% v/v de cloroformo, 2% v/v isoamil alcohol) se agrega a 200 µl de cada uno de los

productos PCR, y se mezcla vigorosamente durante un minuto Después de este tiempo, la mezcla se centrifuga a10 000 X g, y la capa acuosa se recupera y se mezcla con un volumen igual de alcohol cloroformo-isoamilo (96% v/v de cloroformo y 44% v/v de alcohol isoamino), que es de nuevo vigoroso a 10 000 X g y se recupera la capa acuosa Las series de etapas citadas en este párrafo se refieren a en lo sucesivo, como "extracción de fenol")

La precipitación de etanol es entonces desarrollada sobre la capa acuosa recuperada Como se utiliza y se refieree aquí, "precipitación de etanol" consiste en agregar, a una mezcla, un décimo del volumen de acetato de sodio 3M (pH 5,2) y 2,5 volúmenes de 100% de etanol a la solución a ser tratada, y congelar la mezcla utilizando hielo seco La mezcla resultante se centrifuga entonces a 10 000 X g para recuperar el ADN como precipitado

Después de la extracción de fenol la precipitación de etanol el precipitado de ADN resultante se seca por vacío, se disuelve en un mínimo de agua redestilada, y se separa mediante electroforesis de gel de agarosa al 3%

Después de la electroforesis, el gel se tiñe con 1 µg /ml de solución acuosa de bromuro de etidio para permitir la detección de ADN bajo luz UV La banda de ADN que corresponde al ADN del TRA-8 humanizado es cortada utilizando una cuchilla y eluida del gel que utiliza el kit Geneclean Spin (BIO 101, CA, USA) Después de la estación de fenol, el ADN eluido es entonces concentrado mediante centrifugación a 7 500 X g, seguido mediante precipitación de etanol, y finalmente disuelto en 5 µl de agua destilada

El resultante, cada ADN extraído es clonado utilizando un vector Easy pGEM-T (Promega) como sigue

El fragmento recuperado de la reacción de PCR, 5 µl,

Solución de polimerasa Taq 10 X, 1 µl,

Mezcla dNTP, 1 µl,

Polimerasa Taq (5 unidades/ml), 1 µl, y

Agua redestilada hasta un volumen final de 10 µl

Después de lo anterior cada solución reacciona a 70° C durante 30 minutos, cada solución de ADN y de vector Easy pGEM-T son ligados utilizando un kit de ligado de

ADN versión 2 0 (Takara Shuzo Co , Ltd) utilizando el protocolo del fabricante

Después de 4 horas de incubación a 15° C, se mezclan dos µl de solución de reacción incubada con 100 µl de sepa de E Coli competente JM109 a una densidad de célula de 1-2 x 10^9 células/ml (Takara Shuzo Co , Ltd), y la mezcla se mantiene en hielo durante 30 minutos, luego a 42° C durante 30 segundos y de nuevo sobre hielo durante 1 minuto Luego, 500 µl de medio SOC (triptona 2% v/v, 0,5% p/v de extracto de levadura, 0,05% p/v de cloruro de sodio, 2,5 mM p/v de cloruro de potasio, 1 mM de cloruro de magnesio, y 20 mM de glucosa) se agrega a la mezcla, la cual se incuba durante una hora adicional, con agitado Las sepas transformantes son entonces aisladas, y el ADN de plásmido se prepara de las sepas como se describe en "Molecular Cloning A Laboratory Manual" Las secuencias de nucleótido de estos ADN que codifican la cadena pesada del TRA-8 humanizado son confirmadas mediante el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN 3700 (ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

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Los plásmidos resultantes son designados pHB14 (el plásmido lleva un cADN que codifica la cadena pesada del TRA-8 humanizado) La sepa de E Coli transformante que conserva estos plásmidos, designada como E Coli JM109/pHB14 se depositó con el deposito de organismos para patentes internacionales, Ntional Institue of advance Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón el 20 de abril, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7556

(2) Construcción de plásmidos de expresión que llevan el ADN de región variable de cadena pesada del TRA-8 humanizado

Los vectores de expresión recombinante para las células animales son construidos mediante la inserción del ADN que codifica la cadena pesada del TRA-8 humanizado (clonado anteriormente) como sigue

Un µg de plásmido pSRHHH3 (solicitud de patente europea EP 0-909-816 A1) que lleva la región variable de cadena pesada del anticuerpo monoclonal anti-Fas humanizado HFE7A y el ADN genómico de región constante IgG1 humano, un vector de expresión para células de mamífero, es digerido con las enzimas de restricción HindIII y ApaI, y separado mediante electroforesis de gel de agarosa al 3% Después de la electroforesis, el gel se tiñe con una solución acuosa de 1 µg/ml de bromuro de etidio para permitir la detección de ADN bajo luz UV Las bandas de ADN del ADN vector que contienen el ADN genómico de región constante IgG1 humano sin la región variable de cadena pesada del HFE7A humanizado se corta utilizando una cuchilla y se eluye del gel utilizando un kit Geneclean Spin (BIO 101, CA, USA) Después de la estación de fenol, el ADN eluido es entonces concentrado mediante centrifugación a 7 500 X g, seguido mediante precipitación de etanol, y finalmente disuelto en 5 µl de agua destilada y luego se desfosforiliza utilizando CIP El plásmido desfosforilizado digerido resultante (100 ng) se liga con 1 µg de un fragmente de ADN pHB14 que contiene el ADN que codifica la región variable de cadena pesada del TRA-8 humanizado, el cual se había también

digerido con un HindIII y ApaI, utilizando un kit de ligado de ADN versión 2 0 (Takara Shuzo Co , Ltd) la mezcla de ligado se utiliza entonces para transformar el E Coli JM109, el cual es entonces plateado sobre platos de agar LB que contienen 50 µg/ml de ampicilina

Los transformantes obtenidos mediante este método son cultivados en 2 ml de medio LB líquido que contienen 50 µg/ml de ampicilina a 37° C durante toda la noche y el ADN de plásmido se extrae subsecuentemente del cultivo resultante mediante el método alcalino-SDS

El ADN del plásmido extraído se digiere con HindIII y ApaI, y se somete a electroforesis de gel de agarosa al 3% p/v para confirmar la presencia o ausencia del inserto de ADN que codifica la región variable de cadena pesada del TRA-8 humanizado La inserción y orientación del fragmento de ADN deseado en el vector se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de gen (analizador de ADN ABI Prism 3700, Applied Biosystems) El plásmido de expresión resultante que lleva el cADN que codifica la cadena pesada del TRA-8 humanizado se designa como pHB14-1

Ejemplo 19 Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado

(1) Construcción de vectores para la cadena liviana de las versiones humanizadas del anticuerpo TRA-8

Como se muestra en la SEC ID No 46 de la lista de secuencia, al humanizar la secuencia de aminoácido de la cadena liviana del anticuerpo DR5 antihumano de ratón TRA-8, 8vo aminoácido (histidina), 9no aminoácido (glicina), 10mo aminoácido (fenilalanina), 11^{avo} aminoácido (metionina), 13^{avo} aminoácido (treonina), 20^{avo} aminoácido (cerina), el 42^{avo} aminoácido (glutamina), 43^{avo} aminoácido (cerina), 60^{avo} aminoácido (ácido aspártico), 63^{avo} aminoácido (treonina), 77^{avo} aminoácido (asparagina), 78^{avo} aminoácido (valina), 80^{avo} aminoácido (cerina), 83^{avo} aminoácido (leucina), 85^{avo} aminoácido (ácido aspártico), 87^{avo} aminoácido (fenilalanina), y 99^{avo} aminoácido (glicina), 103^{avo} aminoácido (leucina), y 108^{avo} aminoácido (alanina), proveniente del N-terminal de la secuencia de aminoácido de la cadena liviana de TRA-8 so reemplazados con prolina, serina, serina, serina, leucina, alanina,

treonina, lisina, alanina, serina, leucina, prolina, fenilalanina, treonina, tirosina, glutamina, valina y treonina, respectivamente La secuencia resultante se designa LM2

Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo DR5 antihumano TRA-8 se construyen como sigue

1) Síntesis de los iniciadores para la preparación de las regiones variable y constante de la cadena liviana de TRA-8 humanizado

La codificación del ADN para la cadena de polipéptido LM2 (secuencia ID No 46 de la lista de secuencias), cada uno de los cuales es una fusión de al región variable de la cadena liviana TRA-8 del anticuerpo anti-DR5 humanizado y la región constante de la cadena liviana Ig (cadena k), son respectivamente sintetizados al utilizar combinaciones de PCR

Adicionalmente al 7AL1P (SEC ID No 47) y 7ALCN (SEC ID No 48), se sintetizan los siguientes oligonucleótidos para PCR

5'- ccaagttctt tgtctgcatc agtaggagac agggtcacca tcacctgc -3' (HKCDF11, SEQ ID No 50),

5'- agtgtgccgg gtggatgccc agtaaatcag tagtttagga gctttccctg gtttctg -3' (HKCDR12, SEQ ID No 51),

5'- tgggcatcca cccggcacac tggggtccca agcaggttta gtggcagt -3' (HKCDF22, SEQ ID No 52),

5'- atactactta tattgctgac agtaataggt tgcaaaatcc tccggctgca gactagagat ggt -3' (HKCDR22, SEQ ID No 53), y

5'- cagcaatata gcagctatcg gacgttcggt caaggcacca aggtggaaat caaacggact gtg -3' (HKCF12, SEQ ID No 54)

2) Construcción del plásmido pCR3 1/M2-1 (clonación de la cadena liviana TRA-8 humanizada)

El fragmento LM2-F1-ADN como se definió en la SEC ID No 55 de la lista de secuencias codifica para la secuencia de aminoácido como se define en la SEC ID No 46 de la misma que es preparada mediante el desarrollo del PCR de segunda etapa, insertado dentro de un vector plásmido y clonado en E Coli

a) PCR de primera etapa

El fragmento LM2-F1-ADN que codifica para una secuencia de señal de secreción y una porción de la

región FRL_1 con un sitio de escisión de la enzima de restricción HindIII agregada al extremo 5' se prepara bajo las siguientes condiciones Los plásmidos de plantilla, pHSGHM17 y pSRPDHH, se obtienen al seguir la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

ADN pHSGHM17 de plásmido (solicitud de patente europea EP 0 090 816 A1), 25 ng

Iniciador de un oligonucleótido 7AL1P, 50 pmol

Iniciador de un oligonucleótidoHKSPR11, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10xPCR, 5 µl

Polimerasa de ADN ampliTaq (Perkin Elmer), 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en el PCR

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Las condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de los cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 77° C durante dos minutos, seguido por calentamiento a 72° C durante 10 minutos

En el fragmento de LM2-F2-ADN que codifica para una porción del FRL_1, $CDRL_1$, FRL_2, y $CDRL_2$ y se prepara bajo las siguientes condiciones

Composición de la solución de reacción

ADN pL28 de plásmido, 25 ng

Iniciador de oligonucleótido HKCDF11, 50 pmol

Iniciador de oligonucleótido HKCDR12, 50 pmol

Cóctel dNTP, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta al volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento de LM2-FR-ADN que codifica para el $CDRL_2$, y FRL_3, y una porción del $CDRL_3$ se preparan bajo las siguientes condiciones

Composición de la solución de reacción

ADN pSRPDHH de plásmido (solicitud de patente europea EP 0 909 816 A1), 25 ng

Iniciador de oligonucleótido HKCDF22, 50 pmol

Iniciador de oligonucleótido HKCDR22, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y usarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento de LM2-F4-ADN que codifica para el $CDRL_3$, y FRL_4, y la región constante con un sitio de escición de enzima de restricción EcoR I agregada al extremo 3' se prepara bajo las siguientes condiciones

Composición de la solución de reacción

ADN pSRPDHH de plásmido, 25 ng

Iniciador de oligonucleótido HKCF12, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

493

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y usarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN amplificados después de PCR se separan mediante electroforesis de gel de poliacrilamida al 5% El gel después de la electroforesis se tiñe con 1 µg/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas se detectan así y son escisionadas con una cuchilla

b) PCR de segunda etapa

LM2-ADN en el cual los fragmentos anteriormente descritos de LM2-F1-ADN, LM2-F2-ADN, LM2-F3-ADN y LM2-F4-ADN se fusionan se preparan bajo las siguientes condiciones

Composición de la solución de reacción

Fragmento de gel de LM2-F1-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM2-F2-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM2-F3-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM2-F4-ADN preparado en el PCR de primera etapa,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un

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ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento LM2-ADN preparado así se inserta en el PCR 3,1 ADN de plásmido usando un kit de clonación Eukariotic TA (Invitrogen) siguiendo el protocolo del fabricante e introducido en el E Coli TOP10F' competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena de luz del TRA-8 humanizado se confirman mediante el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN 3700 (ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

Los plásmidos resultantes se designan pCR3,1/M2-1 (el plásmido que lleva el cADN que codifica la región variable de cadena liviana del TRA-8 humanizado y una región constante de cadena liviana Ig humano)

El plásmido obtenido PCR3 1/M2-1 que contiene el fragmento LM2-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg de ADN pHSG399 de plásmido de clonación se digiere con las enzimas de restricción HindIII y EcoR I, y luego desfosforilizada con CIP El ADN pHSG399 desfosforilizado resultante y el fragmento LM2-ADN, que se ha digerido con las enzimas de restricción HindIII y EcoR I, son ligadsa utilziando el kit de ligación de ADN versión 2 0 (Takara Shyuzo, Col Ltd) Luego la E Coli DH5α se transforma con el ADN ligado y esparcido sobre el medio de agar que contiene 0,1 mm de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos se cultivan en medio LB líquido que contiene 50 µg/ml de cloramfenicol, y ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS El ADN de plásmido extraído se digiere con HindIII y EcoR I, y luego un clon que lleva el fragmento LM2-ADN se selecciona mediante electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M2-1-4 que lleva un fragmento de fusión de la región variable de la cadena liviana del TRA-8 LM2 humanizado y la región constante de la cadena Igk humana se obtiene La sepa E coli transformante que guarda

estos plásmidos, designada como E coli DH5α/pHSG/M2-2-4 se depositó con el depósito de organismos para patentes internacionales, National Institue of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7563

3) Construcción del plásmido pSR/M2-1 (plásmido de expresión para la cadena liviana del TRA-8 LM2 humanizado)

El plásmido obtenido pHSG/M2-1-4 que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 LM2 humanizada y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado (solicitud de patente europea 0-909-816-A1) se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado

resultante y el fragmento de ADN HindIII-EcoR I, obtenido del pHSG/M2-1-4 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Syuzo, Co Ltd) Entonces, el E coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se cultivan en un medio LB líquido que contiene 100 µg/ml de ampicilina, y de ADN de plásmido y se extraen del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de gen (analizador de ADN ABI Prism 3700, Applied Biosystems)

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del TRA-8 humanizado se designó pSR/M2-1

Ejemplo 20 Producción de anticuerpo humanizado

La transfección de las células COS-7, es decir, una línea de células derivada de un riñón de mono, con los plásmidos de expresión para la cadena pesada del TRA-8 humanizado y la cadena liviana del TRA-8 humanizado

obtenidos anteriormente se condujeron mediante métodos de reactivo de transfección FUGENE6 (Boehringer Mannheim Biochemica) de acuerdo con el manual de instrucción suministrado con el kit

Las células COS-7 (American Type Culture Collection No CRL-1651) crecen a semiconfluente (3 x 10^6 células/plato) en un plato de cultivo (área de cultivo 57 cm^2, Sumitomo Bakelite) que contiene un medio Eagle modificado Dulbecco (en lo sucesivo denominado como "D-MEM", Gibco BRL) suplementado con suero de ternero fetal al 10% (en lo sucesivo abreviado como "FCS", Moregate)

En el entretanto, 10 µg/plato (total cinco platos) se el ADN de plásmido de expresión de cadena pesada DR5 humanizado (pHA15-1) y 10 µg/plato del ADN de plásmido de expresión de cadena liviana DR5 humanizado preparado mediante el método alcalino-SDS y la centrifugación de gradiente de densidad de cloruro de cesio se mezclan, y luego se precipitan con etanol, seguido mediante suspensión en 5 µl/plato de H_2O

Después de 15 µl/plato de reactivo de transfección FUGENE6 se mezcla con 180 µl/plato de D-MEM sin FCS, esta solución FUGENE (185 µl/plato) se mezcla con 5 µl/plato de una solución de ADN que contiene 10 µg/plato de el ADN de plásmido de expresión de cadena pesada DR5 humanizado y 10 µg/plato del ADN del plásmido de expresión de cadena liviana DR5 humanizado Después de 15 minutos de incubación a temperatura ambiente, la suspensión de plásmido obtenida (200 µl) se agrega a los platos COS-7 preparados previamente Después de incubar en CO_2 al 5% a 37° C durante 24 horas, el medio de cultivo se cambia con D-MEM sin FCS Después de incubación en CO_2 al 5% a 37° C durante 72 horas, el supernadante del cultivo se recupera para purificar los productos de expresión en los fluidos supernadantes Mediante el método como se describió anteriormente, las células COS-7 se transfectan con cada una de las siguientes combinaciones de plásmido

(A) sin ADN de plásmido

(B) cotransfección de pHB14-1 y pSR/M2-1

Se centrifuga entonces el cultivo (1 000 r p m , 15 minutos) y se recolecta el supernadante El supernadante

se centrifuga de nuevo (9800 r p m , 15 minutos) y se filtra con un filtro de 0,45 µm (ADVANTEC TOYO DISMIC-25CS, Cat # 25CS045 AS) La purificación del IgG proveniente de los filtrados se logra utilizando cromatografía de afinidad de Protein G-POROS (Applied Biosystems) bajo las siguientes condiciones

HPLC BioCAD 700E (Applied Biosystems)

Columna cartucho sensor ProteinG-ID (tamaño columna 2 1 mmID x 30 mm LD, volumen de lecho 0,1 ml, Cat # 2-1002-00, Applied Biosystems)

Solución de elusión glicina-HCl 0,1 M (pH 2,5)

Solución de neutralización Tris-HCl 1M (pH 8,5)

Detección 280 nm

Proporción de flujo 1 ml/min

Tamaño de fracción 0,5 ml/0,5 min

Tubo de fracción 1,5 ml de microtubo de polipropileno

Temperatura 4° C

Después que todos los filtrados aplicados a la columna, 30 ml de PBS (Sigma, Cat # 1000-3) se utiliza para lavar la columna Cuando se aplica la Solución de

499
502

elusión, se inicia el recolector de fracción Cada microtubo de fracción previamente contenía 55 µl de NaCl 1 M, 110 µl de Solución de neutralización y 74 µl de 2 mg/ml de albúmina de suero de bovino (Sigma, Cat # A-7030) en PBS Las fracciones del número 8 hasta el número 10 se recolectan y se dializan contra un litro de PBS (pH 7,5) a 4° C durante un día utilizando un lizador Slide-A (Pierce, Cat #66450) La solución de diálisis se cambia dos veces

La verificación de la expresión de los anticuerpos humanizados y el ensayo cuantitativo de los productos de expresión en los fluidos de cultivo supernadantes preparados se desarrollan mediante ELISA con un anticuerpo contra IgG antihumano

A cada pozo de un plato de 96 pozos (MaxiSorp, Nunc), se agregaron 100 µl de anticuerpo policlonal específico IgG Fc antihumano de cabra (Kappel) disuelto a la concentración final de 0,5 µg/ml en solución de adsorción (hidrogencarbonato de sodio 0,05 M, azida de sodio 0,02% Ph 9,6) y el plato se incubó a 37° C durante dos horas para originar la adsorción del anticuerpo

500
533

Luego, el plato se lavó con 350 µl de PBS(-) que contiene 0,05% de Tween-20 (BioRad) (en lo sucesivo denominado como "PBS-T") cinco veces A los pozos después de lavados, se agregó el cultivo supernadante diluido con D-MEM que contenía 10% de FCS y se incubó a 37° C durante dos horas Después de lavar de nuevo con PBS-T, 100 µl de anticuerpo monoclonal específico IgG Fc antihumano de cabra etiquetado alcalino fosfatasa (Jakson Inmuno Research Lab) diluido 10 000 veces con PBS-T se agregó a cada pozo y se incubó a 37° C durante dos horas Después de lavar de nuevo con PBS-T, se agregó una solución de sustrato de p-nitrofenilfosfato obtenido del kit Alkalin Phosphatase Substrate (Bio Rad) de acuerdo con el manual de instrucción suministrado con el kit Después de incubar a 37° C durante 0,5 a una hora, se midió la observancia a 405 nm En los presentes experimentos, inmunoglobulina G de plasma de humano subclase 1 (IgG1) (Biopure AG) diluido con D-MEM que contenía 10% de FCS a ciertas concentraciones se utilizó como muestras de referencias de concentración de los anticuerpos DR5 humanizados contenidos en los fluidos supernadantes del cultivo

504

Como resultado, los productos de expresión y purificados en el cultivo supernadante se detectaron específicamente con anticuerpo IgG antihumano La cantidad de anticuerpo IgG humano es 8,96 µg (800 µl)

Ejemplo 21 Actividad que induce apoptosis de anticuerpo humanizado

Las células Jurkat (ATCC No TIB-152), se utilizaron para examinar la actividad que inducía apoptosis del anticuerpo TRA-8 humanizado purificado

Las célula Jurkat cultivadas en medio RPMI1640 con 10% FCS (Gibco BRL) a 37° C durante tres días en la presencia de CO_2 al 5% son suministradas en cada pozo de un microplato de 96 pozos (Sumitomo Bakelite) a 50 µl por pozo El TRA-8 humanizado preparado en el ejemplo 20 se ajustó para que tuviera la concentración del producto final de interés de 100 ng/ml con un medio RPMII1640 que contiene 10% de FCS para estimar sus concentraciones en los fluidos de acuerdo con el método descrito en el ejemplo 20 Cada una de las soluciones de los productos de expresión así ajustada a 100 ng/ml se utilizó para

producir dilusiones seriales al repetir la dilusión serial dos veces con RPMI1640 que contiene 10% de FCS Cada una de las soluciones de TRA-8 humanizadas eluidas se agregó a cada pozo a 50 µl por pozo Después de reaccionar a 37° C durante 12 horas, se agregaron 50 µl de 25 µM de PMS (fenizina metosiulfato, Sigma Chemical Co) que contiene 1 mg/ml XTT sal interior de (2,3-bis[2-metoxi-4-nitro-5-sulfenil]-2H-tetrazolio-5-carboxianirida, Sigma Chemical Co) (concentraciones finales de 250 µg/ml para XTT y 5 µM para PMS), después de incubar durante tres horas, la absorbancia a 450 ml de cada pozo se midió para calcular la viabilidad de las célula al utilizar la capacidad de reducción de las mitocondrias como el índice

La viabilidad de las células en cada pozo se calculó de acuerdo con la siguiente fórmula

$$\text{Viabilidad (\%)} = 100 \times (a-b)/(c-b)$$

En donde "a" se mide como un pozo de ensayo, "b" es la medición de un pozo sin células, y "c" es la medición de un pozo sin agregar anticuerpo

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Como resultado, el producto de expresión preparado en el ejemplo 20 (TRA-8 humanizado) se demostró que inducía la apoptosis en las células de la línea celular del linfoma T que expresa el antigeno DR5 humano

Ejemplo 22 Reactividad de las TRA-8 con varias moléculas DR5

Con el fin de determinar la reactividad de las TRA-8 a varias moléculas DR5 la reactividad del TRA-8 se examinó utilizando linfocitos activados como sigue

Primero, muestras de sangre periférica se toman de un humano (3 ml), titi (2 ml), y mono cinomolgus (20 ml) Las muestras de sangre tenían 1 ml de heparina (Novoheparin, Novo) agregada a ellas y las muestras son entonces puestas en capas lentamente sobre un volumen igual de solución de Ficoll-Paque plus (Amershman Pharmacia Biotech) gravedad especifica 1,077 para todos con excepción del mono cinomolgus, que tuvo una gravedad especifica de 1,072) y se centrifugó a 1700 rpm durante 30 minutos con el fin de obtener una fracción de las células mononucleares de sangre periférica Esta fracción

de células mononucleares se lavó dos veces con solución salina balanceada Hanks y luego se suspendió en un medio RMPI1640 con FCS al 10% v/v a una densidad celular de 1 x 10^6 células/ml Se agregó fitohemaglutinina-p (PHA-P Sigma Chemicals, Co) a la suspensión resultante a una concentración final de 5 µg/ml y la muestra se encubó a 37° C bajo CO_2 al 5% v/v durante 24 horas Después de este tiempo, las células se recubrieron mediante centrifugación, se lavaron y se resuspendieron en un medio RPMI 1640 que contiene FCS al 10% de v/v Luego, para activar las células ¡recuperadas se agregó interleucina-2 (Amershman Pharmacia Biotech) a la suspensión a una concentración final de 10 unidades/ml, y esta se incubó a 37° C bajo CO_2 al 5% v/v durante 72 horas

En la cantidad de la preparación activada calculada por contener 1 x 10^6 células de linfocitos activados se ubicó en el tubo de ensayo y se suspendió en 50 µl de 0,5, 1, 5, 10 µg/ml de TRA-8 en PBS o 50 µl de PBS solo La suspensión resultante se le permitió permanecer en hielo durante una hora, después de lo cual las células se lavaro tres veces con alícuotas de 500 µl de PBS y luego

505
505

se suspendieron en 50 µl de 20 µg/ml de anticuerpo IgG antihumano etiquetado FITC (Bioresource) en PBS Utilizando las células suspendidas en 500 µl de PBS como controles, las intensidades de fluorescencia se midieron, utilizando un citómetro de flujo (FACSCalibur, Becton Dickinson)

Las distribuciones de los números de células mediante intensidad de fluorescencia se obtuvieron y las proporciones de los números de las células teñidas a aquellas de las células totales se calcularon Adicionalmente, cada valor Kd se calcula utilizando la concentración del TRA-8 y las proporciones de los números de las células teñidas a aquellas de las células totales Cada frecuencia de reactividad a linfocitos activados de humano, titi, y monocinomologus es casi la misma De acuerdo con esto, el TRA-8 es capaz de unir un amplio rango de DR5 de primate que incluye un humano contra el cual se prepara originalmente el TRA-8

Ejemplo 23 Estudio de dosis escalada de TRA-8 en titis

Un estudio de toxicidad preliminar de dosis escalada de TRA-8 se desarrolló utilizando un tití macho y un tití hembra Tres conjuntos de dosis intravenosas sencillas las cuales se separaron por un periodo de retiro de siete días, se llevaron a cabo La dosis de TRA-8 se estableció en 50 250, y 1250 µg/cuerpo 48 horas después de cada tratamiento, se recolectó sangre de la vena femoral y se preparó el plasma Se midieron las actividades de aspartato de transferasa y alanina aminotransferasa del plasma utilizando un analizador (FUJI DRI-CHEM Fuji Film Medical Co , Ltd) Toda la sangre se tomó sin ninguna anestesia Como resultado, no se notó evidencia que indicara daño hepático en el ensayo bioquímico del plasma después de cada tratamiento

Ejemplo 24 Estudios farmacológicos in vitro e in vivo de TRA-8 contra células de cáncer

Con el fin de determinar si el TRA-8 tenía la eficacia terapéutica en terapia anticáncer, se examinó la actividad de muerte in vitro de TRA-8 utilizando varias líneas de célula de cáncer

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Varias células de cáncer (2 - 8 x 103 células/50 µl) cultivadas en un medio RPMI 1640 (para Jurkat), medio DMEM (para HCT-116), MEM-R (para WiDr), o DMEM-F12 (para COL2-Jck) obtenida de Gibco BRL con 10% de FCS (Gibco BRL) a 37° C en la presencia de CO2 al 5% se suministraron en cada pozo de un microplato de 96 pozos (Sumitomo Bakelite) El TRA-8 se ajustó para tener una concentración del producto final de interés de 100 ng/ml con un medio que contenía FCS al 10% La solución TRA-8 (100 ng/ml) se utilizó para producir dilusiones seriales a repetir dilusión serial dos veces con un medio que contenía FCS al 10% Cada una de las soluciones de TRA-8 diluidas se agregó a cada pozo a 50 µl por pozo y se incubó a 37° C Después de reaccionar a 97° C durante 72 horas, se agregaron 50 µl de PMS 25 µM (metanosulfato de fenacina, Sigma Chemical Co) que contenía 1 ng/ml de XTT (concentraciones finales de 250 µg/ml para XTT y 50 µM para PMS) Después de incubar durante tres horas, la absorbancia de 450 nm de cada pozo se midió para calcular la viabilidad celular al utilizar la capacidad de reducción de las mitocondrias como el índice

La viabilidad de las células en cada pozo se calculó de acuerdo con la siguiente fórmula

$$\text{Viabilidad (\%)} = 100 \times (a-b)/(c-b)$$

En donde "a" es la medición como un pozo de ensayo, "b" es la medición de un pozo sin células, y "c" es la medición de un pozo sin agregar anticuerpo

Los resultados se muestran en la tabla 3, siguiente

Tabla 3

Células	ED50 (µg/ml)
Jurkat	0,001 - 0,01
HCT-116	0,004 - 0,02
WiDr	0,007 - 0,03
COL2-Jck	2,28

Varias líneas de célula de cáncer son fuertemente inducidas a apoptosis mediante el TRA-8 bajo condiciones in vitro

Adicionalmente, se determinó el efecto antitumoral in vivo de TRA-8 en ratones desnudos, transplantados con

células WiDr, en razón a que TRA-8 no es reactivo en cruce con el DR5 de murino

Se administró anticuerpo DR5 anti-humano TRA-8 a ratones desnudos que llevanan xenoinjertos humanos que expresaban la molécula DR5 humana Los ratones utilizados eran ratones desnudos/desnudos BALb/c de seis semanas (hembra, de Clea Japan Inc), que se transplantaron con líneas celulares de cáncer de colon de humano WiDr (5 mm^3) Un día después del transplante del tumor, estos ratones transplantados son tratados diariamente con la inyección intraarticular de TRA-8 (5 µg/cuerpo) a 14 veces El crecimiento del tumor WiDr se determinó diariamente mediante el tamaño de la masa del tumor Los resultados se muestran en la tabla 4, siguiente

Tabla 4

	8 días	11 días	15 días	18 días	22 días	25 días
Control (BS)	196	259	469	584	833	1193
SD	±55	±77	±149	±230	±274	±419
TRA-8	158	97	155	195	365	530
SD	±78	±30	±60	±58	±91	±135

En este modelo, mientras que todos los animales tratados exhibieron un crecimiento de tumor visible, el crecimiento del tumor en animales tratados con TRA-8 se inhibió como se demostró mediante el tamaño del tumor Este resultado indicó que el TRA-8 es efectivo en la eliminación de células tumorales in vivo

Ejemplo 25 Estudio de combinación de TRA-8

La línea celular de cáncer de próstata de humano PC-3 se obtuvo de American Tissue Culture Collection (ATCC) y se mantuvo en una mezcla de nutrientes F-121K (21127-022, Gibco BRL) que contenía 10% de suero bovino fetal (FBS, Hyclone), 1% de L-glutamina-200 mM (25030-149, Gibco BRL) y solución de penicilina Streptomicina 0,5% (P-7539, Sigma) El medio RPMI1640 (MED-008, IWAKI) suplementado con FBS al 10 % y solución de penicilina estreptomisina 0,5% se utilizó en el siguiente experimento Células PC-3 con crecimiento exponencial se recolectaron mediante tripsinización y se lavaron dos veces con medio fresco Las células son entonces contadas, resuspendidas en un medio fresco, a una densidad de 5 * 10^4 células/ml y distribuidas en triplicado en platos de 96 pozos de fondo plano (3598,

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Corning-Coster) en un volumen total de 100 µl/pozo un día antes de iniciar el experimento Una droga anticáncer representativa, Paclitaxel (169-18611, Wako) disuelta en dimetilsulfóxido (10 mg/ml) se diluyó en un medio fresco y luego se agregó a platos de 96 pozos que contenían células a 50 µl/pozo Las concentraciones finales del dimetilsulfóxido son menores de 0,1% Después de la incubación durante 24 hr a 37° C en una atmósfera de CO_2 al 5%, el TRA-8 diluido en medio fresco se agregó a los pozos Después de la incubación por 24 horas adicionales se agrearon 50 µl de Minimum Essential Medium (11095-098, Gibco BRL) que contenía 1 mg/ml de XTT y 25 mM de PMS a los pozos y los platos son incubados durante 16 h el OD450 es entonces medido mediante SPECTRA MAX 250 (Molecular Devices) y la viabilidad celular se calculó como sigue

Viabilidad celular (%) = (OD450 para el pozo que contenía células tratadas con Taxol y/o TRA-8 (agente (s))-OD450 para el pozo que no contenía ni células ni agentes) x 100 / (OD450 para el pozo que contenía células sin agente - OD450 para el pozo que no contenía ni células ni agentes)

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575

El resultado del ensayo anterior para TRA-8 combinado con una droga anticáncer representativa, Pacitaxel es el que sigue El Paclitaxel redujo la viabilidad de las células PC-3 pero más del 40% de las señales indicó que las células de cáncer viables aún permanecían a concentraciones de hasta 200 nm Notablemente, la adición de 0,1 ng/ml donde TRA-8 disminuyó grandemente la viabilidad de las células de cáncer, hasta el 10%, aún que no se ve reducción en la viabilidad de las células después de una aplicación simple de TRA-8 a esta concentración Este resultado claramente indica que el TRA-8 exhibió actividad anticáncer sinérgicamente cuando se combinó con otras drogas anticáncer

Ejemplo 26 Análisis de otros tipos de anticuerpos humanizados de TRA-8

(1) Designar anticuerpos humanizados

La construcción de una versión humanizada del TRA-8 se desarrolló mediante el método generalmente conocido de injerto CDR Se utilizó anticuerpo mAB58'CL como un

aceptador como se describió en el ejemplo de referencia 2 en las regiones CDR del anticuerpo TRA-8 se injertaron sobre el aceptador En la región marco, algunos aminoácidos se injertan sobre el aceptador del TRA-8 o de las secuencias de consenso humano mediante los criterios dados por Queen et al (Proc Natl Acad Sci USA 86, 10029-10033, (1989)) y las secuencias TRA-8 humanizadas se construyen como se describió a continuación

(2) Construcción del plásmido que lleva ADN de la región variable de cadena pesada de otros tipos de TRA-8 humanizado o de ratón.

Como se muestra en la SEC ID No 56 de la lista de secuencias, la humanización tipo H1 de las secuencias de aminoácidos de la cadena pesada del anticuerpo DR5 antihumano de ratón PR-8 ocasionaron reemplazo del tercer aminoácido (metionina), el 13^{avo} aminoácido (licina), el 19^{avo} aminoácido (glicina), el 40^{avo} aminoácido (treonina), el 42^{avo} aminoácido (ácido glutámico), el 44^{avo} aminoácido (arginina), el 84^{avo} aminoácido (serina), el 88^{avo} aminoácido (serina), el 93^{avo} aminoácido (metionina), el 114^{avo} aminoácido (trionina), el 115^{avo} aminoácido (leucina), con, glutamina, glutamina, arginina, alanina,

glicina, glicina, asparagina, alanina, valina, leucina, y valina, respectivamente

Como se muestra en la SEC ID No 69 de la lista de secuencia, humanización del H3 de las secuencias de aminoácido de la cadena pesada del anticuerpo DR5 antihumano de ratón TRA-8 ocasionó reemplazo del 13^{avo} aminoácido (licina), el 19^{avo} aminoácido (glicina), el 40^{avo} aminoácido (treonina), el 42^{avo} aminoácido (ácido glutámico), el 44^{avo} aminoácido (arginina), el 88^{avo} aminoácido (serina), el 93^{avo} aminoácido (metionina), el 114^{avo} aminoácido (trionina), el 115^{avo} aminoácido (leucina), con, glutamina, arginina, alanina, glicina, glicina, alanina, valina, leucina, y valina, respectivamente

Como se muestra en la SEC ID No 60 de la lista de secuencias, la humanización tipo H4 de las secuencias de aminoácido de la cadena pesada del anticuerpo DR5 antihumano de ratón TRA-8 ocasionó reemplazo del 13^{avo} aminoácido (licina), el 19^{avo} aminoácido (glicina), el 88^{avo} aminoácido (serina), el 93^{avo} aminoácido (metionina), el 114^{avo} aminoácido (trionina), el 115^{avo} aminoácido

(leucina), con, glutamina, arginina, alanina, valina, leucina, y valina, respectivamente

Como se mostró en el SEC ID No 61 de la lista de secuencias, el plásmido que lleva el ADN de región variable de cadena pesada del TRA-8 quimérico se designó como "tipo M" Además, el TRA-8 humanizado descrito en el ejemplo 17 y 18 se designó como "tipo H2"

Los plásmidos que llevan ADN que codifica la región variable de cadena pesada del TRA-8 humanizado o quimérico se construye como sigue

Se utiliza PCR para construir las siguientes secuencias de ADN, cada una de las cuales comprendidas descritas anteriormente

Los siguientes 24 oligonucleótidos se sintetizaron

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5'- ttggataagc ttggcttgac ctcaccatgg gatggagctg tatcatcctc ttcttggtag caacagctac aggtgtccac -3' (A, SEQ ID No 32),

5'- tctgaagtaa tgctggtgga gtctgggggg ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B, SEQ ID No 33),

5'- tctgaagtac agctggtgga gtctgggggg ggcttagtac agcctggagg gtccctgaga ctctcctgtg cagcctctgg -3' (B2, SEQ ID No 57),

5'- tctgaagtaa tgctggtgga gtctgggggg ggcttagtaa agcctggagg gtccctgaaa ctctcctgtg cagcctctgg -3' (B3, SEQ ID No 66),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag gcaccaggga agggtctgga gtgggttgca accattagta -3' (C, SEQ ID No 34),

5'- attcactttc agtagttatg taatgtcttg ggttcggcag actccagaga agaggctgga gtgggttgca accattagta -3' (C2, SEQ ID No 64),

5'- gtggtggtag ttacacctac tatccagaca gtgtgaaggg ccgattcacc atctccagag acaatgccaa gaacaccctg -3' (D, SEQ ID No 35),

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5'- tatctgcaaa tgaacagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E, SEQ ID No 36),

5'- tatctgcaaa tgagcagtct gagagcagag gacacggctg tttattactg tgcaagaagg ggtgactcta tgattacgac -3' (E2, SEQ ID No 62),

5'- tatctgcaaa tgagcagtct gagatctgag gacacggcta tgtattactg tgcaagaagg ggtgactcta tgattacgac -3' (E3, SEQ ID No 67),

5'- ggactactgg ggccaaggga ccctggtcac agtctcctca gcctccacc aagggcccat cggtc -3' (F, SEQ ID No 37),

5' ggactactgg ggccaaggga ccactctcac agtctcctca gcctccacc aagggcccat cggtc -3' (F2, SEQ ID No 68),

5'- ctaccaagaa gaggatgata cagctccatc ccatggtgag gtcaagccaa gcttatccaa -3' (G, SEQ ID No 38),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H, SEQ ID No 39),

5'- tctcagggac cctccaggct gtactaagcc tcccccagac tccaccagct gtacttcaga gtggacacct gtagctgttg -3' (H2, SEQ ID No 58),

5'- tttcagggac cctccaggct ttactaagcc tcccccagac tccaccagca ttacttcaga gtggacacct gtagctgttg -3' (H3, SEQ ID No 69),

5'- tccagaccct tccctggtgc ctgccgaacc caagacatta catcactact gaaagtgaat ccagaggctg cacaggagag -3' (I, SEQ ID No 40),

5'- tccagcctct tctctggagt ctgccgaacc caagacatta catcactact gaaagtgaat ccagaggctg cacaggagag -3' (I2, SEQ ID No 65),

5'- ctctggagat ggtgaatcgg ccctcacac tgtctggata gtaggtgtaa ctaccaccac tactaatggt tgcaacccac -3' (J, SEQ ID No 41),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K, SEQ ID No 42),

5'- ccttcttgca cagtaataaa cagccgtgtc ctctgctctc agactgttca tttgcagata cagggtgttc ttggcattgt -3' (K2, SEQ ID No 63),

5'- ccttcttgca cagtaataca tagccgtgtc ctcagatctc agactgctca tttgcagata cagggtgttc ttggcattgt -3' (K3, SEQ ID No 70),

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5'- gaccgatggg ccctggtgg aggctgagga gactgtgacc agggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L, SEQ ID No 43) y

5 - gaccgatggg ccctggtgg aggctgagga gactgtgaga gtggtcccct ggccccagta gtccgtcgta atcatagagt cacc -3' (L2 SEQ ID No 71)

Los siguientes dos iniciadores se sintetizaron como se describió anteriormente

5'- tggataagc ttggcttgac -3' (P1, SEQ ID No 44), y

5'- gaccgatggg ccctggtgg a -3' (P2, SEQ ID No 45)

La síntesis del ADN tipo H1 que codifica la cadena de polipéptidos que comprende una secuencia de señal de secreción, una región variable de cadena pesada TRA-8 humanizada y los residuos de 8 aminoácido en el n terminal de la región IgG-CH1 se desarrolló utilizando una combinación de PCR respectivamente

El fragmento de ADN tipo H1 se preparó como sigue

Composición de solución de reacción PCR

Oligonucleótido A, 10 pmol,

Oligonucleótido B2, 10 pmol,

Oligonucleótido C, 10 pmol,

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Oligonucleótido D, 10 pmol,

Oligonucleótido E, 10 pmol,

Oligonucleótido F, 10 pmol,

Oligonucleótido G, 10 pmol,

Oligonucleótido H2, 10 pmol,

Oligonucleótido I, 10 pmol,

Oligonucleótido J, 10 pmol,

Oligonucleótido K, 10 pmol,

Oligonucleótido L, 10 pmol,

Iniciador Oligonucleótido P1, 2 µM

Iniciador Oligonucleótido P2, 2 µM

10 X solución Pyrobest II, 10 µl,

Mezcla dNTP, 8 µl,

Polimerasa ADN pirobest, 0,5 µl, y

Agua redestilada a un volumen final de 50 µl

La reacción PCR es conducida como sigue La solución se caliente primero a 94° C durante 5 minutos, después de lo cual se repitió un ciclo de calentamiento siete veces a 98° C durante 10 segundos, 55° C durante 30 segundos y 72° C durante 1 minuto Después de completar este procedimiento, la solución de reacción es calentada a 72° C durante 15 minutos

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Después de extracción de fenol y precipitación de etanol, el precipitado ADN resultante es secado en vacío, disuelto en un mínimo de agua redestilada, y separado por electroforesis de gel de agarosa al 3% Luego de la electroforesis, el gel es marcado con una solución acuosa de 1 µg/ml de bromuro de etidio para permitir la detección del ADN bajo luz ultravioleta Las bandas de ADN correspondientes al tipo H1-ADN se cortan usando una cuchilla de afeitar y eluyendo a partir del gel usando el kit Geneclean Spin (BIO 101, CA, USA) Después de la extracción con fenol, el ADN eluido es entonces concentrado por centrifugación a 7500 x g, seguido por la precipitación con etanol, y finalmente disuelto con 5 µl de agua destilada

La síntesis de ADN tipo H3 que codifica una cadena de polipéptido comprende una secuencia de señal de secreción, una región variable de la cadena pesada TRA-8 humanizada y los residuos de aminoácido 8 en el terminal N de la región IgG-CH1 se lleva a cabo usando una combinación de PCR respectivamente

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El fragmento de ADN tipo H3 es preparado como sigue

Composición de la solución de reacción PCR

Oligonucleótido A, 10 pmol,

Oligonucleótido B, 10 pmol,

Oligonucleótido C, 10 pmol,

Oligonucleótido D, 10 pmol,

Oligonucleótido E2, 10 pmol,

Oligonucleótido F, 10 pmol,

Oligonucleótido G, 10 pmol,

Oligonucleótido H, 10 pmol,

Oligonucleótido I, 10 pmol,

Oligonucleótido J, 10 pmol,

Oligonucleótido K2, 10 pmol,

Oligonucleótido L, 10 pmol,

Oligonucleótido Iniciador P1, 2 µM

Oligonucleótido Iniciador P2, 2 µM

10 X solución Pyrobest II, 10 µl,

Mezcla dNTP, 8 µl,

Polimerasa ADN pirobest, 0,5 µl, y

Agua redestilada a un volumen final de 10 µl

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La reacción PCR es conducida como sigue La solución se caliente primero a 94° C durante 5 minutos, después de lo cual un ciclo de calentamiento a 98° durante 10 segundos, 55° C durante 30 segundos, y 72° C durante 1 minuto, se repite siete veces Después de completar este procedimiento, la solución de reacción es calentada a 72° C durante 15 minutos

Después de extracción de fenol y precipitación de etanol, el precipitado ADN resultante es secado en vacío, disuelto en un mínimo de agua redestilada, y separado por electroforesis de gel de agarosa al 3% Luego de la electroforesis, el gel es marcado con una solución acuosa de 1 µg/ml de bromuro de etidio para permitir la detección del ADN bajo luz ultravioleta Las bandas de ADN correspondientes a ADN tipo H3 se cortan usando una cuchilla de afeitar y eluyendo a partir del gel usando el kit Geneclean Spin Después de la extracción con fenol, el ADN eluido es entonces concentrado por centrifugación a 7500 x g, seguido por la precipitación con etanol, y finalmente disuelto con 5 µl de agua destilada

La síntesis de ADN tipo H4 que codifica una cadena de polipéptido comprende una secuencia de señal de secreción, una región variable de la cadena pesada TRA-8 humanizada y los residuos de aminoácido 8 en el terminal N de la región IgG-CH1 se lleva a cabo usando una combinación de PCR respectivamente

El fragmento de ADN tipo H4 es preparado como sigue

Composición de la solución de reacción PCR

Oligonucleótido A, 10 pmol,

Oligonucleótido B, 10 pmol,

Oligonucleótido C2, 10 pmol,

Oligonucleótido D, 10 pmol,

Oligonucleótido E2, 10 pmol,

Oligonucleótido F, 10 pmol,

Oligonucleótido G, 10 pmol,

Oligonucleótido H, 10 pmol,

Oligonucleótido I2, 10 pmol,

Oligonucleótido J, 10 pmol,

Oligonucleótido K, 10 pmol,

Oligonucleótido G2, 10 pmol,

Oligonucleótido L, 10 pmol,

Oligonucleótido imprimador P1, 2 µM

Oligonucleótido imprimador P2, 2 µM

10 X carga Pyrobest II, 10 µl,

Mezcla dNTP, 8 µl,

Polimerasa ADN pirobest, 0,5 µl, y

Agua redestilada a un volumen final de 50 µl

La reacción PCR es conducida como sigue La solución se caliente primero a 94° C durante 5 minutos, después de lo cual un ciclo de calentamiento a 98° durante 10 segundos, 55° C durante 30 segundos y 72° C durante 1 minuto, se repite siete veces Después de completar este procedimiento, la solución de reacción es calentada a 72° C durante 15 minutos

Después de extracción de fenol y precipitación de etanol, el precipitado ADN resultante es secado en vacío, disuelto en un mínimo de agua redestilada, y separado por electroforesis de gel de agarosa al 3% Luego de la electroforesis, el gel es marcado con una solución acuosa de 1 µg/ml de bromuro de etidio para permitir la detección del ADN bajo luz ultravioleta Las bandas de ADN correspondientes al tipo H4-ADN se cortan usando una

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cuchilla de afeitar y eluyendo a partir del gel usando el kit Geneclean Spin Después de la extracción con fenol, el ADN eluido es entonces concentrado por centrifugación a 7500 x g, seguido por la precipitación con etanol, y finalmente disuelto con 5 µl de agua destilada

La síntesis de ADN tipo M que codifica una cadena de polipéptido comprende una secuencia de señal de secreción, una región variable de la cadena pesada TRA-8 quimérica y los residuos de aminoácido 8 en el terminal N de la región IgG-CH1 se lleva a cabo usando una combinación de PCR respectivamente

El fragmento de ADN tipo M es preparado como sigue

Composición de la solución de reacción PCR

Oligonucleótido A, 10 pmol,
Oligonucleótido B3, 10 pmol,
Oligonucleótido C2, 10 pmol,
Oligonucleótido D, 10 pmol,
Oligonucleótido E3, 10 pmol,
Oligonucleótido F2, 10 pmol,

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Oligonucleótido G, 10 pmol,

Oligonucleótido H3, 10 pmol,

Oligonucleótido I2, 10 pmol,

Oligonucleótido J, 10 pmol,

Oligonucleótido K3, 10 pmol,

Oligonucleótido L2, 10 pmol,

Oligonucleótido Iniciador P1, 2 µM

Oligonucleótido Iniciador P2, 2 µM

10 X carga Pyrobest II, 10 µl,

Mezcla dNTP, 8 µl,

Polimerasa ADN pirobest, 0,5 µl, y

Agua redestilada a un volumen final de 50 µl

La reacción PCR es conducida como sigue La solución se caliente primero a 94° C durante 5 minutos, después de lo cual un ciclo de calentamiento a 98° durante 10 segundos, 55° C durante 30 segundos, y 72° C durante 1 minuto, se repite siete veces Después de completar este procedimiento, la solución de reacción es calentada a 72° C durante 15 minutos

Después de extracción de fenol y precipitación de etanol, el precipitado ADN resultante es secado en vacío,

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disuelto en un mínimo de agua redestilada, y separado por electroforesis de gel de agarosa al 3% Luego de la electroforesis, el gel es marcado con una solución acuosa de 1 µg/ml de bromuro de etidio para permitir la detección del ADN bajo luz ultravioleta Las bandas de ADN correspondientes al tipo M se cortan usando una cuchilla de afeitar y eluyendo a partir del gel usando el kit Geneclean Spin Después de la extracción con fenol, el ADN eluido es entonces concentrado por centrifugación a 7500 x g, seguido por la precipitación con etanol, y finalmente disuelto con 5 µl de agua destilada

El resultado, cada ADN extraído tipo (H1, tipo H3, tipo H4 y tipo M) es clonado usando un vector pGEM-T fácil (Promega) somo sigue

El fragmento de ADN recuperado de la rescción PCR (H1, H3, H4 o M), 5 µl,

10 X carga de polimerasa Taq, 1 µl,

Mezcla dNTP, 1 µl,

Polimeraza Taq (5 unidades/ml), 1 µl, y

Agua redestilada hasta un volumen final de 10 µl

Después de lo anterior cada solución se hace reaccionar a 70° C durante 30 minutos, cada solución de ADN y del vector Easy pGEM-T se ligan usando un kit de ligación de ADN versión 2 0 (Takara Shuzo Co , Ltd), usando el protocolo del fabricante

Luego de cuatro horas de incubación a 15° C, 2 µl de la solución de reacción incubada se mezcla con 100 µl de la sepa E coli competente JM109 a una densidad de células de 1 a 2 x 10^9 células/ml (Takara Shuzo Co , Ltd), y la mezcla se mantiene sobre hielo durante treinta minutos, luego a 42° C durante 30 segundos y nuevamente sobre hielo durante un minuto Entonces, 500 µl de un medio SOC (2% v/v de triptona, 0,5% p/v de extracto de levadura, 0,05% p/v de cloruro de sodio, 2,5 mm p/v de cloruro de potasio, 1 mm de cloruro de magnesio, y 20 mm de glucosa) se agrega a la mezcla, la cual es incubada por una hora adicional, con agitación Las sepas transformantes son entonces aisladas, y el ADN del plásmido es preparado a partir de las sepas como se describió en "Molecular Cloning A Laboratory Manual" La secuencia de nucleótido de este ADN que codifica la

cadena pesada del TRA-8 humanizadoo de ratón es confirmado por le método de dideoxi, respectivamente (Sanger, F S Eta al , (1977), Proc Natl Acad Sci USA, 74 5463 5467 usando el analizador de ADN 3700 ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

El plásmido resultante es designado como pHA15 (el plásmido que lleva el cADN que codifica la cadena pesada tipo H1 del TRA-8 humanizado), el pHC10 (el plásmido que lleva el cADN que codifica en la cadena pesada tipo H3 del TRA-8 humanizado), el pHD21 (el plásmido que lleva el cADN que codifica la cadena pesada tipo H4 del TRA-8 humanizado), y el pM11 (el plásmido que lleva el cADN que codifica la cadena pesada del TRA-8 quimérico) Las sepas de E coli transformantes que llevan estos plásmidos, designadas como JM109/pHA15, E coli, JM109/pHC10, E coli, JM109/pHD21, E coli y JM109/pM11 E coli fueron depositadas junto con el depósiuto de organismos de patentes internacional, el National Institute of Advanced and Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y fue acordado darle el

número FERM BP-7555, FERM BP-7557, FERM BP-7558, y FERM BP-7559, respectivamente

(3) Construcción de plásmidos de expresión que llevan el ADN de la región variable de la cadena pesada de diversos tipos de TRA-8 humanizado o de ratón

El vector de expresión recombinante para células animales son construidos por la inserción del ADN que codifica el tipo H1 de la cadena pesada, tipo H3, y tipo H4 humanizado o tipo M quimérico del TRA-8 (clonado como se mencionó anteriormente,) como sigue

Un µg del ADN pSRHHH3 del plásmido clonado (solicitud de patente europea EP 0-909-816-A1) portaba la cadena pesada de la región variable del anticuerpo monoclonal anti-Fas HFE7A humanizado y la región constante IgG1 humana del ADN genómico, un vector de expresión para células de mamífero, es digerido con enzimas de restricción HindIII y ApaI, y separada por electroforesis de gel de agarosa al 3% Luego de la electroforesis, el gel es marcado con 1 µg/ml de una

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solución acuosa de bromuro de etidio para permitir la detección de ADN bajo luz UV Las bandas del vector de ADN que contienen la región genómica de ADN constante IgG1 humana sin la región variable de la cadena pesada del HFE7A humanizada son cortadas usando cuchillas de afeitar y eluidas con gel usando el kit Geneclean Spin Después de la extracción por fenol, el ADN eluido es entonces concentrado por centrifugación a 7500 x g, seguido por precipitación con etanol, y finalmente disuelto en 5 µl de agua destilada y luego desfosforilada usando CIP El plásmido desfosforilado y digerido resultante (100 ng) es ligado con 1 µg del fragmento de ADN del pHA15, pHC10, pHD21, o pM11 que contienen el ADN que codifica la región variable de la cadena pesada del TRA-8 humanizado o quimérico, que también había sido digerido con HindIII y ApaI, usando la versión 2 0 del kit de ligación de ADN (Takara Shuzo Co , Ltd) La mezcla de ligación es entonces usada para transformar el JM109 de E coli, el cual es plateado sobre placas de agar que contienen 50 µg/ml de ampicilina

Los transformantes obtenidos por este método son cultivados en 2 ml de medio LB líquido que contiene 5

µg/ml de ampicilina a 37° C durante la noche, y el ADN del plásmido es subsecuentemente extraído del cultivo resultante por el método alcalino-SDS

El plásmido de ADN extraído es digerido con HindIII y ApaI, y sometido electroforesis de gel de agarosa al 3% p/v para confirmar la presencia o ausencia del inserto de ADN que codifica la región variable de la cadena pesada del TRA-8 humanizado o quimérico La inserción y orientación del fragmento de ADN deseado en el vector queda confirmado por el secuenciamiento de ADN usando un analizador de secuencia de genes (ABI Prism 3700 DNA Analizer, Applied Biosystems) Los plásmidos de expresión resultantes que llevan el cADN que codifica la cadena pesada del TRA-8 humanizado o quimérico fueron designados pHA15-1, pHC10-3, pHD21-1 y pM-11-1, respectivamente

(4) Construcción de vectores para las cadenas livianas humanizadas

(4 1) Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado (tipo LM1)

Como se muestra en la SEC ID No 72 de la lista de secuencias, otra humanización (tipo LM1) de las secuencias de aminoácido de la cadena liviana del anticuerpo DR5 antiumano TRA-8 del ratón conlleva el reemplazo del tercer aminoácido (valina), el 8vo aminoácido (histidina), el 9no aminoácido (licina), el 10mo aminoácido (fenilalanina), 11^{avo} aminoácido (metionina), 13^{avo} aminoácido (treonina), el 20^{avo} aminoácido (cerina), el 42^{avo} aminoácido (glutamina), el 43^{avo} aminoácido (cerina), el 60^{avo} aminoácido (ácido aspártico), el 63^{avo} aminoácido (treonina), el 77^{avo} aminoácido (asparagina), el 78^{avo} aminoácido (valina), el 80^{avo} aminoácido (cerina), el 83^{avo} aminoácido (leucina), el 85^{avo} aminoácido (ácido aspártico), el 87^{avo} aminoácido (fenilalanina), y el 99^{avo} aminoácido (glicina), el 103^{avo} aminoácido (leucina), y el 108^{avo} aminoácido (alanina), proveniente del N-terminal de la secuencia de aminoácido de la cadena liviana de TRA-8 son reemplazados con glutamina, prolina, serina, serina, leucina, alanina, treonina, lisina, alanina, serina, serina, serina, leucina, prolina, fenilalanina, treonina, tirosina, glutamina, valina y treonina, respectivamente La secuencia resultante se designa LM1

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Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo DR5 antihumano del TRA-8 (tipo LM1, SEC ID No 72 de la lista de secuencias) se construyen como sigue

1) síntesis de cebadores para la preparación de las regiones variable y constante de la cadena liviana del TRA-8 humanizado (tipo LM1)

La codificación del ADN para la cadena de polipéptido LM1 (SEC ID No 72 de la lista de secuencias), cada una de las cuales es una fusión de la región variable del anticuerpo anti-DR5 humanizado del TRA-8 de cadena liviana (tipo LM1) y la región constante de la cadena liviana Ig humana (cadena K), son respectivamente sintetizados usando combinaciones de PCR

Adicionalmente a 7AL1P (SEC ID No 47), 7ALCN (SEC ID No 48), HKCDF11 (SEC ID No 50), HKCDR12 (SEC ID No 51), HKCDF22 (SEC ID No 52), HKCDR22 (SEC ID No 53), y HKCF12 (SEC ID No 54)

Los siguientes cebadores de oligonucleótido son sintetizados por PCR

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5'- gtcccccaca gatgcagaca aagaactigg agattgggtc atctgaatgt caccagtgga -3'

(HKSPR12, (SEC ID No 77)

2) Construcción del plásmido pCR3 1/LM1-2 (clonación de la cadena liviana del TRA-8 humanizado tipo LM1)

El fragmento LM1-ADN que codifica la secuencia de aminoácido como se dfinió en la SEC ID No 72 del mismo se preparó llevando a cabo 2 etapas PCR insertadas dentro de un vector plásmido y clonando en el E coli

a) primera etapa de PCR

El fragmento de ADN del LM1-F1 que codifica para una secuencia de señal de secreción y una porción de la región FRL_1 con una enzima de restricción HindIII en un sitio de escisión agregado al extremo 5' es preparado bajo las siguientes condiciones En los plásmidos de plantilla, pHSGHM17 y pSRPDHH, son obtenidos siguiendo la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

ADN del Plásmido pHSGHM17, 25 ng

Iniciador oligonucleótido 7AL1P, 50 pmol

Iniciador oligonucleótido HKSPR12, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10xPCR, 5 µl

Polimerasa de ADN ampliTaq (Perkin Elmer), 2,5 unidades

La solución de reacción que tiene la anterior composición es ajustada a un volumen final de 50 µl agregando agua redestilada y usada en PCR

Condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces, seguido por calentamiento a 72° C durante 10 minutos

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El fragmento de LM1-F2-ADN que codifica para una porción del FRL_1, $CDRL_1$, FRL_2, y $CDRL_2$ se prepara bajo las siguientes condiciones

Composición de la solución de reacción

ADN pL28 de plásmido, 25 ng

Iniciador de oligonucleótido HKCDF11, 50 pmol

Iniciador de oligonucleótido HKCDR12, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta al volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento de LM2-FR-ADN que codifica para el $CDRL_2$, y FRL_3, y una porción del $CDRL_3$ se preparan bajo las siguientes condiciones

Composición de la solución de reacción

ADN pSRPDHH de plásmido 25 ng

Iniciador de oligonucleótido HKCDF22, 50 pmol

Iniciador de oligonucleótido HKCDR22, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la anterior composición es ajustada a un volumen final de 50 µl agregando agua redestilada y usado en PCR

Condiciones térmicas del PCR calentamiento a 94° C durante dos minutos, después de los cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido treinta veces, seguido por calentamiento a 72° C durante 10 minutos

El fragmento de LM2-F4-ADN que codifica para una porción del $CDRL_3$, FRL_4, y la región constante con un sitio de escisión de enzima de restricción EcoR I agregado en el extremo 3' es preparado bajo las siguientes condiciones

Composición de la solución de reacción

ADN pSRPDHH de plásmido, 25 ng

Iniciador de oligonucleótido HKCDF12, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta al volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C

durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN aplicado después de PCR son separados por electroforesis de gel de acrilamida al 5% El gel después de la electroforesis es marcado con 1µl/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas así detectadas son cortadas con una cuchilla

b) segunda etapa PCR

ADN-LM1 en los cuales se describe anteriromente los fragmentos de LM1-F1-ADN, LM1-F2-ADN, LM1-F3ADN y LM1-F4-ADN son fusionados se prepara bajo las siguientes condiciones

Composición de la solución de reacción

Fragmento de gel de LM1-F1-ADN preparado en la primera etapa PCR,

Fragmento de gel de LM1-F2-ADN preparado en la primera etapa PCR,

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Fragmento de gel de LM1-F3-ADN preparado en la primera etapa PCR,

Fragmento de gel de LM1-F4-ADN preparado en la primera etapa PCR,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la anterior composición se ajusta a un volumen final de µl agregando agua redestilada y usada en PCR

Las condiciones térmicas del PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

Los fragmentos de LM1-ADN así preparado insertado en el plásmido pCR3 1ADN usando el kit de clonación eucariótico TA (in vitro gen) siguiendo el protocolo del

fabricante e introducido en el TOP10F' del E coli competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena liviana del TRA-8 humanizado (tipo LM1) son confirmadas por el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN 3700 (ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

Los plásmidos resultantes son designados pCR3 1/LM1-2 (el plásmido que lleva el cADN que codifica la cadena liviana de la región variable del TRA-8 humanizado (tipo LM1) y un Ig humano de región constante de cadena liviana)

El plásmido obtenido pCR3 1/LM1-2 contiene el fragmento de LM1-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg del plásmido de clonación del ADN pHSG399 es digerido con las enzimas de restricción HindIII y EcoR I, y luego desfosforilado con CIP EL ADN Phsg399 desfosforilizado resultante y el fragmento de LM1-ADN,

que han sido digeridos con las enzimas de restricción HindIII y EcoR I, son ligados bajo la versión 2 0 del kit de ligado de ADN (Takara Shyuzo Co , Ltd) Luego el DH5α del E coli es transformado con el ADN ligado y distribuido en un medio de agar LB que contiene 0,1 mM de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos son cultivados en un medio líquido de LB que contiene 50 µg/ml de cloramfenicol, y el ADN del plásmido es extraído del cultivo resultante de acuerdo con el método alcalino-SDS El ADN del plásmido es digerido con HindIII y EcoR I y luego un clon que lleva el fragmento de LM1-ADN se selecciona por electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M1-2-2 lleva un fragmento de fusión de la región variable de la cadena liviana de TRA-8 LM1 humanizado y la región constante de la cadena Igk se obtiene La sepa E coli transformante que produce estos plásmidos, designada como E coli DH5α/pHSG/M1-2-2 fue depositada con el depositario de organismos de patente internacional, National Institue of Advanced Industrial

Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7562

3) Construcción del plásmido pSR/LM1-2 (plásmido de expresión para la cadena liviana del TRA-8 LM2 humanizado)

El plásmido obtenido pHSG/M1-2-2 obtenido que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 LM1 humanizada y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado (solicitud de patente europea EP 0-909-816-A1) se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado resultante y el fragmento de ADN HindIII-EcoR I, obtenido del pHSG/M1-2-2 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Syuzo, Co Ltd) Entonces, el E

Coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se cultivan en un medio LB líquido que contiene 100 µg/ml de ampicilina, y de ADN de plásmido y se extraen del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de genes

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del LM1 TRA-8 humanizado se designó pSR/LM1-2

(4 2) Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado (LM3)

Como se muestra en la SEC ID No 73 de la lista de secuencias, otras de las secuencias de aminoácido de humanización (tipo LM3) de la cadena liviana del anticuerpo DR5 antihumano de ratón TRA-8 ocasionaron reemplazo del 8vo aminoácido (histidina), 9no aminoácido (licina), el 10mo aminoácido (fenilalanina), el 11^{avo} aminoácido (metionina), el 13^{avo} aminoácido (treonina), el

20avo aminoácido (serina), el 42avo aminoácido (glutamina), el 43avo aminoácido (serina), el 77avo aminoácido (asparagina), el 78avo aminoácido (valina), el 80avo aminoácido (serina), el 83avo aminoácido (leucina), el 85avo aminoácido (ácido aspártico), el 87avo aminoácido (fenilalanina), el 99avo aminoácido (glicina), el 103avo aminoácido (leucina), y 108avo aminoácido (alanina) a partir del terminal N de la secuencia de aminoácido de la cadena liviana del TRA-8 son reemplazados con prolina, serina, serina, leucina, alanina, trionina, licina, alanina, serina, leucina, prolina, fenilalanina, treonina, tirosina, glutamina, valina, y treonina, respectivamente Las secuencias resultantes se designa como LM3

Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo antihumano DR5 del TRA-8 (tipo LM3, SEC ID No 73 del listado de secuencias) es construido como sigue

1) síntesis de los iniciadores para preparar las regiones variable y constante de la cadena liviana del LM3 humanizado del TRA-8

El ADN que codifica para la cadena de polipéptido LM3 (SEC ID No 73 del listado de secuencias), cada uno de los cuales es una fusión de la región variable del anticuerpo anti-DR5 humanizado del TRA-8 de cadena liviana y la región constante de la cadena liviana del Ig humano (cadena K), son sintetizados respectivamente usando combinaciones de PCR

Adicionalmente al 7AL1P (SEC ID No 47) y 7ALCN (SEC ID No 48), los siguientes iniciadores de oligonucleótido son sintetizados para PCR

5'- atctagttct cagagatgga gacagacaca atcctgctat gggtgctgct gctctgggtt ccagg

3' (MOD1F1, SEC ID No 78),

5 - cagcacccat agcaggattg tgtctgtctc catctctgag aactagatga gaggatgctt cttaagctt -3 (MOD1R1, SEQ ID No 79),

5'- ctccactggt gacattgtga tgacccaatc tccaagttct ttgtctgcat ctgtggggga cagggtc -3' (MOD1F22, SEQ ID No 80),

5'- acttggagat tgggtcatca caatgtcacc agtggagcct ggaacccaga gcag-3' (MOD1R22, SEQ ID No 81),

5'-accatcacct gcaaggccag tcaggatgtg ggtactgctg tagcctggta ccaacagaaa ccaggaa -3' (MOD1F3, SEQ ID No 82),

5'- tacagcagta cccacatcct gactggcctt gcaggtgatg gtgaccctgt cccccacaga tgcagacaaa ga -3' (MOD1R3, SEQ ID No 83),

5'- aagcacccaa actcctcatc tattgggcat ccacccggca cactggggtc ccagataggt ttacaggcag t -3' (MOD1F42, SEQ ID No 84),

5'- cccagtgtgc cgggtggatg cccaatagat gaggagtttg ggtgcttttc ctggtttctg ttggtaccag gc -3' (MOD1R4, SEQ ID No 85),

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcaacc tat -3'(MOD1F5, SEQ ID No 86),

5'- actagagatg gtgagggtga agtctgtccc agacccactg cctgtaaacc tatctgggac -3' (MOD1R52, SEQ ID No 87),

5'- tactgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3' (MOD1F6, SEQ ID No 88),

5'- cgtccgatag ctgctatatt gctgacagta ataggttgca aaatcctccg gctgcac -3' (MOD1R6, SEQ ID No 89)

5'- aaacggactg tggctgcacc atctgtcttc atcttcccgc catctgatga g -3' (MOD1F7, SEQ ID No 90),

5'- gaagatgaag acagatggtg cagccacagt ccgttttgatt tccaccttgg tgccttgacc gaa -3' (MOD1R7, SEQ ID No 91), and

5'- agatttcaac tgctcatcag atggcgggaa (LR17, SEQ ID No 101)

2) Construcción de plásmido pCR3 1/LM3-3-44 (clonación del tipo LM3 de cadena liviana del TRA-8 humanizado)

El fragmento de ADN LM3 que codifica para la secuencia de aminoácido como se definió en la SEC ID No 73 del mismo se prepara llevando a cabo dos etapas PCR, insertado en un vector de plásmido y clonado en E Coli

a) Primera etapa PCR

El fragmento de LM3-F31B-ADN que codifica para una señal de secreción de la región de secuencia con un sitio de escisión de enzima de restricción HindIII agregado en el extremo 5' FRL_1, $DCRL_1$, FRL_2, y $CDRL_2$, FRL_3, $CDRL_3$, y FRL_4 y una porción de la región constante se preparan bajo las siguientes conduciones

Composición de la solución de reacción

Iniciador de oligonucleótido MOD1F1,5 pmol
Iniciador de oligonucleótido MOD1R1,5 pmol
Iniciador de oligonucleótido MOD1F22,5 pmol
Iniciador de oligonucleótido MOD1R22,5 pmol

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Iniciador de oligonucleótido MOD1F3,5 pmol

Iniciador de oligonucleótido MOD1R3,5 pmol

Iniciador de oligonucleótido MOD1F42,5 pmol

Iniciador de oligonucleótido MOD1R4,5 pmol

Iniciador de oligonucleótido MOD1F5,5 pmol

Iniciador de oligonucleótido MOD1R52,5 pmol

Iniciador de oligonucleótido MOD1F6,5 pmol

Iniciador de oligonucleótido MOD1R6,5 pmol

Iniciador de oligonucleótido MOD1F7,50 pmol

Iniciador de oligonucleótido MOD1R7,5 pmol

Iniciador de oligonucleótido 7AL1P,50 pmol

Iniciador de oligonucleótido LR17,50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la anterior composición es ajustada a un volumen final de 50 µl agregando agua redestilada y usada en PCR

Condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante

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un minuto y 72° C durante dos minutos, repetido 30 veces, seguido por calentamiento a 72° C durante 10 minutos

El fragmento de LM3-F31C-ADN que codifica para una porción de la región constante con un sitio de escisión de enzima de restricción EcoR I agregado en el extremo 3' preparado bajo las siguientes condiciones

Los plásmidos de plantilla pSRPDHH, es obtenido siguiendo la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

Plásmido pDRPDHH ADN, 25 ng
Iniciador de oligonucleótido MOD1F7,50 pmol
Iniciador de oligonucleótido 7ALCN,50 pmol
Cóctel dNTPs, 5,0 µl
Solución 10 X PCR, 5,0 µl
Polimerasa de ADN ampliTaq 2,5 unidades

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La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en el PCR

Las condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces seguido por calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN amplificados después del PCR son separados por electroforesis del gel de poliacrilamida al 5% El gel después de la electroforesis es marcado con 1 µg/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas así detectadas son cortadas con una cuchilla

b) segunda etapa PCR

El LM3-ADN en el cual se escribió anteriormente los fragmentos LM3-F31B-ADN, y LM3-F31C-ADN son fusionados se preparan bajo las siguientes condiciones

Composición de la solución de reacción

Fragmento de gel de LM3-F31B-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM2-F31C-ADN preparado en el PCR de primera etapa,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento LM3-ADN preparado así se inserta en el PCR 3,1 ADN de plásmido usando un kit de clonación Eukariotic TA (Invitrogen) siguiendo el protocolo del fabricante e introducido en el E Coli TOP10F' competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena liviana del TRA-8 humanizado se confirman mediante el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN 3700 (ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

Los plásmidos resultantes se designan pCR3,1/LM3-3-44 (el plásmido que lleva el cADN que codifica la región variable de cadena liviana del LM3 TRA-8 humanizado y una región constante de cadena liviana Ig humano)

El plásmido obtenido PCR3 1/LM3-3-44 que contiene el fragmento LM3-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg de ADN pHSG399 de plásmido de clonación se digiere con las enzimas de restricción HindIII y EcoR I, y luego desfosforilizada con CIP El ADN pHSG399

desfosforilizado resultante y el fragmento LM3-ADN, que se ha digerido con las enzimas de restricción HindIII y EcoR I, son ligadas utilizando el kit de ligación de ADN versión 2 0 (Takara Shyuzo, Co Ltd) Luego la E Coli DH5α se transforma con el ADN ligado y esparcido sobre el medio de agar LB que contiene 0,1 mm de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos se cultivan en medio LB líquido que contiene 50 µg/ml de cloramfenicol, y ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS El ADN de plásmido extraído se digiere con HindIII y EcoR I, y luego un clon que lleva el fragmento LM3-ADN se selecciona mediante electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M3-3-22 que lleva un fragmento de fusión de la región variable de la cadena liviana del TRA-8 LM3 humanizado y la región constante de la cadena Igk humana se obtiene La sepa E coli transformante que guarda estos plásmidos, designada como E coli DH5α/pHSG/M3-3-22 se depositó con el depósito de organismos para patentes

internacionales, National Institue of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7564

3) Construcción del plásmido pSR/LM3-3-44-10 (plásmido de expresión para la cadena liviana del TRA-8 LM3 humanizado)

El plásmido obtenido pHSG/M3-3-22 que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 LM3 humanizada y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado (solicitud de patente europea EP 0-909-816-A1) se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado resultante y el fragmento de ADN HindIII-EcoR I, obtenido

del pHSG/M3-3-22 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Shyuzo, Co Ltd) Entonces, el E Coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se cultivan en un medio LB líquido que contiene 100 μg/ml de ampicilina, y de ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de gen (analizador de ADN ABI Prism 3700, Applied Biosystems)

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del LM3 TRA-8 humanizado se designó pSR/LM3-3-44-10

(4 3) Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado (tipo LM4)

Como se muestra en la SEC ID No 74 de la lista de secuencias, otras humanización de las secuencias de aminoácido (tipo LM4) de la cadena liviana del anticuerpo DR5 antihumano de ratón TRA-8 fabricado reemplaza el 8vo

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aminoácido (histidina), 9no aminoácido (glicina), 10mo aminoácido (fenilalanina), 11^{avo} aminoácido (metionina), 13^{avo} aminoácido (treonina), 20^{avo} aminoácido (cerina), el 42^{avo} aminoácido (glutamina), 43^{avo} aminoácido (cerina), 77^{avo} aminoácido (asparagina), 78^{avo} aminoácido (valina), 80^{avo} aminoácido (cerina), 83^{avo} aminoácido (leucina), 85^{avo} aminoácido (ácido aspártico), 99^{avo} aminoácido (glicina), 103^{avo} aminoácido (leucina), y 108^{avo} aminoácido (alanina), proveniente del N-terminal de la secuencia de aminoácido de la cadena liviana de TRA-8 son reemplazados con prolina, serina, serina, leucina, alanina, treonina, lisina, alanina, serina, leucina, prolina, fenilalanina, treonina, glutamina, valina y treonina, respectivamente La secuencia resultante se designa LM4

Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo DR5 antihumano TRA-8 se construyen (tipo LM4) (SEC ID No 74 del listado de secuencias) como sigue

1) Síntesis de los iniciadores para la preparación de las regiones variable y constante de la cadena liviana de LM4 TRA-8 humanizado

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La codificación del ADN para la cadena de polipéptido LM4 (secuencia ID No 74 de la lista de secuencias), cada uno de los cuales es una fusión de la región variable de la cadena liviana TRA-8 del anticuerpo anti-DR5 humanizado y la región constante de la cadena liviana Ig humana (cadena k), son respectivamente sintetizados al utilizar combinaciones de PCR

Adicional a

7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79), MOD1F22 (SEQ ID No 80), MOD1R22 (SEQ ID No 81) MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1F5 (SEQ ID No 86), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), y MOD1R7 (SEQ ID No 91), LR17 (SEQ ID No 101),

Los siguientes iniciadores de oligonucleótido se sintetizaron para PCR

5 - ttctgtcagc aatatagcag ctatcggacg ttcggtcaag gcaccaaggt ggaaatc -3
(MOD1F62, SEQ ID No 92)

5'- cgtccgatag ctgctatatt gctgacagaa ataggttgca aaatcctccg gctgcag -3
(MOD1R62, SEQ ID No 93)

2) Construcción del plásmido pCR3 1/LM4-5-3 (clonación de la cadena liviana TRA-8 humanizada tipo LM4)

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El fragmento ADN-LM4-F41B que codifica para la secuencia de aminoácido como se definió en la SEC ID No 74 del mismo es preparado llevando a cabo dos etapas PCR, insertado en un vector de plásmido y clonado en E coli

a) PCR de primera etapa

El fragmento LM4-F41B-ADN que codifica para una secuencia de señal de secreción y una porción de la región con un sitio de escisión de la enzima de restricción HindIII agregada al extremo 5', FRL_1, CDRL1, FRL_2, y $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 y una porción de la región constante es preparado bajo las siguientes condiciones

Composición de la solución de reacción

Iniciador de oligonucleótido MOD1F1,5 pmol
Iniciador de oligonucleótido MOD1R1,5 pmol
Iniciador de oligonucleótido MOD1F22,5 pmol
Iniciador de oligonucleótido MOD1R22,5 pmol
Iniciador de oligonucleótido MOD1F3,5 pmol
Iniciador de oligonucleótido MOD1R3,5 pmol
Iniciador de oligonucleótido MOD1F42,5 pmol

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Iniciador de oligonucleótido MOD1R4,5 pmol

Iniciador de oligonucleótido MOD1F5,5 pmol

Iniciador de oligonucleótido MOD1R52,5 pmol

Iniciador de oligonucleótido MOD1F62,5 pmol

Iniciador de oligonucleótido MOD1R62,5 pmol

Iniciador de oligonucleótido MOD1F7,50 pmol

Iniciador de oligonucleótido MOD1R7,5 pmol

Iniciador de oligonucleótido 7AL1P,50 pmol

Iniciador de oligonucleótido LR17,50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la anterior composición es ajustada a un volumen final de 50 µl agregando agua redestilada y usada en PCR

Condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces, seguido por calentamiento a 72° C durante 10 minutos

El fragmento de LM4-F41C-ADN que codifica para una porción de la región constante con un sitio de escisión de enzima de restricción EcoR I agregado en el extremo 3' es preparado bajo las siguientes condiciones

Los plásmidos de plantilla pSRPDHH, es obtenido siguiendo la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

Plásmido pSRPDHH ADN, 25 ng
Iniciador de oligonucleótido MOD1F7,50 pmol
Iniciador de oligonucleótido 7ALCN,50 pmol
Cóctel dNTPs, 5,0 µl
Solución 10 X PCR, 5,0 µl
Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en el PCR

Las condiciones térmicas del PCR calentamiento 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces seguido por calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN amplificados después del PCR son separados por electroforesis del gel de poliacrilamida al 5% El gel después de la electroforesis es marcado con 1 µg/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas así detectadas son cortadas con una cuchilla de afietar

b) segunda etapa PCR

El LM4-ADN en el cual se escribió anteriormente los fragmentos LM4-F41B-ADN, y LM4-F41C-ADN son fusionados se preparan bajo las siguientes condiciones

Composición de la solución de reacción

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Fragmento de gel de LM4-F41B-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM4-F41C-ADN preparado en el PCR de primera etapa,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento LM4-ADN preparado así se inserta en el PCR 3,1 ADN de plásmido usando un kit de clonación Eukariotic TA (Invitrogen) siguiendo el protocolo del

fabricante e introducido en el E Coli TOP10F' competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena liviana del LM4 TRA-8 humanizado se confirman mediante el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN 3700 (ABI PRISM, Perkin Elmer Applied Biosystems, Japón)

Los plásmidos resultantes se designan pCR3,1/LM4-5-3 (el plásmido que lleva el cADN que codifica la región variable de cadena liviana del LM4 TRA-8 humanizado y una región constante de cadena liviana Ig humano)

El plásmido obtenido PCR3 1/LM4-5-3 que contiene el fragmento LM4-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg de ADN pHSG399 de plásmido de clonación se digiere con las enzimas de restricción HindIII y EcoR I, y luego desfosforilizada con CIP El ADN pHSG399 desfosforilizado resultante y el fragmento LM4-ADN, que se ha digerido con las enzimas de restricción HindIII y EcoR I, son ligadas utilizando el kit de ligación de ADN

versión 2 0 (Takara Shyuzo, Co Ltd) Luego la E Coli DH5α se transforma con el ADN ligado y esparcido sobre el medio de agar LB que contiene 0,1 mm de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos se cultivan en medio LB líquido que contiene 50 µg/ml de cloramfenicol, y ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS El ADN de plásmido extraído se digiere con HindIII y EcoR I, y luego un clon que lleva el fragmento LM4-ADN se selecciona mediante electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M4-5-3-1 que lleva un fragmento de fusión de la región variable de la cadena liviana del TRA-8 LM4 humanizado y la región constante de la cadena Igk humana se obtiene La sepa E coli transformante que guarda estos plásmidos, designada como E coli DH5α/pHSG/M4-5-3-1 se depositó con el depósito de organismos para patentes internacionales, National Institue of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de

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acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7565

3) Construcción del plásmido pSR/LM4-5-3-3 (plásmido de expresión para la cadena liviana del TRA-8 LM4 humanizado)

El plásmido obtenido pHSG/M4-5-3-1 que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 LM4 humanizada y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado (solicitud de patente europea EP 0-909-816-A1) se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado resultante y el fragmento de ADN HindIII-EcoR I, obtenido del pHSG/M4-5-3-1 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Shyuzo, Co Ltd) Entonces, el E Coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se

cultivan en un medio LB líquido que contiene 100 µg/ml de ampicilina, y el ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de gen (analizador de ADN ABI Prism 3700, Applied Biosystems)

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del LM4 TRA-8 humanizado se designó pSR/LM4-5-3-3

(4 4) Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado (tipo LM5)

Como se muestra en la SEC ID No 75 de la lista de secuencias, otras humanización de las secuencias de aminoácido (tipo LM5) de la cadena liviana del anticuerpo DR5 antihumano de ratón TRA-8 fabricado reemplaza el 8vo aminoácido (histidina), 9no aminoácido (glicina), 10mo aminoácido (fenilalanina), 11^{avo} aminoácido (metionina), 13^{avo} aminoácido (treonina), 20^{avo} aminoácido (cerina), el 42^{avo} aminoácido (glutamina), 43^{avo} aminoácido (cerina),

77^{avo} aminoácido (asparagina), 78^{avo} aminoácido (valina), 80^{avo} aminoácido (cerina), 83^{avo} aminoácido (leucina), 103^{avo} aminoácido (leucina), y 108^{avo} aminoácido (alanina), proveniente del N-terminal de la secuencia de aminoácido de la cadena liviana de TRA-8 son reemplazados con prolina, serina, serina, leucina, alanina, treonina, lisina, alanina, serina, leucina, prolina, fenilalanina, valina y treonina, respectivamente La secuencia resultante se designa LM5

Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo DR5 antihumano TRA-8 se construyen (tipo LM5) (SEC ID No 75 del listado de secuencias) como sigue

1) Síntesis de los iniciadores para la preparación de las regiones variable y constante de la cadena liviana de LM5 TRA-8 humanizado

La codificación del ADN para la cadena de polipéptido LM5 (secuencia ID No 75 de la lista de secuencias), cada uno de los cuales es una fusión de la región variable de la cadena liviana TRA-8 del anticuerpo anti-DR5 humanizado y la región constante de la cadena

liviana Ig humana (cadena k), son respectivamente sintetizados al utilizar combinaciones de PCR

Adicionalmente a

7AL1P (SEQ ID No 47), 7ALCN (SEQ ID No 48), MOD1F1 (SEQ ID No 78), MOD1R1 (SEQ ID No 79) MOD1F22 (SEQ ID No 80), MOD1R22 (SEQ ID No 81), MOD1F3 (SEQ ID No 82), MOD1R3 (SEQ ID No 83), MOD1F42 (SEQ ID No 84), MOD1R4 (SEQ ID No 85), MOD1R52 (SEQ ID No 87), MOD1F7 (SEQ ID No 90), y LR17 (SEQ ID No 101),

Los siguientes iniciadores de oligonucleótidose sintetizaron para PCR

5'- gggtctggga cagacttcac cctcaccatc tctagtctgc agccggagga ttttgcagat tat -3'
(MOD1F52, SEQ ID No 94)

5'- ttctgtcagc aatatagcag ctatcggacg ttcggtggag gcaccaaggt ggaaatc -3'
(MOD1F63, SEQ ID No 95)

5'- cgtccgatag ctgctatatt gctgacagaa ataatctgca aaatcctccg gctgcag -3'
(MOD1R63, SEQ ID No 96)

5'- gaagatgaag acagatggtg cagccacagt ccgtttgatt tccaccttgg tgccttccacc gaa-3'
(MOD1R72, SEQ ID No 102)

2) Construcción del plásmido pCR3 1/LM5-3-42 (clonación de la cadena liviana TRA-8 humanizada tipo LM5)

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El fragmento ADN-LM5 que codifica para la secuencia de aminoácido como se definió en la SEC ID No 75 del mismo es preparado llevando a cabo dos etapas PCR, insertado en un vector de plásmido y clonado en E coli

a) PCR de primera etapa

El fragmento LM5-F51B-ADN que codifica para una secuencia de señal de secreción y una porción de la región con un sitio de escisión de la enzima de restricción HindIII agregada al extremo 5', FRL_1, $CDRL_1$, FRL_2, y $CDRL_2$, FRL_3, $CDRL_3$, FRL_4 y una porción de la región constante es preparado bajo las siguientes condiciones

Composición de la solución de reacción

Iniciador de oligonucleótido MOD1F1,5 pmol
Iniciador de oligonucleótido MOD1R1,5 pmol
Iniciador de oligonucleótido MOD1F22,5 pmol
Iniciador de oligonucleótido MOD1R22,5 pmol
Iniciador de oligonucleótido MOD1F3,5 pmol
Iniciador de oligonucleótido MOD1R3,5 pmol
Iniciador de oligonucleótido MOD1F42,5 pmol
Iniciador de oligonucleótido MOD1R4,5 pmol

Iniciador de oligonucleótido MOD1F52,5 pmol

Iniciador de oligonucleótido MOD1R52,5 pmol

Iniciador de oligonucleótido MOD1F63,5 pmol

Iniciador de oligonucleótido MOD1R63,5 pmol

Iniciador de oligonucleótido MOD1F7,50 pmol

Iniciador de oligonucleótido MOD1R72,5 pmol

Iniciador de oligonucleótido 7AL1P,50 pmol

Iniciador de oligonucleótido LR17,50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la anterior composición es ajustada a un volumen final de 50 µl agregando agua redestilada y usada en PCR

Condiciones térmicas del PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces, seguido por calentamiento a 72° C durante 10 minutos

El fragmento de LM5-F51C-ADN que codifica para una porción de la región constante con un sitio de escisión de enzima de restricción EcoR I agregado en el extremo 3' es preparado bajo las siguientes condiciones

Los plásmidos de plantilla pSRPDHH, es obtenido siguiendo la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

Plásmido pSRPDHH ADN, 25 ng
Iniciador de oligonucleótido MOD1F7,50 pmol
Iniciador de oligonucleótido 7ALCN,50 pmol
Cóctel dNTPs, 5,0 µl
Solución 10 X PCR, 5,0 µl
Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en el PCR

Las condiciones térmicas del PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, repetido 30 veces, seguido por calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN amplificados después del PCR son separados por electroforesis del gel de poliacrilamida al 5% El gel después de la electroforesis es marcado con 1 µg/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas así detectadas son cortadas con una cuchilla de afietar

b) segunda etapa PCR

El LM5-ADN en el cual se escribió anteriormente los fragmentos LM5-F51B-ADN, y LM5-F51C-ADN son fusionados se preparan bajo las siguientes condiciones

Composición de la solución de reacción

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Fragmento de gel de LM5-F51B-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM5-F51C-ADN preparado en el PCR de primera etapa,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento LM5-ADN preparado así se inserta en el pCR3,1 ADN de plásmido usando un kit de clonación Eukariotic TA (Invitrogen) siguiendo el protocolo del

fabricante e introducido en el E Coli TOP10F' competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena liviana del LM5 TRA-8 humanizado se confirman mediante el método dideoxi (Sanger, F S , et al , (1977), Proc Natl Acad Sci USA, 74 5463-5467) utilizando un analizador de ADN

Los plásmidos resultantes se designan pCR3,1/LM5-3-42 (el plásmido que lleva el cADN que codifica la región variable de cadena liviana del LM5 TRA-8 humanizado y una región constante de cadena liviana Ig humano)

El plásmido obtenido PCR3 1/LM5-3-42 que contiene el fragmento LM5-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg de ADN pHSG399 de plásmido de clonación se digiere con las enzimas de restricción HindIII y EcoR I, y luego desfosforilizada con CIP El ADN pHSG399 desfosforilizado resultante y el fragmento LM5-ADN, que se ha digerido con las enzimas de restricción HindIII y EcoR I, son ligadas utilizando el kit de ligación de ADN versión 2 0 (Takara Shyuzo, Col Ltd) Luego la E Coli

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DH5α se transforma con el ADN ligado y esparcido sobre el medio de agar LB que contiene 0,1 mm de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos se cultivan en medio LB líquido que contiene 50 µg/ml de cloramfenicol, y ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS El ADN de plásmido extraído se digiere con HindIII y EcoR I, y luego un clon que lleva el fragmento LM2-ADN se selecciona mediante electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M5-3-27 que lleva un fragmento de fusión de la región variable de la cadena liviana del TRA-8 LM5 humanizado y la región constante de la cadena Igk humana se obtiene

3) Construcción del plásmido pSR/LM5-3-27-1 (plásmido de expresión para la cadena liviana del TRA-8 LM5 humanizado)

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El plásmido obtenido pHSG/M5-3-27 que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 LM5 humanizada y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado (solicitud de patente europea 0-909-816-A1) se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado resultante y el fragmento de ADN HindIII-EcoR I, obtenido del pHSG/M3-3-27 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Shyuzo, Co Ltd) Entonces, el E Coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se cultivan en un medio LB líquido que contiene 100 µg/ml de ampicilina, y de ADN de plásmido y se extraen del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de gen (analizador de ADN ABI Prism 3700, Applied Biosystems)

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del LM5 TRA-8 humanizado se designó pSR/LM5-3-27-1

(4 5) Construcción de un vector de expresión para la cadena liviana del anticuerpo humanizado (tipo quimera)

La secuencia mostrada en la SEC ID No 76 de la lista de secuencias, la secunecia de aminoácido de la cadena liviana de TRA-8 tipo quimera se designa como LM6

Los plásmidos de expresión que llevan este tipo de secuencias de aminoácido de cadena liviana humanizada del anticuerpo DR5 antihumano TRA-8 se construyen (tipo LM6) (SEC ID No 75 del listado de secuencias) como sigue

1) Síntesis de los iniciadores para la preparación de las regiones variable y constante de la cadena liviana de LM6 TRA-8 humanizado

La codificación del ADN para la cadena de polipéptido LM6 (secuencia ID No 76 de la lista de secuencias), cada uno de los cuales es una fusión de la región variable de la cadena liviana TRA-8 del anticuerpo

anti-DR5 humanizado y la región constante de la cadena liviana Ig humana (cadena k), son respectivamente sintetizados al utilizar combinaciones de PCR

Adicional a 7AL1P (SEC ID No 47) y 7ALCN (SEC ID No 48), los siguientes iniciadores de oligonucleótidos son sintetizados para PCR

5 - tgatgtggac atgaatttgt gagactgggt catcacaatg tcaccagtgg a -3' (HKSPR13, SEQ ID No 97),

5 - tgggttccag gctccactgg tgacattgtg atgacccagt ctcacaaatt c -3' (MVF11, SEQ ID No 98),

5'- aagacagatg gtgcagccac agcccgtttg atttccagct tggtgcctc -3 (MVR11, SEQ ID No 99), y

5'- aagctggaaa tcaaacgggc tgtggctgca ccatctgtct tcatc -3'(MCF11 SEQ ID

2) Construcción del plásmido pCR3 1/LM6-1-16 (clonación de la cadena liviana TRA-8 humanizada tipo LM6)

El fragmento LM6-ADN como se definió en la SEC ID No 75 de la lista de secuencias codifica para la secuencia de aminoácido como se define en la SEC ID No. 46 de la misma que es preparada mediante el desarrollo

del PCR de segunda etapa, insertado dentro de un vector plásmido y clonado en E Coli

a) PCR de primera etapa

El fragmento LM6-F1-ADN que codifica para una secuencia de señal de secreción y una porción de la región FRL_1 con un sitio de escisión de la enzima de restricción HindIII agregada al extremo 5' se prepara bajo las siguientes condiciones Los plásmidos de plantilla, pHSGHM17 y pSRPDHH, se obtienen al seguir la descripción en una solicitud de patente europea EP 0 909 816 A1

Composición de la solución de reacción

ADN pHSGHM17 de plásmido, 25 ng

Iniciador de un oligonucleótido 7AL1P, 50 pmol

Iniciador de un oligonucleótido HKSPR11, 50 pmol

Cóctel dNTPs, 5 µl

Solución 10xPCR, 5 µl

Polimerasa de ADN ampliTaq (Perkin Elmer), 2,5 unidades

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La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en el PCR

Las condiciones térmicas del PCR calentamientoa 94° C durante dos minutos, después de lo cual se repite un ciclo térmico de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por calentamiento a 72° C durante 10 minutos

En el fragmento de LM6-F2-ADN que codifica para una porción del FRL_1, $CDRL_1$, FRL_2, $CDRL_2$ FRL3, CDRL3, FRL4 y una porción de la región constante se prepara bajo las siguientes condiciones

Composición de la solución de reacción

ADN pL28 de plásmido, 25 ng
Iniciador de oligonucleótido MVF11, 50 pmol
Iniciador de oligonucleótido MVR12, 50 pmol
Cóctel dNTP, 5 µl
Solución 10 X PCR, 5 µl

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Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta al volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento de LM6-FR3-ADN que codifica para una porción del FRL4 y la región constante con un sitio de escisión de enzima de restricción EcoR I agregado en ele extremo 3' se prepara bajo las siguientes condiciones

Composición de la solución de reacción

ADN pSRPDHH de plásmido 25 ng
Iniciador de oligonucleótido MCF11, 50 pmol
Iniciador de oligonucleótido 7ALCN, 50 pmol
Cóctel dNTPs, 5 µl

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Solución 10 X PCR, 5 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y usarla en PCR

Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

Los fragmentos de ADN amplificados después de PCR se separan mediante electroforesis de gel de poliacrilamida al 5% El gel después de la electroforesis se tiñe con 1 µg/ml de bromuro de etidio para detectar el ADN producido bajo luz UV Las bandas de ADN respectivas se detectan así y son escisionadas con una cuchilla

b) PCR de segunda etapa

LM6-ADN en el cual los fragmentos anteriormente descritos de LM6-F1-ADN, LM6-F2-ADN, y LM6-F3-ADN son fusionados y se preparan bajo las siguientes condiciones

Composición de la solución de reacción

Fragmento de gel de LM6-F1-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM6-F2-ADN preparado en el PCR de primera etapa,

Fragmento de gel de LM6-F3-ADN preparado en el PCR de primera etapa,

Iniciador de oligonucleótido 7AL1P, 50 pmol

Iniciador de oligonucleótido 7ALCN, 50 pmol

Cóctel dNTPs, 5,0 µl

Solución 10 X PCR, 5,0 µl

Polimerasa de ADN ampliTaq 2,5 unidades

La solución de reacción que tiene la composición anterior se ajusta a un volumen final de 50 µl al agregar agua redestilada y utilizarla en PCR

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Condiciones térmicas PCR calentamiento a 94° C durante dos minutos, después de lo cual se repite un ciclo térmico 30 veces de 94° C durante un minuto, 55° C durante un minuto y 72° C durante dos minutos, seguido por el calentamiento a 72° C durante 10 minutos

El fragmento LM6-ADN preparado así se inserta en el pCR3,1 ADN de plásmido usando un kit de clonación Eukariotic TA (Invitrogen) siguiendo el protocolo del fabricante e introducido en el E Coli TOP10F' competente contenido en el kit Las secuencias de nucleótido de estos ADN que codifican la cadena de luz del TRA-8 humanizado se confirman mediante el método dideoxi utilizando un analizador de ADN

Los plásmidos resultantes se designan pCR3,1/LM6-1-16 (el plásmido que lleva el cADN que codifica la región variable de cadena liviana del TRA-8 de ratón y una región constante de cadena liviana Ig humano)

El plásmido obtenido PCR3 1/LM6-1-16 que contiene el fragmento LM6-ADN es digerido con las enzimas de restricción HindIII y EcoR I

Un µg de ADN pHSG399 de plásmido de clonación se digiere con las enzimas de restricción HindIII y EcoR I, y luego desfosforilizada con CIP El ADN pHSG399 desfosforilizado resultante y el fragmento LM6-ADN, que se ha digerido con las enzimas de restricción HindIII y EcoR I, son ligadas utilziando el kit de ligación de ADN versión 2 0 (Takara Shyuzo, Col Ltd) Luego la E Coli DH5α se transforma con el ADN ligado y esparcido sobre el medio de agar que contiene 0,1 mm de IPTG, 0,1% de X-Gal y 50 µg/ml de cloramfenicol (concentraciones finales) Los transformantes blancos obtenidos se cultivan en medio LB líquido que contiene 50 µg/ml de cloramfenicol, y ADN de plásmido se extrae del cultivo resultante de acuerdo con el método alcalino-SDS El ADN de plásmido extraído se digiere con HindIII y EcoR I, y luego un clon que lleva el fragmento LM6-ADN se selecciona mediante electroforesis de gel de agarosa al 1%

Como resultado del procedimiento anterior, el plásmido pHSG/M6-1-4-1 que lleva un fragmento de fusión de la región variable de la cadena liviana del TRA-8 de ratón y de la región constante de la cadena Igk humana se obtiene La sepa E coli transformante que guarda estos

plásmidos, designada como E coli DH5α/pHSG/M6-1-4-1 se depositó con el depósito de organismos para patentes internacionales, National Institue of Advanced Industrial Science and Technology, 1-1, Higashi 1 chome Tsukuba-shi, Ibaraki-ken, 305-5466, Japón en abril 20, 2001, de acuerdo con el tratado de Budapest para el depósito de microorganismos, y se acordó el número de acceso FERM BP-7566

3) Construcción del plásmido pSR/LM6-1-4-6 (plásmido de expresión para la quimera tipo LM6 de la cadena liviana TRA-8)

El plásmido obtenido pHSG/LM6-1-4-1 que lleva un fragmento de la fusión de la región variable de la cadena liviana TRA-8 de ratón y la región constante de la cadena Igk humana se digiere con las enzimas de restricción HindIII y EcoR I

Un µg del ADN pSRPDHH del plásmido clonado se digiere con las enzimas de restricción HindIII y EcoR I, luego se desfosforiliza con CIP El ADN pSRPDHH desfosforilizado resultante y el fragmento de ADN

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HindIII-EcoR I, obtenido del pHSG/LM6-1-4-1 se ligan utilizando un kit de ligado de ADN versión 2 0 (Takara Syuzo, Co Ltd) Entonces, el E Coli DH5α se transforma con el ADN ligado y se esparce sobre el agar LB Los transformantes obtenidos se cultivan en un medio LB líquido que contiene 100 µg/ml de ampicilina, y el ADN de plásmido y se extraen del cultivo resultante de acuerdo con el método alcalino-SDS La inserción y orientación del fragmento de ADN deseado en el vector pSRPDHH se confirma mediante la secuenciación de ADN utilizando un analizador de secuencia de genes

El plásmido de expresión resultante que lleva el cADN que codifica la cadena liviana del TRA-8 tipo quimera (se designa pSR/LM6-1-4-6)

(5) Producción de diversos anticuerpos de TRA-8 tipos quimérico o humanizado

La transfección de las células COS-7, es conducida por los métodos de reactivo de transfección FUGENE6 (Boehringer Mannheim Biochemica) de acuerdo con el manual de instrucción suministrado con el kit

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Las células COS-7 (American Type Culture Collection No CRL-1651) crecen a semiconfluente (3 x 10^6 células/plato) en un plato de cultivo (área de cultivo 57 cm^2, Sumitomo Bakelite) que contiene un medio Eagle modificado Dulbecco (en lo sucesivo denominado como "D-MEM", Gibco BRL) suplementado con suero de ternero fetal al 10% (en lo sucesivo abreviado como "FCS", Moregate)

En el entretanto, 10 µg/plato (total cinco platos) del ADN de plásmido de expresión de cadena pesada DR5 humanizado (pHA15-1) y 10 µg/plato del ADN de plásmido de expresión de cadena liviana DR5 humanizado preparado mediante el método alcalino-SDS y la centrifugación de gradiente de densidad de cloruro de cesio se mezclan, y luego se precipitan con etanol, seguido mediante suspensión en 5 µl/plato de dH_2O

Después de 15 µl/plato de reactivo de transfección FUGENE6 se mezcla con 180 µl/plato de D-MEM sin FCS, esta solución FUGENE (185 µl/plato) se mezcla con 5 µl/plato de una solución de ADN que contiene 10 µg/plato del ADN de plásmido de expresión de cadena pesada DR5 humanizado

y 10 µg/plato del ADN del plásmido de expresión de cadena liviana DR5 humanizado Después de 15 minutos de incubación a temperatura ambiente, la suspensión de plásmido obtenida (200 µl) se agrega a los platos COS-7 preparados previamente Después de incubar en CO_2 al 5% a 37° C durante 24 horas, el medio de cultivo se cambia con D-MEM sin FCS Después de incubación en CO_2 al 5% a 37° C durante 72 horas, el supernadante del cultivo se recupera para purificar los productos de expresión en los fluidos supernadantes Mediante el método como se describió anteriormente, las células COS-7 se transfectan con cada una de las siguientes combinaciones de plásmido

(A) cotransfección de pHA15-1 y pSR/LM1-2 (H2L2)

(B) cotransfección de pHB14-1 y pSR/M2-1 (H2L2)

(C) cotransfección de pHB14-1 y pSR/M3-3-44-10 (H2L3)

(D) cotransfección de pHB14-1 y pSR/LM4-5-3-3 (H2L4)

(E) cotransfección de pHC10-3 y pSR/M2-1 (H3L2)

(F) cotransfección de pHC10-3 y pSR/LM3-3-44-10 (H3L3)

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(G) cotransfección de pHC10-3 y pSR/LM4-5-3-3 (H3L4)

(H) cotransfección de Phd21-1 y pSR/LM5-3-27-1 (H4L5)

(I) cotransfección de pM11-1 y pSR/LM6-1-4-6 (quimera)

Se centrifuga entonces el cultivo (3 500 r p m , 15 minutos) y se recolecta el supernadante El supernadante se filtra con un filtro de 0,45 µm (ADVANTEC TOYO DISMIC-25CS, Cat # 25CS045 AS) La purificación del IgG proveniente de los filtrados se logra utilizando cromatografía de afinidad de Protein G-POROS (Applied Biosystems) bajo las siguientes condiciones

Sistema HPLC BioCAD 700E (Applied Biosystems)

Columna cartucho sensor ProteinG-ID (tamaño columna 2 1 mmID x 30 mm LD, volumen de lecho 0,1 ml, Applied Biosystems)

Solución de elusión glicina-HCl 0,1 M (pH 2,5)

Solución de neutralización Tris-HCl 1M (pH 8,5)

Detección 280 nm

Proporción de flujo 1 ml/min

Tamaño de fracción 0,5 ml/0,5 min

Tubo de fracción 1,5 ml de microtubo de polietileno

Temperatura 4° C

Después que todos los filtrados aplicados a la columna, 30 ml de PBS (Sigma, Cat # 1000-3) se utiliza para lavar la columna Cuando se aplica la Solución de elusión, se inicia el recolector de fracción Cada microtubo de fracción previamente contenía 55 µl de NaCl 1 M, 110 µl de Solución de neutralización y 74 µl de 2 mg/ml de albúmina de suero de bovino (Sigma, Cat # A-7030) en PBS Las fracciones del número 7 hasta el número 8 se recolectan

La verificación de la expresión de los anticuerpos humanizados y el ensayo cuantitativo de los productos de expresión en los fluidos de cultivo supernadantes preparados se desarrollan mediante ELISA con un anticuerpo contra IgG antihumano

A cada pozo de un plato de 96 pozos (MaxiSorp, Nunc), se agregaron 100 µl de anticuerpo policlonal específico IgG Fc antihumano de cabra (Kappel) disuelto a

la concentración final de 0,5 µg/ml en solución de adsorción (hidrogencarbonato de sodio 0,05 M, azida de sodio 0,02% Ph 9,6) y el plato se incubó a 37° C durante dos horas para originar la adsorción del anticuerpo Luego, el plato se lavó con 350 µl de PBS-T cinco veces A los pozos después de lavados, se agregó el cultivo supernadante diluido con D-MEM que contenía 10% de FCS y se incubó a 37° C durante dos horas Después de lavar de nuevo con PBS-T, 100 µl de anticuerpo policlonal específico de IgG Fc antihumano de cabra etiquetado alcalino fosfatasa (Jakson Inmuno Research Lab) diluido 10 000 veces con PBS-T se agregó a cada pozo y se incubó a 37° C durante dos horas Después de lavar de nuevo con PBS-T, se agregó una solución de sustrato de p-nitrofenilfosfato obtenido del kit Alkalin Phosphatase Substrate (Bio Rad) de acuerdo con el manual de instrucción suministrado con el kit Después de incubar a 37° C durante 0,5 a una hora, se midió la observancia a 405 nm En los presentes experimentos, inmunoglobulina G de plasma de humano subclase 1 (IgG1) (Biopure AG) diluido con D-MEM que contenía 10% de FCS a ciertas concentraciones se utilizó como muestras de referencias

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de concentración de los anticuerpos DR5 humanizados contenidos en los fluidos supernadantes del cultivo

Como resultado, los productos de expresión y purificados en el cultivo supernadante se detectaron específicamente con anticuerpo IgG antihumano La cantidad de anticuerpo IgG humano es 44,03 µg (H1-L1), 39,8 µG/ml (H2L2), 26,7 µg/ml (H2L3), 41 0 µg/ml (H2L4), 39,3 µg/ml (H3L2), 24,7 µg/ml (H3L3), 21,5 µg/ml (H3L4), 16,7 µg/ml (H4L5) y 18,3 µg/ml (quimera), respectivamente

(6) Actividad inducida por apoptosis de diversos tipos de anticuerpo humanizado o de anticuerpo quimérico

Las células Jurkat (ATCC No TIB-152), se utilizaron para examinar la actividad que inducía apoptosis del anticuerpo TRA-8 humanizado purificado

Las célula Jurkat cultivadas en medio RPMI1640 con 10% FCS (Gibco BRL) a 37° C durante tres días en la presencia de CO_2 al 5% son suministradas en cada pozo de un microplato de 96 pozos (Sumitomo Bakelite) a 50 µl por

pozo El TRA-8 humanizado preparado en este ejemplo 26 se ajustó para que tuviera la concentración del producto final de interés de 100 ng/ml con un medio RPMI1640 que contiene 10% de FCS para estimar sus concentraciones en los fluidos de acuerdo con el método descrito en el ejemplo 26 Cada una de las soluciones de los productos de expresión así ajustada a 100 ng/ml se utilizó para producir dilusiones seriales al repetir la dilusión serial dos veces con RPMI1640 que contiene 10% de FCS Cada una de las soluciones de TRA-8 humanizadas eluidas (H1L1, H2L2, H2L3, H2L4, H3L3, H3L4 o H4L5) se agregó a cada pozo a 50 µl por pozo Después de reaccionar a 37° C durante 12 horas, se agregaron 50 µl de 25 µM de PMS que contiene 1 ng/ml de XTT es agregado (concentraciones finales de 250 µg/ml para XTT y 5 µM para PMS) Después de incubar durante tres horas, la absorbancia a 450 nM de cada pozo se midió para calcular la viabilidad de la célula al utilizar la capacidad de reducción de las mitocondrias como el índice

La viabilidad de las células en cada pozo se calculó de acuerdo con la siguiente fórmula

Viabilidad (%) = 100 x (a-b)/(c-b)

En donde "a" se mide como un pozo de ensayo, "b" es la medición de un pozo sin células, y "c" es la medición de un pozo sin agregar anticuerpo

Como resultado, los anticuerpo humanizados ensayados demostraron que inducen la apoptosis en las células de la línea de células linfoma T que expresan el antígeno DR5 humano

Más aún, la actividad inducida por apoptosis del TRA-8 humanizado al PC-3 es examinada agregando taxol de acuerdo con el método descrito en el ejemplo 25

La línea celular de cáncer de próstata de humano PC-3 se obtuvo (ATCC No CRL-435) de American Tissue Culture Collection (ATCC) y se mantuvo en una mezcla de nutrientes F-12K (21127-022, Gibco BRL) que contenía 10% de suero bovino fetal (FBS, Hyclone), 1% de L-glutamina-200 mM (25030-149, Gibco BRL) y solución de penicilina Streptomicina 0,5% (P-7539, Sigma) El medio RPMI1640 (MED-008, IWAKI) suplementado con FBS al 10 % y solución de penicilina estreptomisina 0,5% se utilizó en el

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siguiente experimento Células PC-3 con crecimiento exponencial se recolectaron mediante tripsinización y se lavaron dos veces con medio fresco Las células son entonces contadas, resuspendidas en un medio fresco, a una densidad de 5 * 10^4 células/ml y distribuidas en triplicado en platos de 96 pozos de fondo plano (3598, Corning-Coster) en un volumen total de 100 µl/pozo un día antes de iniciar el experimento Una droga anticáncer representativa, Paclitaxel (169-18611, Wako) disuelta en dimetilsulfóxido (10 mg/ml) se diluyó en un medio fresco y luego se agregó a platos de 96 pozos que contenían células a 50 µl/pozo Las concentraciones finales del dimetilsulfóxido son menores de 0,1% Después de la incubación durante 24 hr a 37° C en una atmósfera de CO_2 al 5%, el TRA-8 humanizado (H1L1, H2L2, H2L3, H2L4, H3L2, H3L3 H3L4 o H4L5) diluido en medio fresco se agregó a los pozos Después de la incubación por 24 horas adicionales se agrearon 50 µl de Minimum Essential Medium (11095-098, Gibco BRL) que contenía 1 mg/ml de XTT y 25 mM de PMS a los pozos y los platos son incubados durante 6 h El OD450 es entonces medido mediante el contador multimarca ARVO HTS 1420 (Wallac Berthold) y la viabilidad celular se calculó como sigue

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Viabilidad celular (%) = (OD450 para el pozo que contenía células tratadas con Taxol y/o TRA-8 (agente (s))-OD450 para el pozo que no contenía ni células ni agentes) x 100 / (OD450 para el pozo que contenía células sin agente - OD450 para el pozo que no contenía ni células ni agentes)

Como resultado, los anticuerpos humanizados ensayados demostraron que inducen apoptosis en las células de cáncer de próstata que expresan el anticuerpo DR5 humano

Cualesquier patentes o publicaciones mencionadas en la especificación son indicativas del nivel de aquellos versados en la técnica a la cual pertenece la invención Estas patentes y publicaciones son incorporadas aquí como referencia al mismo grado como si cada publicación individual fuera expeecifia e individualmente que se incorpora como referencia

La presente invención no está limitada en alcance por los anteriores depósitos o modalidades descritos en los ejemplos que tienen el propósito de ser ilustraciones

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de unos pocos aspectos de la invención y cuales quier modalidades que sean equivalentes funcionalmente están dentro del alcance de la invención Varias modificaciones de la invención en adición a aquellas mostradas y descrita aquí serán aparentes o serán evidentes para aquellos versados en la técnica y tienen el propósito de caer dentro del alcance de las reivindicaciones adjuntas

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REIVINDICACIONES

1. Un anticuerpo purificado que liga un receptor DR5 TRAIL, en donde dicho anticuerpo, en su forma soluble a bajas concentraciones, tiene in vivo e in vitro actividad de muerte de células inducidas en las células objetivo que expresan el DR5

2 El anticuerpo purificado de la reivindicación 1, en donde el anticuerpo induce la muerte de células in vitro en la ausencia de un reticulador de anticuerpo secundario

3 El anticuerpo purificado de la reivindicación 1 en donde el anticuerpo no induce una muerte de células significante de células de fibroblasto no transformado

4 El anticuerpo purificado de la reivindicación 1 donde la actividad de inducir muerte de células está caracterizada por menos de 40% de viabilidad

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de célula objetivo en concentraciones de anticuerpo de menores que 5 µg/ml

5 El anticuerpo purificado de la reivindicación 1 donde el anticuerpo específicamente liga el receptor DR5 TRAIL y lo liga los receptors DR4, DcR1 o DcR2 TRAIL luego de la rutina de análisis Western blot

6 El anticuerpo purificado de la reivindicación 1 en donde dicho anticuerpo es un anticuerpo monoclonal

7 El anticuerpo purificado de la reivindicación 1 en donde el DR5 es DR5 humano

8 El anticuerpo purificado de la reivindicación 1 en donde dichas células objetivo son células sinoviales inflamatorias

9 El anticuerpo purificado de la reivindicación 1 en donde dichas células objetivo son células de cáncer

10. El anticuerpo purificado de la reivindicación 1 en donde dicha célula objetivo es una célula inmune activada

11 El anticuerpo purificado de la reivindicación 10 en donde dicha célula inmune es un linfocito activado

12 El anticuerpo purificado de la reivindicación 10 en donde dicha célula inmune es una célula NK activada.

13 El anticuerpo purificado de la reivindicación 10 en donde dicha célula inmune es una célula dentrítica activada

14 Un anticuerpo monoclonal que tiene la misma especificidad del epítope que un hibridoma de ratón de TRA-8 que tiene un número de acceso ATCC PTA-1428

15 Un anticuerpo monoclonal producido por el hibridoma de ratón de TRA-8 que tiene un número de acceso ATCC PTA-1428

16 El anticuerpo purificado de la reivindicación 1, en donde dicho anticuerpo es humanizado

17 Un método para inducir selectivamente la muerte de células en células objetivo que expresan el DR5, comprende la etapa de poner en contacto las células objetivo con una cantidad terapéutica de un anticuerpo de al reivindicación 1.

18 El método de la reivindicación 17, en donde el poner en contacto se hace in vivo

19 El método de la reivindicación 17, en donde el poner en contacto se hace in vitro

20 El método de la reivindicación 17, en donde las células objetivo que expresan el DR5 son células de cáncer

21. El método de la reivindicación 17, en donde las células objetivo que expresan el DR5 son células sinoviales inflamatorias

22 El método de la reivindicación 17, en donde las células objetivo que expresan el DR5 son células inmune activadas

23 El método de la reivindicación 22, en donde las células inmune son linfocitos activados

24. El método de la reivindicación 22, en donde las células inmune son células nk activadas

25 El método de la reivindicación 22, en donde las células inmune son células dentríticas activadas

26 Un método para inducir selectivamente la muerte en células objetivo que expresan DR5, comprende la etapa de poner en contacto las células objetivo con una cantidad terapéutica del anticuerpo de la reivindicación 16

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27. Un proceso para inhibir la proliferación de células objetivo que expresan DR5, comprende la etapa de poner en contacto las células con una cantidad terapéutica del anticuerpo de la reivindicación 1

28 El proceso de la reivindicación 26, en donde el poner en contacto es in vivo

29. El proceso de la reivindicación 27, en donde el poner en contacto es in vitro

30 El proceso de la reivindicación 27, en donde las células objetivo que expresan el DR5 son células de cáncer

31 El proceso de la reivindicación 27, en donde las células objetivo que expresan el DR5 son células sinoviales inflamatorias

32 El proceso de la reivindicación 27, en donde las células objetivo que expresan el DR5 son células inmune activadas

33 El proceso de la reivindicación 32, en donde las células inmune activadas son linfocitos activados

34 El proceso de la reivindicación 21, en donde las células inmune activada son células nk activadas

35 El proceso de la reivindicación 21, en donde las células inmune activadas son células dentríticas activadas

36 El proceso de la reivindicación 21, que comprende adicionalmene poner en contacto las células objetivo con un agente terapéutico

37 El método de la reivindicación 36, en donde el agente terapéutico es un agente quimiterapéutico

38 El método de la reivindicación 37, en donde el agente terapéutico se selecciona a partir del grupo que consiste de bleomicina, carboplatina, clorambucil, cisplatina, colchicina, ciclofosfamida, daunorobicina, actinomicina, dietilestilbestrol, doxorubicin, etopocida, 5-

fluorouracil, floxuridina, melfalan, metotrexato, mitomicina, 6-mercaptopurina, paxlitaxel, teniposida, 6-tioguanina, vincristina y vinblastina

39 El método de la reivindicación 37, en donde el agente terapéutico es doxirubicina

40 El método de la reivindicación 37, en donde el agente terapéutico es metotrexato

41 El método de la reivindicación 37, en donde el agente terapéutico es paclitaxel

42 Un proceso para inhibir la proliferación de células objetivo que expresan DR5, comprende la etapa de poner en contacto las células con una cantidad terapéutica del anticuerpo de la reivindicación 16

43 El proceso de la reivindicación 42, en donde el poner en contacto se hace in vivo

44 El proceso de la reivindicación 42, en donde el contacto se hace in vitro

45 El proceso de la reivindicación 42, en donde las células objetivo que expresan DR5 son células de cáncer

46 El proceso de la reivindicación 42, en donde las células objetivo que expresan DR5 son células sinoviales inflamatorias

47 El proceso de la reivindicación 42, en donde las células objetivo que expresan DR5 son células inmune activadas

48 El proceso de la reivindicación 47, en donde las células inmune son linfocitos activados

49 El proceso de la reivindicación 47, en donde las células inmune son células NK activadas

50 El proceso de la reivindicación 47, en donde las células inmune son células dentríticas activadas

51 El proceso de la reivindicación 42, que comprende adicionalmente poner en contacto las células objetivo con un agente terapéutico

52 Método de la reivindicación 51, en donde el agente terapéutico es un agente quimioterpéutico

53 El método de la reivindicación 52, en donde el agente terapéutico se selecciona a partir del grupo que consiste de bleomicina, carboplatina, clorambucil, cisplatina, colchicina, ciclofosfamida, daunorobicina, actinomicina, dietilestilbestrol, doxorubicin, etopocida, 5-fluorouracil, floxuridina, melfalan, metotrexato, mitomicina, 6-mercaptopurina, paxlitaxel, teniposida, 6-tioguanina, vincristina y vinblastina

54 El método de la reivindicación 52, en donde el agente terapéutico es doxorubicina

55 El método de la reivindicación 52, en donde el agente terapéutico es metotextrato

56 El método de la reivindicación 52, en donde el agente terapéutico es paxlitaxel

57 Una composición que comprende una cantidadd efectiva farmacéuticamente del anticuerpo de la reivindicación 1 y un portador farmacéuticamente aceptable

58 Uso del anticuerpo de la reivindicación 1 para preparar un terapéutico para la muerte selectiva de células objetivo o de células disreguladas

59 Un proceso para tratar un sujeto con una enfermedad relacionada con apoptosis que comprende la etapa de poner en contacto un tejido objetivo del sujeto que expresa la enfermedad relacionada con apoptosis con una cantidad terapoéutica con un primer anticuerpo de acuerdo con la reivindicación 1

60 Un proceso para tratar un proceso con una enfermedad relacionada con apoptosis que comprende la etapa de poner en contacto un tejido objetivo del sujeto que expresa la enfermedad

relacionada con apoptosis con una cantidad terapéutica de un primer anticuerpo de acuerdo con la reivindicación 16

61 Un método para tratar un sujeto con cáncer, que comprende administrar al sujeto una cantidad farmacéuticamente efectiva del anticuerpo en la reivindicación 1, en donde la cantidad farmacéuticamente efectiva del anticuerpo induce selectivamente la muerte celular de las células de cáncer

62 El método de la reivindicación 61, que comprende adicionalmente administrar al sujeto un segundo anticuerpo que promueve la muerte celular de las células de cáncer

63 El método de la reivindicación 61, que comprende adicionalmente administrar al sujeto un agente terapéutico

64 El método de la reivindicación 63, en donde el agente terapéutico es un agente quimioterapéutico

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65 El método de la reivindicación 64, en donde el agente terapéutico se selecciona a partir del grupo que consiste de bleomicina, carboplatina, clorambucil, cisplatina, colchicina, ciclofosfamida, daunorobicina, actinomicina, dietilestilbestrol, doxorubicin, etopocida, 5-fluorouracil, floxuridina, melfalan, metotrexato, mitomicina, 6-mercaptopurina, paxlitaxel, teniposida, 6-tioguanina, vincristina y vinblastina

66 El método de la reivindicación 64, en donde el agente es doxorubicina

67 El método de la reivindicación 64, en donde el agente es metotextrato

68 El método de la reivindicación 64, en donde el agente es paclitaxel

69 El método de la reivindicación 64, quecomprende adicionalmente administrar al sujeto una terapia de radiación

70 El método de la reivindicación 64, que comprende adicionalmente administrar al sujeto un agente terapéutico y una terapia de radiación

71 Un método para tratar un sujeto con cáncer, que comprende administrar al sujeto una cantidad farmacéuticamente efectiva del anticuerpo de la reivindicación 16, en donde la cantidad farmacéuticamente efectiva del anticuerpo induce selectivamente la muerte celular de las células de cáncer.

72 Un método de tratar un sujeto que tiene una enfermedad inflamatoria o una enfermedad autoinmune, que comprende administrar al sujeto una cantidad farmacéuticamente efectiva del anticuerpo de la reivindicación 1, en donde la cantidad farmacéuticamente efectiva del anticuerpo selectivamente induce la muerte celular de las células objetivo que tienen receptores DR5

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73. El método de la reivindicación 72, en donde las células objetivo son células inmune inmunoactivadas

74. El método de la reivindicación 73, en donde las células inmunoactivadas son linfocitos activados

75. El método de la reivindicación 73, en donde las células inmunoactivadas son células NK activadas

76. El método de la reivindicación 73, en donde las células inmunoactivadas son células dendríticas activadas

77. El método de la reivindicación 72, en donde las células objetivo son células sinoviales inflamatorias

78. El método de la reivindicación 72, que comprende adicionalmente suministrar al sujeto una cantidad farmacéuticamente efectiva de un segundo anticuerpo que promueve la muerte celular de las células objetivo

79 El método de la reivindicación 72, que comprende adicionalmente administrar al sujeto una cantidad farmacéuticamente efectiva de un agente terapéutico

80 El método de la reivindicación 79, en donde el agente terapéutico es bisindolilmaleimida

81 El método de la reivindicación 79, en donde el agente terapéutico es metotextrato

82 Un método para tratar un sujeto que tiene una enfermedad inflamatoria o una enfermedad autoinmune, que comprende administrar al sujeto una cantidad farmacéuticamente efectiva del anticuerpo de la reivindicación 16 en donde la cantidad farmacéuticamente efectiva del anticuerpo induce selectivamente la muerte célular de las células objetivo

83 Un método de inducir de manera selectiva la muerte célular en células objetivo que comprende las etapas de

(a) transfectar las células objetivo con un vector que comprende una secuencia de ácido nucleico receptor de TRAIL expresible,

(b) expresar en dichas células un receptor TRAIL codificado por dicha secuencia de ácido nuclieco receptor de TRAIL, y

(c) poner en contacto dichas células con un anticuerpo inductor de muerte célular selectivo para ligar dicho receptor TRAIL

84. El método de la reivindicación 83, en donde el receptor TRAIL es un receptor DR5

85. El método de la reivindicación 83, en donde dicho anticuerpo es un anticuerpo monoclonal que tiene la misma especificidad epítope que el hibridoma de ratón TRA-8 de ratón que tiene el número de acceso ATCC PTA-1428

86 Un ácido nucléico aislado, que comprende una secuencia de nucleótido que codifica una cadena pesafa de inmunoglobulina de un anticuerpo capaz de ligar el receptor DR5 del TRAIL y capaz de

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inducir la muerte celular de una célula con receptores DR5

87 Un ácido nucléico aislado de la reivindicación 86, en donde la secuencia nucleótida codifica la cadena pesada de inmunoglobulina expresada por el hibridoma TRA-8 de ratón que tiene un número de acceso ATCC PTA-1428

88 El ácido nucleico aislado de la reivindicación 86, en donde la secuencia de nucleótido codifica la cadena pesada de inmunoglobulina expresada por el hibridoma del TRA-8 de ratón que tiene un número de acceso TCC PTA-1428 con uno o más sustituciones de aminoácido conservativas

89 El ácido nucleico aislado de la reivindicación 86, en donde la secuencia nucleótida codifica la cadena pesada de inmunoglobulina expresada por el hibridoma TRA-8 de ratón que tiene el número de acceso a TCC PTA-1428 con uno o más eliminaciones de aminoácido

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90 El ácido nucleico aislado de la reivindicación 86, que comprende los nucleótidos 1-1398 de la SEC ID No 21

91 El ácido nucleico aislado de la reivindicación 86, en donde la cadena pesada codificada de inmunoglobulina es humanizada

92 El ácido nucleico aislado de la reivindicación 91, en donde la cadena pesada de inmunoglobulina humanizada codificada comprende residuos de aminoácido 1-119 de la SEC ID No. 31.

93 Un vector que comprende el ácido nucleico de la reivindicación 86 y un elemento regulatorio operablemente ligado con el ácido nucleico

94 Una célula cultivada que comprende el vector de la reivindicación 93

95 Un polipéptido purificado que comprende una secuencia de aminoácido de una cadena pesada de inmunoglobulina de un anticuerpo capaz de ligar

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el recpetor DR5 del TRAIL y capaz de inducir la muerte celular de una célula con receptores DR5

96 El polipéptido purificado de la reivindicación 95, en donde la secuencia de aminoácido es la secuencia de aminoácido de la cadena pesada de inmunoglobulina expresada por el hibridoma de ratón TRA-8 de ratón que tiene el número de acceso ATCC No PTA-1428

97 El polipéptido purificado de la reivindicación 95, en donde la secuencia de aminoácido es el aminoácido de la inmunoglobulina de cadena pesada expresada por el hibridoma TRA-8 de ratón que tiene el número de acceso ATTC PTA-1428 con uno o más sustituciones de aminoácido conservativas

98 El polipéptido purificado de la reivindicación 95, en donde la secuencia de aminoácido es el aminoácido de la inmunoglobulina de cadena pesada expresada por el hibridoma TRA-8 de ratón que tiene el número de acceso ATCC PTA-1428 con un o más eliminaciones de aminoácido

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99 El polipéptido purificado de la reivindicación 95, en donde la secuencia de aminoácido comprende los residuos de aminoácido 1-464 de la SEC ID No 23

100 El péptido purificado de la reivindicación 95, en donde la inmunoglobulina de cadena pesada es humanizada

I01 El péptido purificado de la reivindicación 100 en donde la secuencia de aminoácido de la inmonuglobulina de cadena pesada humanizada comprende residuos de aminoácido 1-119 de la SEC ID No 31

102 El péptido purificado de la reivindicación 100, en donde la secuencia de aminoácido de la inmunoglobulina de cadena pesada humanizada comprende residuos de aminoácido 1-119 de la SEC ID No 31 con uno o más sustituciones conservativas

103 El péptido purificado de la reivindicación 100, en donde la secuencia de aminoácido de la

inmunoglobulina de cadena pesada humanizada comprende residuos de aminoácido 1-119 de la SEC ID No 31 con un o más eliminaciones de aminoácido

104 Un ácido nucleico aislado, que comprende una secuencia de nucleótido que codifica una inmunoglobulina de cadena liviana de un anticuerpo capaz de ligar recetpores DR5 de TRAIL y capaz de inducir la muerte celular de una célula con receptores DR5

105 El ácido nucleico aislado de la reivindicación 104, en donde la secuencia de nucleótido codifica la inmunoglobulina de cadena liviana expresada por el híbrido TRA-8 de ratón que tiene un número de acceso ATCC PTA-1428

106 El ácido nucleico aislado de la reivindicación 104, en donde la secuencia de nucleótido codifica la inmunoglobulina de cadena liviana expresada por el híbrido TRA-8 de ratón que tiene un número de acceso ATCC PTA-1428 con un o más sustituciones conservativas de aminoácido

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107 El ácido nucleico aislado de la reivindicación 104, en donde la secuencia de nucleótido codifica la inmunoglobulina de cadena liviana expresada por el híbrido TRA-8 de ratón que tiene un número de acceso ATCC PTA-1428 con una o más eliminaciones de aminoácido

108 El ácido nucleico aislado de la reivindicación 104, que comprende los nucleótidos 1-705 de la SEC ID No. 22

109 El ácido nucleico aislado de la reivindicación 104, en donde la inmunoglobulina de cadena liviana codificada es humanizada

110 El ácido nucleico aislado de la reivindicación 104, en donde la inmunoglobulina de cadena liviana humanizada codificada comprende residuos de aminoácido 1-213 de la SEC ID No 46

111 Un vector que comprende el ácido nucleico de la reivindicación 104 y un elemento regulatorio operablemente ligado al ácido nucleico

112 Una célula cultivada que comprende un vector de la reivindicación 111

113 Un polipéptido purificado que comprende una secuencia de aminoácido de una inmunoglobulina de cadena liviana de un anticuerpo capaz de ligar el receptor DR5 TRAIL y capaz de inducir la muerte celular de una célula con receptores DR5

114 El polipéptido purificado de la reivindicación 113, en donde la secuencia de aminoácido es la secuencia de aminoácido de la inmunoglobulina de cadena liviana expresada por el hibridoma de ratón TRA-8 que tiene el número de acceso ATCC PTA-1428

115 El polipéptido purificado de la reivindicación 113, en donde la secuencia de aminoácido es la secuencia de aminoácido de la inmunoglobulina de cadena liviana expresada por el hibridoma de ratón TRA-8 que tiene el número de acceso ATCC PTA-1428 con uno o más sustituciones conservativas de aminoácido

116 El polipéptido purificado de al reivindicación 113, en donde la secuencia de aminoácido es el aminoácido de la inmunoglobulina de cadena liviana expresada por el hibridoma de ratón TRA-8 que tiene el número de acceso ATCC PTA-1428 con uno o más eliminaciones de aminoácido

117 El polipéptido purificado de la reivindicación 113, en donde la secuencia de aminoácido comprende residuos de aminoácido 1-234 de la SEC ID No 24

118 El péptido purificado de la reivindicación 113, en donde la inmunoglobulina de cadena liviana es humanizada

119 El péptido purificado de la reivindicación 118 en donde la secuencia de aminoácido de la inmunoglobulina de cadena liviana humanziada comprende residuos de aminoácido 1-213 de la SEC ID No 46

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120 El péptido purificado de la reivindicación 118, en donde la secuencia de aminoácido de la inmunoglobulina de cadena liviana humanizada comprende residuos de aminoácido 1-213 de la SEC ID No 46 con uno o más sustituciones conservativas

121 El péptido purificado de la reivindicación 118, en donde la secuencia de aminoácido de la inmunoglobulina de cadena liviana humanizada comprende residuos de aminoácido 1-213 de la SEC ID No. 46 con uno o más eliminaciones de aminoácido

122 Un proceso para producir el anticuerpo humanizado de la reivindicación 16, que comprende las etapas de

(a) transformar una célula huésped con un primer vector que codifica una inmunoglobulina humanizada de cadena liviana, una inmunoglobulina humanizada de cadena pesada, o ambos,

(b) opcionalmente, transformar la célula huésped con un segundo vector que codifica la cadena liviana o la cadena pesada de la etapa (a) si cualquiera está ausente de dicho primer vector,

(c) incubar la célula huésped transformada bajo condiciones que permita la expresión del vector o de los vectores, y

(d) aislar el anticuerpo humanizado de la célula huésped o del medio de la célula de huésped

123 Un kit comercial para inducir apoptosis que comprende el anticuerpo de la reivindicación 1 en un contenedor

124 Un kit comercial para inducir apoptosis que comprende el anticuerpo de la reivindicación 16 en un contenedor

125 Un proceso para incrementar niveles de NFkB en una célula que comprende la etapa de poner en contacto la célula con una cantidad efectiva del anticuerpo de la reivindicación 1

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126 Un proceso para incrementar los niveles de NFkB en una célula que comprende la etapa de poner en contacto la célula con una cantidad efectiva del anticuerpo de al reivindicación 16

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RESUMEN

Un anticuerpo de la invención interactúa con el DR5 humano para producir efectos agonísticos o antagonísticos aguas abajo del receptor incluyendo la inhibicoón de la proliferación celular y apoptosis Las secuencias de ácido nucleico y secuencias de aminoácido de los anticuerpos anti-DR5 han sido elusidados y los vectores y células que contienen y expresan estas secuencias han sido generados Los métodos y usos para los anticuerpos son detallados incluyendo el tratamiento de enfermedades relacionadas con apoptosis y el tratamiento de crecimiento celular disregulado

Industria y Comercio

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Dependencia 2020 DIVISION DE NUEVAS CREACIONES

NIT 800176089-2

ECIBO OFICIAL DE CAJA 02 - 75,599
ECHA NOVIEMBRE 13 DE 2002

***** C O N S I G N A C I O N *****

EPOSITANTE	TIPO PAGO	BANCO	CUENTA	No PAGO	FECHA PGO	Vr PAGO
AVELIER ABOGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	3136955	13/11/2002	15,849,560 00

***** C O N C E P T O *****

ANT	RENTISTICO	CONCEPTO		TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL	DE PATENTE DE IN	378,000 00
			TOTAL $	378,000 00

ON TRESCIENTOS SETENTA Y OCHO MIL PESOS

ESPONSABLE _____

ECIBO DE CAJA APLICADO AL EXPEDIENTE No _____

Carrera 13 No 27-00 Pisos 5 7 y 10
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Clo 13529-BU1_001-4

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REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

	USUARIO	RADICADOR
PATENTE DE INVENCIÓN MODELO DE UTILIDAD	( )	( )
- Indicación de que se solicita la concesión de una patente.	( )	( )
- Datos de identificación del solicitante o de la persona que se presenta la solicitud.	( )	( )
- Descripcion de la invención.	( )	( )
- Dibujos de ser estos pertinentes.	( )	( )
- Comprobante de Pago de las tasas establecidas.		
COMPLETA ( ) INCOMPLETA	( )	( )
DISEÑO INDUSTRIAL ( )		
- Indicación de que se solicita el registro de Diseño Industrial	( )	( )
- Datos de identificación del solicitante o de la persona que presenta	( )	( )
- la solicitud		
- Representación gráfica y fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) INCOMPLETA	( )	( )
ESQUEMA DE TRAZADO ( )		
- Indicación de que solicita el registro de un Esquema de trazado	( )	( )
- Datos de identificación del solicitante o de la persona que presenta La solicitud.	( )	( )
Representación gráfica del esquema de trazado	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) INCOMPLETA	( )	( )

Firma Responsable

Fecha

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Industria y Comercio
SUPERINTENDENCIA

2020
Bogotá, D C ,

Doctor
Emilio Ferrero
Apoderado
UAB Research Foundation.

Oficio N°
095 10

ASUNTO	Radicación N°	02-108186
	Solicitud Internacional	PCT/US2001/14151
	Fecha inicio fase nacional	02/12/2002
	Trámite	011
	Evento	379
	Actuación	430
	Folios	001

Como resultado del examen de forma, realizado con fundamento el el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Unica de la Superintendencia de Industria y Comercio, se le comunica que:

- ☐ La solicitud no contiene la traducción de las reivindicaciones tal como fueron presentadas. (Numeral 5.2.5, capítulo quinto del título décimo de la Circular Unica de la SIC)

- ☐ La solicitud no contiene el certificado de depósito del material biológico. (Art. 26-j) Decisión 486)

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Unica N° 10 de 2001

ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

31 OCT 2003

El anterior requerimiento fue notificado por fijación en lista de fecha,_____

202002