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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. 06.019.270
Solicitud de patente a nombre de **TRUBION
PHARMACEUTICALS, INC**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **TRUBION PHARMACEUTICALS, INC**, domiciliada en Seattle, Estados Unidos, solicitante de la patente de la referencia, en respuesta al oficio No. 13177 notificado por fijación en lista el 26 de agosto de 2011 relacionado con el concepto técnico No. 2337, nos permitimos informar lo siguiente:

Nos permitimos presentar ante ese Despacho copia informal del siguiente documento:

Hayden, et al. "Single mono- and bispecific antibody derivatives with novel biological properties and antitumor activity from COS cell transient expression system". *Therapeutic immunology*. 1994. Vol 94:3-15.

Con la presentación de este documento damos cabal cumplimiento al requerimiento realizado por esa oficina mediante el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina.

Señor Superintendente,

DILIA MARIA RODRIGUEZ D'ALEMAN
CC. 41.654.882
TP 20824 CSJ

Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumour activity from a COS cell transient expression system

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Summary

Single-chain antibody molecules were expressed from modified eukaryotic expression vectors as individual protein domains encoded on interchangeable cDNA cassettes. Two different single-chain antibody derivatives were constructed by linking individual light- and heavy-chain variable domains. The first was specific for the L6 tumour-associated antigen and the second was specific for human CD3. Each single-chain variable domain was genetically fused with an Fc 'tag' and expressed as a fusion protein in a COS cell transient transfection system. These single-chain antibody derivatives demonstrated specific binding to cells expressing appropriate antigen and bound with affinities similar to native antibody. The CD3 single chain molecule mediated stronger activation of PLC γ 1 and similar levels of T-cell proliferation compared with native antibody. A bispecific Fv single-chain cassette was created by fusing the expression cassettes encoding the binding domains for L6 and CD3 single-chain molecules using oligonucleotide primers encoding a short 27-residue 'helical' peptide linker. The CD3-L6 variable domains were fused to the Fc tag and expressed in COS cells. The CD3-L6Fvlg bispecific fusion protein mediated adhesion between T cells and L6-positive tumour cells, and stimulated potent T-cell proliferation and cytotoxicity against tumour cells expressing the L6 antigen.

Keywords

bispecific, cancer therapy, single-chain antibodies

Introduction

The use of murine mAb for the treatment of human disease has been most successful in the diagnosis and therapy of cancer and in the induction of immunosuppression for treatment of autoimmunity, graft versus host disease, and allograft rejection. Patient immune responses against murine mAb initiated a widespread effort to design proteins using recombinant DNA technology that are better suited for *in vivo* applications in humans. For example, humanized

antibodies are less immunogenic than their murine counterparts (1,2) as only the antigen-binding complementarity determining regions are of rodent origin (3). Chimerized and humanized mAb also exhibit longer survival rates *in vivo* (4). In addition, genetic engineering has been used to create single-chain Fv fragments expressed from DNA encoding a single immunoglobulin light chain variable region (V_L) and a short peptide linker followed by heavy chain variable region (V_H) sequence. These smaller molecules have shown increased accessibility to tumour cells *in vivo* and may therefore be better than native mAb for use in drug, radionuclide or hormone delivery systems (5,6).

Of particular interest to us is the design and expression of small bispecific protein fragments containing anti-CD3 and anti-tumour antigen specificities for use in targeting T cells to kill tumour cells. Initially, bispecific molecules for target-

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ing were relatively large in size, made by chemically coupling mAb specific for the CD3/TCR complex with mAb against a target cell specificity. *In vitro* studies showed that in the presence of these heteroconjugates, cytotoxic T lymphocytes killed target cells in a non-MHC restricted manner (7–10). Truly bispecific antibodies (monovalent for two specificities) produced from hybrid hybridomas (11–15), have demonstrated properties *in vitro* similar to those observed for heteroconjugates, while possessing the added advantage of smaller size. *In vivo*, heteroconjugated or bispecific mAbs with appropriate target specificities have been reported to kill murine B lymphoma cells (16–18) and significantly reduce the growth of pulmonary metastases (19) following intravenous injection of tumour cells in murine models. In addition, significant killing of xenografts of human ovarian (20,21), renal (22), or colon carcinoma cells (23,24) in human T-cell-reconstituted nude or athymic mice has been reported using heteroconjugated or bispecific mAb. In humans, therapeutic regimens involving bispecific molecules to target destruction of tumour cells has been reported for the treatment of ovarian carcinoma (20) and malignant glioma (25).

Despite the promising results obtained using bispecific antibodies for therapy, several factors make them impractical for large scale therapeutic applications including the labour-intensive techniques required for their generation, the low yields of protein recovered, and the need for extensive purification of the bispecific antibody from a mixture of molecules formed by random association of both parental immunoglobulin heavy and light chains. Bacterial expression of genetically engineered proteins has partially circumvented these problems. Although bacterially expressed Fab and single-chain antibody derivatives provide a means of generating large quantities of small, antigen-specific molecules (26–31), the protein must often be recovered from inclusion bodies and refolded *in vitro*, or is toxic to the cell when expressed behind a leader sequence that targets the protein to the periplasm (32). Bacteria may not be the best host for expression of bispecific proteins because (a) four homologous variable region repeats may lead to deletion of at least a portion of the DNA sequence and (b) difficulty may be encountered in renaturing functional molecules from the complex protein precipitates found in inclusion bodies.

Fv fragments have also been expressed in myeloma cells (33), suggesting that mammalian expression systems are potentially suitable for expression of single chain bispecific molecules. Although no reports exist in the literature to date describing the expression of two genetically linked antibody variable regions, Trautnecker *et al.* (34) described a bispecific molecule containing CD4 receptor domains genetically fused to CD3 Fv that induced CTL killing of HIV1-infected target cells. In the present study, we describe the adaptation of an existing mammalian expression system for the rapid production, expression, and screening of single-chain mono- and bispecific antibody derivatives. Tran-

sient expression of these molecules in COS cells avoided problems associated with the recovery of biologically active molecules from bacteria and allowed rapid recovery of bispecific Fv protein that was functional for both parental mAb specificities.

Methods

Modification of expression vectors: The plasmids pCDM8 (35, 36) and pLNX (B. Seed and A. Aruffo, personal communication) were modified as follows (Fig. 1): the *Hind*III site at the 5' end of the stuffer region was used to insert a *Hind*III–*Sal*I cassette containing the anti-L6 immunoglobulin light chain leader sequence for secretion of fusion proteins. This sequence was encoded on complementary oligonucleotides with *Hind*III and *Sal*I cohesive end overhangs (the sense oligonucleotide was AGCTTATGGATT-TTCAAGTGCAGATTTCAGCTTCCTGCTAA-TCAGTGCTTCAGTCATAATGTCCAGAGGAG, while the complementary oligonucleotide was TCGACTCTCTGGACATTATGACTGAAGCACTGATT-AGCAGGAAGCTGAAAATCTGCACCTTGAAAAT-CCATA. The sense oligonucleotide was phosphorylated

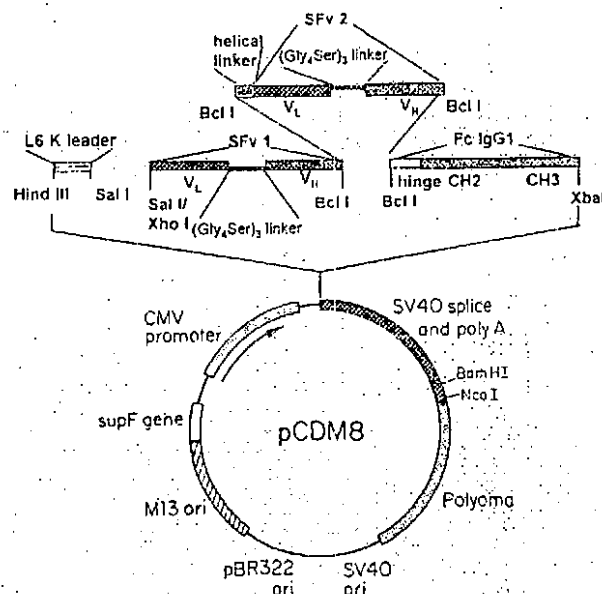


Fig. 1 Modification of pCDM8 for expression of single chain antibody derivatives. The mammalian expression vector is diagrammed with the alterations and additions shown along the top portion of the vector. The stuffer fragment containing the polylinker was replaced by a *Hind*III–*Sal*I leader peptide cassette at the amino terminus of the sFv insertion and a *Bcl*I–*Xba*I molecular tag at the carboxyl end. A single (*Sal*I–*Xho*I) to (*Bcl*I–*Bam*HI–*Bgl*II) V_L–V_H fusion cassette was fused in frame with the two flanking cassettes to create the single chain antibody derivatives for L6Fv–Ig and CD3Fv–Ig. To create the bispecific gene fusion, a second binding domain was inserted into the vector adjacent to the first, with a short 'helical' peptide linker cassette separating the two antibody binding domains from one another.

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with polynucleotide kinase (Boehringer-Mannheim, Indianapolis, ID) and annealed to the complement prior to ligation. In addition, the *Xba*I site at the 3' end of the stuffer region was used to insert a molecular tag (an Fc fragment from human IgG1) or a simple stop codon, flanked by *Bcl*I and *Xba*I restriction sites at the carboxyl terminus. The human IgG1 sequences were isolated from the RNA of the chimeric anti-L6 transfectoma (37) by coupled reverse transcription (Avian Myeloblastosis Virus; Life Sciences, St. Petersburg, FL) and PCR reactions or by secondary PCR of full-length anti-L6 immunoglobulin heavy-chain cDNA.

A so-called 'helical' peptide linker (DQSNSEEAKEEAKKEEAKKSNSLESL) was used to make a bispecific fusion cassette between the anti-CD3 and anti-L6 variable region domains. The sequence motif (EEAKK)_n was chosen because it is particularly hydrophilic due to an abundance of charged amino acids, but overall is neutral. The peptide can form stabilizing (*i* + 3) and (*i* + 4) ionic interactions if helical conformation is adopted, as may be expected for helical sequences of about thirty residues or more. It was encoded on complementary oligonucleotides with GATC cohesive end overhangs: the sense oligonucleotide was GATCAATCCAACTCTGAAGAAGCAAAGAAAGAGGAGGCCAAAAAGGAGGAAGCCAAGAATCTAACAGGCTCGAGAGC and the antisense oligonucleotide was GATCGCTCTCGAGGCTGTTAGATTTCTTTGGCTTCCTCCTTTTGGCCTCCTCTTTCTTTGCTTCTTCAGAGTTGGATT.

PCR samples (100 µl per reaction) included *Taq* polymerase buffer (Stratagene, Torrey Pines, CA), 20 µmol of each dNTP, 50–100 pmol primers, 1–10 ng template, and *Taq* polymerase (Stratagene, Torrey Pines, CA). Reactions were carried out in a Perkin-Elmer Cetus Thermal Cycler with a 30-cycle programme, typically consisting of 1 min steps at 94°C, at 55°C, and at 72°C. Ligation products were transformed into MC1061/p3 (Invitrogen, San Diego, CA) and colonies were screened for the appropriate insertion plasmids. Positive clones were verified by DNA sequencing and minitransfection.

Isolation of V regions

Anti-L6 mAb is specific for a recently cloned tumour antigen (38, 39) that is expressed at high levels on certain human tumours (40). RNA from the chimeric anti-L6 transfectoma was isolated by rapid lysis in NP40 (41) and full-length cDNA cassettes for both heavy and light chain were amplified by PCR using primers based on the published sequences for anti-L6 variable regions (37) and human constant regions (42) but with restriction sites attached for cloning. These full-length cDNAs were used as templates for secondary PCR reactions to subclone variable domains or the Fc region for human IgG1. RNA from G19-4 anti-CD3 hybridoma cells (43), producing mAb against the ε-chain of the human CD3/TCR complex (44) was also extracted using NP40 lysis. First strand cDNA syn-

thesis was performed using AMV reverse transcriptase and primers complementary to 18 bases of CH1 or Cκ, annealing approximately 100 bases from the J–C junction. First strand cDNA products were poly-G tailed using terminal transferase (Stratagene, Torrey Pines, CA), dGTP and a 3' nested primer annealing approximately 50 bases from the J–C junction. Anchor-tailed PCR was then performed with 1–2 µl of purified G-tailed cDNA and a forward primer containing poly-C sequences (45), and modified reverse primers that eliminated the remaining constant region sequences. Secondary PCR was used to add restriction sites for cloning.

Immunostaining and FACS analysis

Binding of the antibody derivatives to Jurkat T cells or to L6-positive tumour cells (H2981) was analysed by indirect immunostaining. Cells were incubated with native mAb or with single chain derivatives at various concentrations in binding buffer (GIBCO-BRL, Grand Island, NY) for 40 min at 4°C. Cells were then washed and incubated with a FITC-conjugated second step reagent for an additional 30–40 min at 4°C. Second step antibody was one of the following: goat anti-mouse Ig for murine mAb, goat anti-human IgG (Tago, Burlingame, CA) for single chain Fvlg fusions, or anti-idiotypic directed against the L6 mAb binding site (46) for L6Fv-Ig or chimeric L6-mAb. Fluorescence was analysed on a FACS IV cell sorter (Becton Dickinson and Co., Mountain View, CA) equipped with a four-decade logarithmic amplifier.

Cell culture, transfections, and purification of fusion proteins

COS cells were transfected with expression plasmids as previously described (36,47). Plasmid DNA was added to transfection media at 1 µg ml⁻¹ in a total volume of 12 ml per 150 mm plate. Spent serum free culture media from three batches of transfected COS cells was pooled and used for isolation of fusion proteins with Ig molecular tags. Cellular debris was removed by low-speed centrifugation and the clarified supernatant was applied to a column of immobilized protein A (Repligen Corp, Cambridge, MA) equilibrated with 50-mM sodium citrate (pH 8.0) as described previously (47). The concentration of purified proteins was determined using a commercially available protein assay kit (BioRad, Richmond, CA).

Cell adhesion assays

Adhesion assays were performed essentially as described (48), in the presence of 10-mM EDTA. Jurkat T cells were labelled with ⁵¹Cr, incubated with antibody stimuli, washed, and incubated with L6-positive H2981 tumour cells. To prevent potential nonspecific binding to H2981 cells, the Jurkat cells and H2981 monolayers were incu-

bated with an irrelevant antibody to saturate Fc receptors prior to addition of the CD3Fv-Ig, L6Fv-Ig, or CD3-L6FvIg antibody derivatives. After the adhesion reactions were complete, monolayers were washed five times with ice-cold RPMI media, solubilized by addition of 0.5-M NaOH, and radioactivity was quantitated by gamma counting. Numbers of bound cells were calculated by dividing total bound radioactivity (c.p.m.) by the specific activity (c.p.m. per cell) of labelled cells.

SDS-PAGE and western blotting with alkaline phosphatase

Acrylamide gels forming a linear 6–15% gradient with a 4% stacker were run at 200 V for 4–5 h. Gels were immunoblotted to nitrocellulose membranes using a western semi-dry transfer apparatus (BioRad, Richmond, CA) at 20 V (3 mA cm⁻²) for 1 h. Blots were blocked with 2% nonfat milk plus 0.1% Tween in PBS (blocking buffer) for 1–2 h and then incubated with alkaline-phosphatase conjugated goat antihuman IgG (Boehringer-Mannheim, Indianapolis, IN) at a 1:1500 dilution in blocking buffer. Blots were washed three times in blocking buffer and were developed in Western Blue (Promega, Madison, WI) for 5–15 min before stopping the reaction by rinsing in distilled water.

Immunoprecipitation and western blotting with anti-P-tyr

Jurkat T cells were unstimulated (0) or stimulated with either native G19-4 mAb or with CD3Fv-Ig for 1 or 3 min at the concentrations indicated. Cells were lysed in modified RIPA buffer (49) containing phosphatase and protease inhibitors (1-mM sodium orthovanadate, 1-mM PMSF, 2-mM EGTA, 0.5% aprotinin and 10 µg ml⁻¹ leupeptin). Cell lysates were cleared (10 min at 14 000 r.p.m.) and immunoprecipitated with either rabbit anti-P-tyr (50) or PLCγ1 antiserum (51). Immune complexes were recovered with protein A-Sepharose beads (Pharmacia, Piscataway, NJ) and washed. The proteins were separated by SDS-PAGE (8%) and were transferred to PVDF Immobilon (Millipore, Bedford, MA) for 2 h at 4°C. The immunoblots were blocked before addition of 0.5 µg ml⁻¹ of affinity purified rabbit anti-P-tyr in blocking buffer. Proteins were detected with 1 mCi µl⁻¹ high specific activity ¹²⁵I-labelled protein A (ICN Biomedicals, Costa Mesa, CA) and autoradiography.

Measurement of intracellular free calcium ([Ca²⁺]_i)

Cytoplasmic free calcium was measured using the calcium-binding dye indo-1 (Molecular Probes, Eugene, OR) and a model 50 HH/2150 cell sorter (Ortho Diagnostic Systems, Westwood, MA) as described (52). When laser light at 355 nm is used to excite the indo-1 within loaded cells, the fluorescence emission in the violet increases while blue decreases as Ca²⁺ is bound. The ratio of violet/blue is proportional to the intracellular calcium concentration and mean [Ca²⁺]_i is determined by plotting the indo-1 violet/blue

fluorescence ratio versus time. The percentage of responding cells versus time is calculated using programs that determine the values of the indo-1 ratio 2 SD above the mean ratio of control cells and plotted as the percentage of cells above this threshold versus time. There are 80 data points determined during an 8-minute response.

Proliferation assays

Lymphocytes obtained from peripheral blood were washed several times in serum free RPMI 1640, and the cell concentration was adjusted to 1 × 10⁶ cells ml⁻¹ in RPMI containing 10% FCS. Cells were cultured in 96-well, Xat-bottom plates (5 × 10⁴ cells well⁻¹ in a volume of 0.2 ml). Proliferation was measured in triplicate samples by uptake of [³H] thymidine at 1 µCi ml⁻¹ during the last 6–8 h of a 3-day culture. PHA activated T cells were prepared by culturing PBL with 1 µg ml⁻¹ PHA (Wellcome, Charlotte, NC) for 5 days, and resting 1 day in media lacking PHA. L6-positive H2981 tumour cells were exposed to 10 000 rads prior to use in proliferation assays. Fusion proteins were either prebound to the tumor cells and excess was removed by washing before incubating with PBL ('prebound' samples) or fusion proteins were added in solution ('solution' samples) to the PBL with the tumor cells. PBL and irradiated tumor cells were co-cultured at 5:1, 25:1 and 625:1 PBL:tumour cells per well.

Cytotoxicity assays

L6-positive H2981 tumour cells were incubated with [⁵¹Cr] for 2 hours prior to incubation with the fusion proteins (5 µg ml⁻¹ and 0.5 µg ml⁻¹) and PBL (at several eVector: target ratios ranging from 100:1 to 10:1). Cells were cultured in RPMI containing 10% FCS in a total volume of 0.2 ml for 5 h prior to harvesting of the cultures and determination of counts released by gamma counting.

Results

Adaptation of mammalian expression vectors

The vectors pCDM8 and pILNX are derivatives of the vector H3M (36) and use either the cytomegalovirus or the AMV promoter and enhancer to achieve high level expression of genes inserted into the polylinker/stuffer region located downstream of these control regions. In addition, control sequences for splicing and polyadenylation are located adjacent to the polylinker, facilitating gene expression from cDNA rather than genomic sequences. The polylinker was altered to contain two cDNA cassettes flanking the restriction sites used for variable region insertion. The first was a HindIII-Sall fragment containing the leader peptide from the anti-L6 immunoglobulin kappa light chain that was inserted at the 5' end of the polylinker to achieve secretion of the molecules fused to it. The second was a

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BclI-XbaI fragment encoding the hinge, CH2 and CH3 domains of human IgG1 that was fused in frame at the 3' end of the polylinker to facilitate detection and purification of antibody derivatives. Single-chain antibody cassettes encoding the variable domains of the heavy and light chains were connected to one another by a short peptide linker and inserted as *Sall-BclI* fragments (other compatible ends were sometimes used) between these two short flanking cassettes so that a single open reading frame was formed, as shown in Fig. 1.

Construction and expression of single-chain monospecific L6Fv-Ig, CD3-Fv-Ig and bispecific CD3-L6FvIg antibody derivatives

The variable domain sequences encoding the heavy and light chains of antibodies directed against the L6 tumour antigen or the CD3 T-cell surface receptor were isolated. The V_L and V_H of anti-L6 or of G19-4 were linked into a single coding region by using overlap extension PCR to create a (Gly/Ser)₃ linker (53) between the carboxyl end of V_L and the amino terminus of V_H . The V_L - V_H cassette was inserted into the adapted vector so that a single open reading frame was created encoding the anti-L6 light-chain signal peptide, the variable region containing the antibody binding specificity, and a mutant derivative of the human IgG1 Fc domain that had hinge cysteines (residues 220, 226 and 229; EU index) changed to serines to reduce intrachain disulfide bonding. Upon sequencing, this affinity tag was found to contain another sequence change which resulted in the substitution of a serine to proline at residue 238 in the hinge-link region. The CD3-L6 bispecific fusion cassette was created by fusing the anti-CD3 and anti-L6 V_L - V_H cassettes using sequence encoding a short 'helical' peptide linker. The bispecific fusion cassette was inserted into the expression vector using the same restriction sites as for the monospecific constructs. Each of the three single-chain FvIg constructs was transfected into COS cells and the fusion proteins were purified from culture supernatants by affinity chromatography on immobilized protein A. Yields of purified protein were typically about 2 mg l⁻¹ for the L6Fv-Ig fusion protein, about 10 mg l⁻¹ for CD3Fv-Ig, and about 0.5 mg l⁻¹ for the CD3-L6FvIg bispecific molecule.

Western blots of proteins subjected to reducing or nonreducing SDS-PAGE were probed with alkaline-phosphatase conjugated goat anti-human IgG as shown in Fig. 2. Panels A and B illustrate that the L6Fv-Ig and CD3Fv-Ig fusion proteins migrated as a single species of M_r 55 000 under both reducing and nonreducing conditions, the approximate size expected for these single chain antibody derivatives. Similarly, the CD3-L6FvIg bispecific molecule migrated as a single species of M_r ~83 000 under either of these conditions. By comparison, chimeric L6 mAb or L6 wild-type dimer (WTD) consisting of mouse variable regions fused to wild-type sequences for human hinge-CH2-CH3, exhibited significant mobility differ-

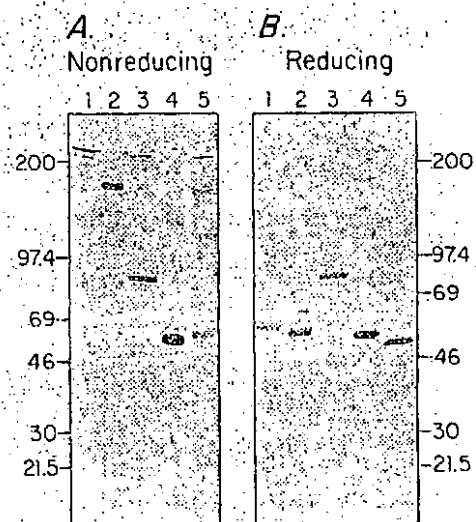


Fig. 2 Western blots of single-chain anti-L6 and anti-CD3 monospecific and anti-CD3/L6 bispecific molecules. Serum free spent culture medium from COS cell transfections was collected and purified by protein A affinity chromatography. Protein was resuspended in loading buffer and subjected to SDS-PAGE gradient gel (5–16%) electrophoresis under (A) nonreducing or (B) reducing conditions, blotted to nitrocellulose, and detected with alkaline phosphatase conjugated anti-human IgG. (A) Lane 1 = Chimeric L6 mAb (0.5 µg); lane 2 = anti-L6 WTD (0.5 µg); lane 3 = CD3-L6FvIg bispecific (0.5 µg); lane 4 = L6FvIg (0.6 µg); and lane 5 = CD3Fv-Ig (0.4 µg). (B) lane 1 = CD3Fv-Ig (0.4 µg); lane 2 = L6FvIg (0.6 µg); lane 3 = CD3-L6FvIg bispecific (0.5 µg); lane 4 = anti-L6 WTD (0.5 µg); and lane 5 = Chimeric L6 mAb (0.5 µg).

ences depending on reduction and on the degree of denaturation, indicating that the heavy chain constant regions of these molecules associated by disulfide bonding to form dimers. Occasionally, western blots from nonreducing SDS-PAGE exhibited a very faint band for the monospecific molecules migrating at M_r > 100 000 or for the bispecific molecule migrating at M_r > 200 000. Further characterization of the single-chain molecules by gel exclusion chromatography showed that while the L6Fv-Ig, CD3Fv-Ig and CD3-L6FvIg molecules were present predominantly in the monomer form, the molecules did associate via the CH3 domain and/or by nonspecific interactions to form homodimers (~30%) and a small percentage (10% or less) of higher order oligomers (data not shown). Native gel electrophoresis confirmed these observations and indicated that the bispecific single chain molecule did not aggregate to the same degree as the monospecific derivatives (data not shown). If aggregation was caused by exposure of hydrophobic residues in CH2 and CH3 in the absence of CH1 and C_H , the inclusion of the hydrophilic 'helical' linker sequence and/or addition of a second variable region may have driven the bispecific molecule to fold differently from the monospecific molecules thereby decreasing its tendency to oligomerize.

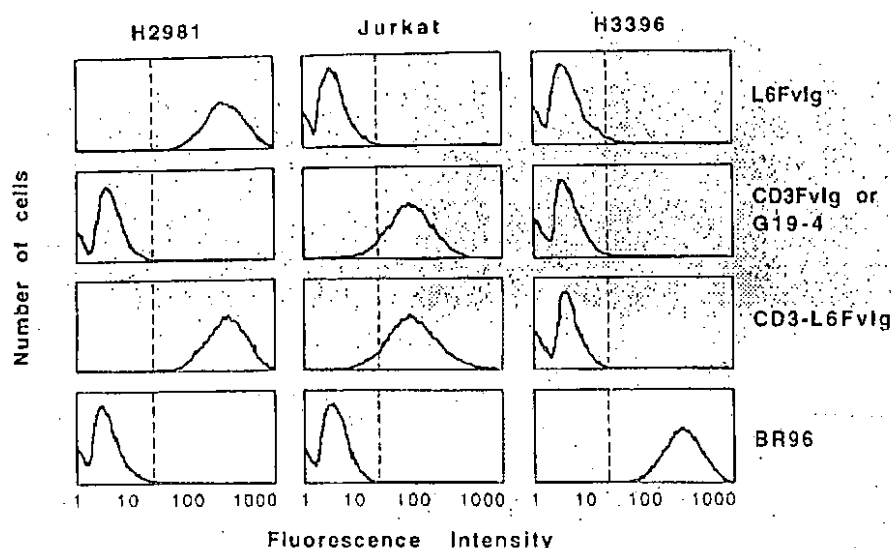


Fig. 3 L6Fv-Ig, CD3Fv-Ig and CD3-L6Fv-Ig bind to cells expressing target antigen. Antibody derivatives at $10 \mu\text{g ml}^{-1}$ were incubated with H2981 tumour cells (L6-positive), Jurkat cells (CD3-positive) or H3396 tumour cells (BR96-positive). The cells were washed and incubated with FITC-conjugated goat anti-human IgG as second step reagent. A total of 10 000 stained cells was analysed by FACS.

Binding activities of L6Fv-Ig, CD3Fv-Ig and CD3-L6Fv-Ig fusion proteins

Antigen specific binding of the single-chain Fv-Ig derivatives was tested by binding to cells expressing target antigen. The human tumour cell line H2981 expresses high levels of L6 target antigen but no CD3 or BR96 molecules, while the H3396 human tumor cell line expresses high levels of BR96 antigen (54) but no L6 or CD3, and the human Jurkat T-cell line expresses CD3 receptors, but no L6 or BR96 antigens. Binding of the single chain molecules was detected by fluorescence activated cell sorter analysis using FITC-conjugated goat anti-human Ig as second step reagent. As shown in Fig. 3, the fusion proteins bound specifically to cells expressing target antigen, similar to the reactivity of native anti-L6 mAb and G19-4 mAb binding to these cells. The CD3-L6Fv-Ig bispecific fusion protein bound to both Jurkat and H2981 cells and could be detected with either goat anti-human IgG or a FITC-conjugated anti-L6 idiotype mAb (46), an indirect indication that this derivative contained dual specificity.

The anti-L6 binding domain was fused to several Fc molecular tag sequences containing mutations at critical residues reported to interact with human Fc γ R (55,56). The expressed L6Fv-Ig proteins were then compared to chimeric L6 mAb to determine which molecules best retained the antigen binding characteristics of native antibody. Although all of these proteins were capable of Fc-receptor binding, the Fc mutant containing the Pro to Ser mutation at residue 238 in the hinge-link region failed to mediate ADCC and complement killing (data not shown), while preserving anti-L6 binding affinity at a level comparable to native L6 mAb. The mutant Fc was therefore used as the recipient for cloning other variable region cassettes.

CD3 single-chain antibodies exhibit biological activities qualitatively similar to but quantitatively distinct from native antibody

Several different CD3 fusion proteins were constructed, including an -Ig fusion, a V_L - V_H sFv (no tag), and the CD3-L6Fv-Ig bispecific molecule. Figure 4 shows the sequence of the anti-CD3 variable domains including the junction sequences for the mono- and bispecific derivatives. The Fv-Ig fusion proteins were purified by affinity chromatography using immobilized protein A and the sFv was used as filtered culture supernatant. The concentration of sFv was estimated by titrating the ability of the transfection supernatant to block the binding of known concentrations of native G19-4 mAb to Jurkat T cells.

The signalling activity of CD3Fv-Ig was compared to native G19-4 mAb in assays which measure induction of tyrosine phosphorylation on cellular proteins, including PLC γ 1. Jurkat T cells were stimulated with CD3Fv-Ig or G19-4 mAb and proteins from cell lysates were separated by SDS-PAGE, transferred to Immobilon, and probed with either anti-P-tyr or anti-PLC γ 1. Surprisingly, we found that CD3Fv-Ig induced stronger tyrosine phosphorylation of cellular proteins including PLC γ 1 in whole cell lysates and that a greater amount of the pp35/36 phosphoprotein was associated with PLC γ 1 than that observed with equivalent amounts of native G19-4 mAb (Fig. 5).

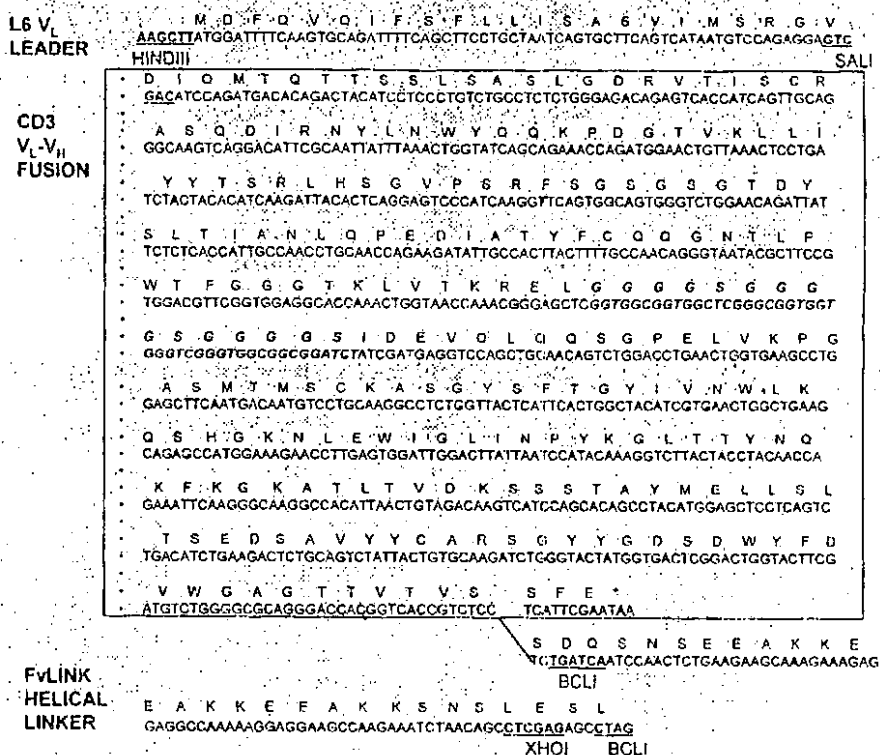
Calcium mobilization was induced by all of the CD3 single-chain antibody derivatives (Table 1). At equivalent concentrations of protein, CD3Fv-Ig induced calcium fluxes in PBL similar to native G19-4 mAb and greater than that observed with G19-4 Fab fragments. Similar results were observed when stimulations were carried out in the presence of 10-mM EGTA to chelate extracellular calcium. Interestingly, the CD3 sFv protein from transfected COS cell supernatants also mediated strong calcium fluxes in

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Fig. 4 Sequence of CD3Fv variable region gene fusion. Variable regions from the heavy and light chains of anti-CD3 mAb G19-4 were cloned by PCR. The nucleotide and protein sequence of the gene fusion constructed from these cDNA cassettes is shown with the (Gly₃Ser)₃ linker in bold typeface. The amino terminus of the V_L-V_H cassette was fused at the *Sali* site to the L6 light-chain variable region leader peptide, and the carboxy terminus was fused directly to the hinge region of the Fc domain at the *Bcl* site or to a short 'helical' peptide linker to construct the bispecific CD3-L6FvIg antibody derivative. The variable domains for anti-L6 were fused in frame to the opposite end of the 'helical' linker as shown in Fig. 1.



T cells. This suggests that aggregation mediated by the affinity tag is not a factor in early signalling events using CD3-FvIg or bispecific CD3-L6FvIg.

In proliferation assays of peripheral blood lymphocytes and purified T cells responding to stimulation with the CD3 antibody derivatives at various concentrations, we found that all of the CD3 derivatives activated T cells. In Table 2, the proliferative responses measured at antibody treatments of 1 $\mu\text{g ml}^{-1}$ of a 3-day culture are shown. A similar pattern of proliferative responses was observed when fusion proteins were used at either 0.2 or 5 $\mu\text{g ml}^{-1}$. The responses were also found to be similar but weaker in intensity if measured on day 2 or 4 following exposure to the

stimuli (data not shown). The data indicate that binding of the CD3 derivatives elicited proliferative responses in T cells similar to those observed with either native G19-4 (compared to the CD3Fv-Ig) or the Fab fragment (compared to the sFv) of the murine antibody. Proliferation of PBL induced by CD3Fv-Ig but not by CD3 sFv or the nonmitogenic CD3 mAb BC3 (57) suggests that during a 3-day culture there is an Fc-mediated component involved in CD3Fv-Ig signalling. In general, the genetically engineered molecules stimulated slightly stronger levels of proliferation from purified T cells, and slightly weaker levels from PBL. The only exception was CD3Fv-Ig acting in synergy with PMA to increase proliferative responses in purified T cells

Fig. 5 CD3Fv-Ig stimulates strong tyrosine phosphorylation and activation of PLC γ 1 in T cells, and induces the association of PLC γ 1 with pp35/36. Jurkat cells (10^7 per sample) were stimulated for 1 min or 3 min with native anti-CD3 mAb G19-4 at 5, 20 or 80 $\mu\text{g ml}^{-1}$ or with CD3Fv-Ig at 1, 5, or 20 $\mu\text{g ml}^{-1}$, as indicated. Proteins from cell lysates were immunoprecipitated with (A) rabbit anti-P-tyr or with (B) PLC γ 1 antiserum and were recovered with protein A sepharose, separated by SDS-PAGE, transferred to nitrocellulose and detected with purified rabbit anti-P-tyr and ^{125}I -labelled protein A.

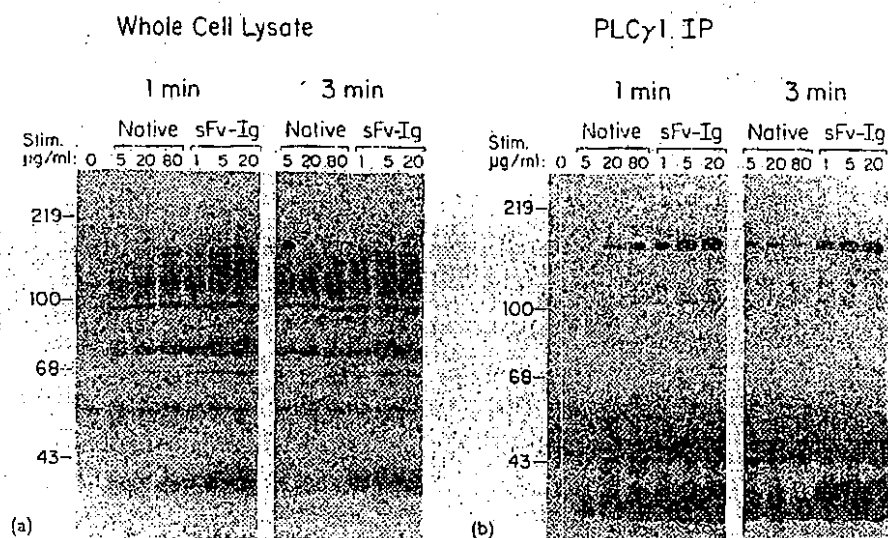


Table 1 CD3 single-chain molecules mobilize intracellular calcium in T cells. PBL were loaded with indo-1 at 2×10^7 cells ml^{-1} for 45 min to an intracellular indo-1 concentration of approximately $50 \mu\text{M}$. B cells were excluded from the analysis by staining with PE-conjugated anti-CD20 mAb 2H7 and restricting data acquisition to unstained cells. For each measurement, 1×10^6 cells were warmed to 37°C for 5 min before addition of the stimulus at concentrations indicated. Calcium fluxes were monitored for 8 min per stimulus. Measurements in parentheses were in the presence of $10\text{-}\mu\text{M}$ EGTA to chelate extracellular calcium. The intracellular calcium concentration for resting cells is 130 nM

Stimulus	Amount	Peak mean $[\text{Ca}^{2+}]_i$ (nM)	Percentage of cells responding
G19-4	1 μg	575 (190)	100 (25)
G19-4 Fab	1 μg	175 (130)	15 (0)
CD3sFv	25 μl	335 (165)	100 (20)
CD3Fv-Ig	1 μg	335 (165)	65 (15)
CD3-L6Fvlg	1 μg	185 (147)	30 (10)
L6Fv-Ig	1 μg	130 (n.d.)	0 (n.d.)
G19-4	0.2 μg	280	65
G19-4 Fab	0.2 μg	130	0
CD3sFv	5 μl	225	40
CD3Fv-Ig	0.2 μg	280	60
CD3-L6Fvlg	0.2 μg	175	25
L6Fv-Ig	0.2 μg	n.d.	n.d.

Table 2 CD3 single-chain molecules stimulate T-cell proliferation in the presence of accessory cells and are synergistic for activation of T cells in the presence of PMA or α -CD28 mAb. PBMC or purified T cells (monocytes and B cells were removed by two rounds of plastic adherence followed by passage over nylon wool columns) at 5×10^4 cells well^{-1} were cultured for 3 days in the presence of stimulus, as indicated. A second activation signal (immobilized anti-CD28 mAb 9.3 or PMA) was provided to the purified T cells. Anti-CD28 mAb was added to the wells at $2 \mu\text{g ml}^{-1}$ and incubated $> 1 \text{ h}$ at 37°C then washed extensively with PBS prior to addition of the stimuli. PMA was used at 0.5 ng ml^{-1} in solution. Stimuli were used at $1 \mu\text{g ml}^{-1}$ for all experiments except in the anti-CD28 experiments where stimuli were used at $0.2 \mu\text{g ml}^{-1}$. In addition to native CD3 mAb G19-4, other controls included a Fab fragment of G19-4, two CD3 mAb of different isotypes (OKT3, mlgG2a and BC3, mlgG2b) and non-T cell binding L6-Fvlg fusion protein. Values are for triplicate cultures (day 3, c.p.m. $\times 10^3$; SEM $< 20\%$)

Stimulus	PBMC + medium	Purified T cells + $2 \mu\text{g ml}^{-1}$ α -CD28	Purified T cells + 0.5 ng ml^{-1} PMA
Medium	2.7	0.8	6.0
G19-4	49.0	11.2	83.6
CD3Fv-Ig	55.6	9.3	112.5
G19-4 Fab	2.1	58.4	n.d.
OKT3	77.0	6.2	76.0
BC3	0.8	3.0	87.3
CD3sFv (25 μl)	3.4	55.0	65.0
L6Fv-Ig	2.6	0.5	5.7

over those observed with native G19-4. All CD3 mAb and CD3 mAb derivatives induced proliferation of purified T cells in the presence of PMA. Interestingly, in the presence of second signal delivered via CD28, the CD3sFv and G19-4 Fab fragments induced the highest levels of proliferation, suggesting that the smaller molecules may have greater access to the binding site on CD3 ϵ -chain.

CD3-L6 bispecific fusion protein mediates adhesion between Jurkat and H2981⁺ tumour cells

A cell adhesion assay (48) was used to test whether the bispecific molecule was capable of mediating adhesion between cells expressing CD3 or L6 antigens. Jurkat T cells were first incubated with anti-CD3 or with CD3-L6Fvlg. Cells were washed and added to adherent L6-positive H2981 cells that had been preincubated with or without L6Fv-Ig. Microscopic examination of the H2981 monolayers demonstrated that CD3-L6Fvlg protein mediated adhesion between Jurkat cells and H2981 cells in the presence of EDTA. Preblocking the CD3 and L6 receptors with L6 mAb or with G19-4 mAb inhibited CD3-L6Fvlg-mediated

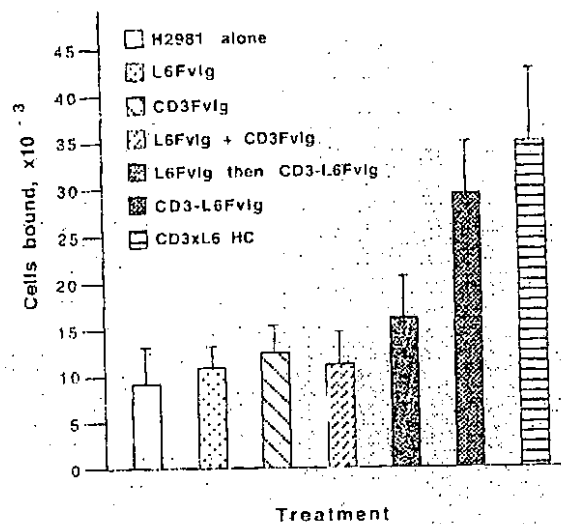


Fig. 6 CD3-L6Fvlg mediates adhesion between H2981 and Jurkat cells. Monolayers of H2981 tumour cells were plated at a density of 10^5 cells well^{-1} in 48 well plates, fixed in 0.1% paraformaldehyde for 20 min at 23°C , washed, blocked in complete RPMI + 10% FBS, and preincubated alone or with the antibody indicated for 1 h at 37°C . A heteroconjugate of G19-4 anti-CD3 mAb and anti-L6 mAb (CD3xL6 HC) was used as a positive control. All antibodies were used at $20 \mu\text{g ml}^{-1}$ except where indicated. Jurkat cells were labelled with ^{51}Cr , preincubated in 10-mM EDTA, and added to the tumour cells and antibodies. Adhesion was initiated by spinning the plates at 1000 r.p.m. for 2 min. Reactions were incubated for 45 min at 37°C , washed five times in ice-cold RPMI, and lysed in 0.5-N NaOH. Counts released were determined by gamma counting. Data represent the number of cells bound ($\times 10^3$). Mean and standard deviation (error bars) of three replicate determinations are shown.

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ated adhesion. Further assays confirmed these results. When Jurkat T cells were prelabelled with ^{51}Cr and incubated with the bispecific fusion protein and H2981 tumour cells as described above, greater binding of the Jurkat cells to the unlabelled monolayer was observed when receptors on either cell type were not preblocked by incubation with unconjugated G19-4 or L6 mAb (Fig. 6).

CD3-L6 bispecific fusion protein stimulates high levels of T-cell proliferation in vitro

We investigated whether triggering of the CD3-TCR complex by the bispecific CD3-L6Fvlg fusion protein could induce T-cell proliferation in the presence of tumour cells expressing the L6 antigen. Resting PBL were either incubated with irradiated H2981 tumour cells and the fusion protein in solution, or stimulating proteins were prebound to the irradiated tumour cells and unbound protein was removed by several washes. An irrelevant antibody

was incubated with the H2981 cells before addition of the stimuli to prevent nonspecific binding of the tumour cells to PBL. Figure 7 displays the results of both the solution and prebinding proliferation experiments. The levels of proliferation were markedly enhanced in the presence of the CD3-L6Fvlg bispecific fusion protein at $5\text{ }\mu\text{g ml}^{-1}$. Significant levels of proliferation were also observed at $0.5\text{ }\mu\text{g ml}^{-1}$ (data not shown). Prebinding L6Fv-Ig to the tumour cells before addition of the bispecific molecule eliminated T-cell proliferation. Inhibition of proliferation by engagement of CD3 with CD3Fv-Ig was more difficult to demonstrate because CD3Fv-Ig in solution stimulated significant levels of proliferation, independent of the presence or absence of the tumour cells. In the prebinding assays, excess CD3Fv-Ig was removed during the washing steps, thus eliminating proliferation due to the soluble molecules. Taken together, these results demonstrate the ability of the CD3-L6Fvlg bispecific fusion protein to stimulate T-cell proliferation in the presence of L6-positive H2981 tumour cells.

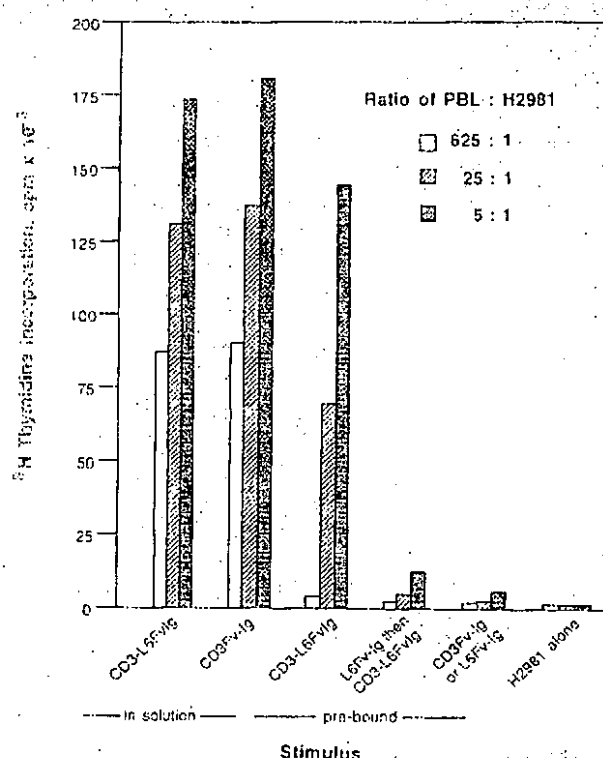


Fig. 7 CD3-L6Fvlg bispecific protein stimulates high levels of T-cell proliferation when bound to H2981 tumour cells. PBL were isolated and cultured in the presence of irradiated H2981 tumour cells and the stimuli indicated (added in solution at either 1 or $10\text{ }\mu\text{g ml}^{-1}$). For prebinding experiments, H2981 tumour cells were irradiated and incubated with $10\text{ }\mu\text{g ml}^{-1}$ antibody derivative for 1 h on ice, washed several times to remove unbound antibody, and added at varying ratios to PBL at 5×10^4 cells well $^{-1}$. After 3 days of culture, proliferation was measured by 6 hour's uptake of [^3H]thymidine. Values shown are for quadruplicate cultures for each treatment (SEM < 15%).

CD3-L6 bispecific fusion protein targets T-cell cytotoxicity to H2981 tumour cells

Resting PBL were used as effector cells to determine if naive T cells could be activated to kill H2981 tumour cells (Fig. 8). At effector to target ratios of 10:1, the CD3-L6Fvlg molecule mediated 30% specific lysis, while at ratios

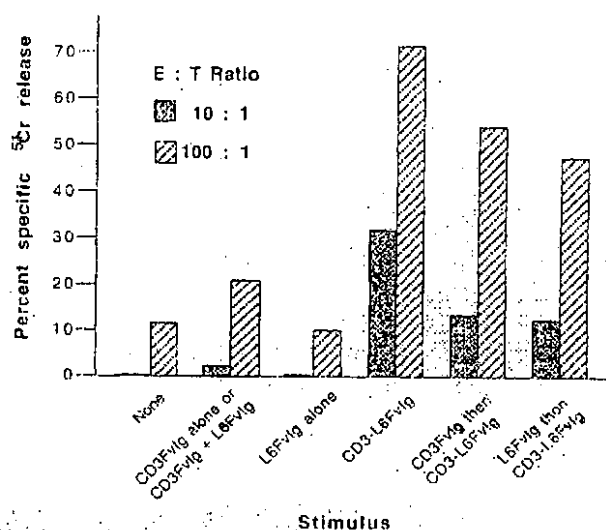


Fig. 8 CD3-L6Fvlg bispecific protein targets T-cell cytotoxicity to H2981 tumour cells. H2981 tumour cells were labelled for 2 h with ^{51}Cr and incubated with antibody stimuli ($5\text{ }\mu\text{g ml}^{-1}$) and PBL at effector to target ratios of 10:1 and 100:1. Chromium release was measured and specific lysis was tabulated for each antibody derivative from triplicate cultures (SEM < 12%). For inhibition assays, CD3Fv-Ig or L6Fv-Ig were incubated with the appropriate cell type prior to addition of the CD3-L6Fvlg bispecific molecule. Preincubation with either monospecific reagent ($10\text{ }\mu\text{g ml}^{-1}$) failed to block cytotoxicity mediated by the bispecific protein.

of 100:1, specific lysis rose to 71%. The level of specific lysis for the two monospecific molecules when mixed together, or for either molecule alone ranged from 3% to 5% at an effector to target ratio of 10:1, and 9% to 20% at an effector to target ratio of 100:1. The CD3Fv-Ig and L6Fv-Ig antibody derivatives failed to completely block the targeted cytotoxicity mediated by the CD3-L6 fusion protein in a 5-h assay where the mAb derivatives repeatedly associate and dissociate at equilibrium.

Discussion

In the present study, we describe the adaptation of an existing mammalian expression system for the rapid production, expression, and screening of single-chain monospecific and bispecific antibody derivatives. The COS cell transient expression system has been used successfully by previous investigators to express soluble cell surface receptors by creating fusion proteins between the extracellular domain of the surface receptor and the heavy chain of human IgG1 coded by genomic hinge, CH2 and CH3 sequences (36,47). As opposed to bacterial expression systems where recovery of biologically active molecules is problematic, even for molecules possessing a single binding specificity, proteins are secreted and can be easily recovered in active form from culture supernatants of transfected COS cells. In initial experiments, it was determined that COS cells were capable of expressing and secreting functional intact IgG molecules using cDNA rather than genomic sequences. Full length anti-L6 kappa and gamma cDNA cassettes were ligated separately to pCDM8 and the insertion vectors were cotransfected into COS cells by the DEAE-Dextran procedure. Culture supernatants contained 100–500 ng ml⁻¹ antibody with specific binding activity for L6-positive tumour cells, indicating that COS cells assembled and secreted native antibody from cDNA expression cassettes (data not shown). Once production of L6 antibody in COS cells was verified, we modified the expression vectors pCDM8 and pILNX for expression of single chain antibody derivatives using interchangeable cassettes containing DNA encoding individual protein domains. This allowed us to generate novel combinations, make rapid exchanges, and screen different domains for their efficacy in performing a desired function. Only those molecules exhibiting the desired subset of characteristics from this screening would require shuttling into a secondary expression system for large-scale production, eliminating lengthy or complicated production and isolation procedures for the majority of molecules generated.

The modified expression vectors were tested by insertion and expression of the variable domains for two different antibody binding specificities, the human L6 tumour antigen and human CD3-E. The single chain antibody derivatives bound antigen with varying avidities and affinities, depending on the molecular tag to which they were fused. The Fc domain of human IgG1 was the best tag for repro-

ducing the binding characteristics of the native antibody, despite the absence of CH1 and Cκ domains. Several other tags were constructed and tested, but the Cκ (34) and the FLAG peptide (International Biotechnologies, Inc., New Haven, CT) both failed to function reliably (data not shown). The peptide RKSIRIQRGPGRAFVTIGKI from the V3 loop of gp110 encoded by human immunodeficiency virus was also used as an affinity tail, recognized by the peptide-specific mAb 110.3. This tag gave variable results depending on the Fv to which it was fused, failing to function properly when fused to L6 Fv, but performing successfully when fused to CD3 Fv (data not shown). Two other peptides to which we have specific mAb are currently being tested as possible replacements for the Fc domain affinity tail.

Our initial goal was to find an affinity tag that could be used with any variable region cassette such that rapid screening of the transfection supernatants was possible. The Fc portion of human IgG1 was a good candidate for this purpose, however, we wanted a monomeric tag that did not mediate Fc-related effector functions (58) which could potentially mask activity mediated by specific variable region binding. Therefore we produced several mutant forms of the hinge-CH2-CH3 cassette that had various combinations of mutations in the hinge (Cys to Ser) and CH2 domains (55,56). We found that most of the Fc mutants retained binding characteristics similar to native L6 mAb and to control anti-L6 WTD (M. S. Hayden, unpublished data). We chose the mutant containing the single point mutation at residue 238 (Pro to Ser) for further variable domain insertions because it failed to mediate both ADCC and complement killing in standard assays, while preserving antigen binding. This mutant also had Cys to Ser mutations in the hinge which prevented disulfide bond formation in this region. Despite the absence of hinge disulfides, we found that a proportion of the proteins did form dimers or higher order oligomers, presumably through interactions of the CH3 domains. However, the bispecific molecule appeared to be less able to oligomerize, perhaps due in part to the presence of the hydrophilic helical linker. We also noted that the affinity tail did appear to interact with FcR over a time frame longer than the five hour chromium release assay. In three day proliferation assays, CD3Fv-Ig induced activation of PBMC, whereas CD3 sFv without a tail and the nonmitogenic BC3 mAb did not induce this response. We do not know if the FcR interaction detected here is capable of mediating ADCC at these later time points. Further experiments aimed at resolving these questions are currently underway.

Anti-L6 and anti-CD3 single-chain derivatives lacking Fc tags may be useful in therapy. For example, L6sFv could be chemically attached to a radionuclide or genetically fused to a toxin such as PE40. Initial studies of an IL2R-PE40 fusion protein have shown specific toxicity towards IL2-receptor-bearing cells *in vivo* (29). However, the L6 antigen is expressed at low levels on some normal cells including the

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vascular endothelium of the lung (59). Because smaller sFv fragments are better able to penetrate tumor mass and have shown improved localization at tumour sites (5,6), smaller doses of L6 sFv may be effective in targeting therapeutic agents to tumor cells that express high levels of L6 antigen. CD3 sFv may be well suited for induction of immunosuppression *in vivo* since delivery of a T-cell receptor signal in the absence of a second signal such as ligation of CD28 can lead to T-cell anergy. It has been demonstrated that F(ab')₂ fragments of OKT3 are immunosuppressive while markedly reducing the cytokine toxicity associated with whole antibody OKT3-induced immunosuppression (60).

Interestingly, the CD3 single-chain monospecific antibody derivatives exhibited some functional properties distinct from native antibody. The CD3Fv-Ig derivative potently induced tyrosine phosphorylation of PLC γ 1 and increased the association of PLC γ 1 with pp35/36 in comparison to native anti-CD3 mAb. This association has been shown to increase as T cells are maximally stimulated (51, 61). Additionally, stimulation of T cells with CD3Fv-Ig resulted in greater overall tyrosine phosphorylation of cellular proteins following activation. It is possible that because the molecules are smaller than native mAb, the binding site on CD3-E is more accessible and improves the interaction with TCR/CD3-associated tyrosine kinases by an unknown mechanism. This hypothesis may explain the ability of CD3sFv and CD3Fv-Ig to induce strong calcium responses in T cells. These stimuli were repeatedly stronger than equivalent concentrations of G19-4 Fab fragments and sometimes stronger than native G19-4 mAb in calcium fluxing experiments. Further studies are being carried out to identify the mechanism by which the single-chain derivatives elicit potent early T-cell activation signals.

In addition to single chain monospecific antibody derivatives, bispecific single-chain molecules may also be useful in the treatment of human disease. In animal models and in clinical trials in humans, heteroconjugated or bispecific mAb direct the activity of cytotoxic T lymphocytes or of lymphocyte activated killer cells to kill malignant (7-10, 12) or virally infected target cells (62-64). Although anti-CD3 mAb can induce potent cytokine toxicity *in vivo* (65), patients treated with bispecific mAb have not developed cytokine toxicity because their T cells were pretreated with the reagent *in vitro* and then readministered (20,25). In murine models, molecules smaller than native mAb such as F(ab')₂ fragments show increased tumor localization for both monospecific antitumour and for bispecific anti-CD3 plus antitumour specificities (22,23).

The bispecific CD3-L6Fvlg molecule is potentially useful for human cancer therapy. The molecule contains binding specificity for both CD3 and the tumor antigen L6, and has been shown *in vitro* to induce T-cell proliferation in the presence of L6-positive tumour cells, and to direct CTL killing toward these cells. It is unclear whether the bispecific molecule would be better suited for *in-vivo* applications with or without an Fc tail. Although the Ig portion served

simply as an affinity tail in these studies, it could also increase the half-life of the bispecific protein *in vivo*. In one report, a chimeric mouse anticolorectal cancer mAb variable region fused to human IgG1 constant region (66) administered to 10 patients with metastatic colon cancer showed a six-fold increase in circulation time (4). The size of the bispecific CD3-Lvlg fusion protein is approximately two-thirds the size of native IgG. Since the tag is of human origin, the overall immunogenicity of the molecule would be less than whole murine IgG. The protein could be engineered to decrease further the immunogenicity by humanizing the framework regions and constructing the solvent-accessible portion of the helical linker to resemble helices from known human proteins. Additional modifications may be necessary to ensure that the Fc portion does not mediate Fc-related effector functions.

We are currently constructing a variant of the bispecific CD3-L6Fv molecule that has a short (10-amino-acid) peptide tail replacing the human Ig tail. Protein obtained from COS cell supernatants will be purified on affinity columns using mAb specific for that peptide and we will then be able to assess the activity of even smaller bispecific molecules consisting essentially of the two sFv connected by the short 'helical' linker. Finally, although the bispecific molecule is expressed from transient transfection of COS cells, the protein may be potentially expressed in a stable transfection system. Stable transfectants for the single-chain monospecific molecules have been generated and attempts to express stably transfected bispecific CD3-L6Fvlg are currently underway.

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