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Chimeric (Mouse/Human) Anti-Colon Cancer Antibody c30.6 Inhibits the Growth of Human Colorectal Cancer Xenografts in *scid/scid* Mice¹

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ABSTRACT

The mouse monoclonal antibody, m30.6 (IgG2b), detects an antigenic determinant expressed predominantly on the surface of colorectal adenocarcinoma cells and has been shown previously to be a potentially useful therapeutic and diagnostic reagent for human colon cancer. We report the production and characterization of a mouse/human chimeric antibody, c30.6, with potent *in vitro* and *in vivo* antitumor activity. The genes encoding the variable domains for heavy and light chains were amplified by thermal cycling using degenerate oligonucleotide primers complementary to conserved immunoglobulin framework sequences. The gene segments were sequenced, subcloned into eukaryotic expression vectors containing human constant region genes (IgG1 and κ), and cotransfected into nonsecreting Sp2/0 mouse myeloma cells. There were significant differences in the biological activities of the murine and chimeric antibodies. The i.p. administration of c30.6 but not of m30.6 produced a marked growth inhibition of s.c. 30.6⁺ COLO 205 tumors in *scid/scid* mice (~40% reduction in tumor size, measured 21 days after tumor inoculation). Reduced tumor growth was not due to altered binding characteristics of c30.6 because: (a) the chimeric antibody was shown by flow cytometry to bind exclusively to cell lines that expressed the 30.6 determinant; (b) c30.6 was able to completely inhibit the binding of m30.6 on 30.6⁺ cells; and (c) the affinity of binding of the two antibodies was the same (K_{a} , $\sim 1.50 \times 10^8$). Up to 15% of the total injected antibody dose/g tissue was localized in 30.6⁺ tumors at 24 h, ~13% was present in the tumors at 48 h, and ~10% was present at 72 h. Furthermore, c30.6 demonstrated a shorter circulating half-life (53 h; m30.6, 72 h) when given i.p. to C57BL/6 $\times$ BALB/c F₁ mice. Unlike m30.6, c30.6 was also strongly active in antibody-dependent cell-mediated cytotoxicity against a range of 30.6⁺ tumor target cells *in vitro*. Up to 80% specific ⁵¹Cr release was achieved using either freshly isolated human peripheral blood mononuclear cells or 2-day-old interleukin 2-stimulated human peripheral blood mononuclear cells as effectors. The enhanced antitumor activity of c30.6 suggests that it might be a useful immunotherapeutic reagent for colorectal carcinoma.

INTRODUCTION

CRC³ is among the most common causes of cancer death in many Western countries. Presently available options for combating the high mortality and the morbidity associated with CRC are inadequate, and only those cases diagnosed at an early stage (tumor confined to the wall of the colon) are likely to be cured by surgical resection. Once a tumor has spread beyond the colonic wall, the prognosis is much poorer (1, 2), albeit that treatment with the cytotoxic agents 5-fluorouracil and levamisole in an adjuvant setting has been

demonstrated to afford some prolongation of survival and some increase in the cure rate in this group (3). Unfortunately, there is no reliable screening test for early CRC.

One strategy subject to much investigation has been the use of mAbs directed against CRC-associated antigens. Apart from their usefulness as the basis for screening tests and in diagnostic immunoscintigraphy, mAbs are potentially useful therapeutic reagents that are occasionally effective when used alone (4), or they can be used to deliver toxins (5), drugs (6), or radioactive isotopes (7) to tumors. Until recently, mAbs have almost invariably been of rodent origin, resulting in a HAMA response in patients, which accelerates the clearance of the mAb and precludes its repeated administration due to the risk of hypersensitivity reactions. To overcome this problem, recombinant DNA technology has been used to produce chimeric Abs combining the variable domains of the parental mouse antibodies and the human constant domain (8-11). Alternatively, CDR-grafted antibodies have used minimal antigen-binding domains to further reduce the mouse sequences in the recombinant product and to more faithfully mimic the original 3-dimensional antigen-binding domains (12). As well as reducing immunogenicity, these approaches enable the acquisition of improved effector functions, including antigen-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity.

The mouse mAb designated 30.6 (m30.6) (13) recognizes an antigen that is expressed on a high proportion of CRCs and has shown diagnostic and therapeutic potential. Immunoscintigraphy has shown that it is capable of localizing to primary and secondary tumor deposits in humans (14, 15). Furthermore, ¹³¹I-radioimmunoconjugates (16) and methotrexate (17) immunoconjugates of 30.6 produced regression of human CRC xenografts in nude mice. However, in a phase I clinical trial, administration of m30.6 provoked a HAMA response that precluded the possibility of its repeated administration (18). We now present the production and characterization of a chimeric mAb, c30.6. The specificity and affinity of c30.6 are identical to those of m30.6, and its ability to inhibit the growth of 30.6⁺ tumors in *scid/scid* (scid) mice demonstrates that this chimeric antibody is a good candidate for further development as a therapeutic reagent for CRC.

MATERIALS AND METHODS

Mice. Studies involving mice were carried out at the Biological Research Laboratory, Austin Research Institute. mAbs were purified from ascites fluid derived from CBA $\times$ BALB/c F₁ mice (m30.6) or BALB/c scid mice (c30.6) bearing i.p. tumors. m30.6 and c30.6 were purified by affinity chromatography on protein A-Sepharose (Pharmacia).

Cells and Cell Lines. The human CRC cell lines COLO 205, HT 29, LIM 1899, and LOVO and the human leukemia cell lines Sultan, Daudi, and K562 were maintained in RPMI supplemented with 10% (v/v) fetal calf serum (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 units/ml penicillin (Commonwealth Serum Laboratories), and 100 μ g/ml streptomycin (Glaxo, Melbourne, Australia). PBMC were isolated from healthy donors by centrifugation through Ficoll-Paque (Pharmacia, Piscataway, NJ) and resuspended in medium supplemented with 100 units/ml recombinant human IL-2, a kind gift of Dr. Mark Smyth, Biological Response Modifiers Program, National Cancer Institute, Frederick, MD.

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³ The abbreviations used are: CRC, colorectal carcinoma; ADCC, antibody-dependent cell-mediated cytotoxicity; V_H, immunoglobulin heavy chain variable domain; V_L/V_K, immunoglobulin lambda/kappa chain variable domain; HAMA, human anti-mouse antibody; CDR, complementarity-determining region; PBMC, peripheral blood mononuclear cells; E/T, effector/target; FITC, fluorescein isothiocyanate; scid, *scid/scid*; mAb, monoclonal antibody; PBS, phosphate-buffered saline; IL-2, interleukin 2.

Isolation of 30.6 Variable Region Genes. Thermal cycling was used to isolate mouse variable region gene segments. Cytoplasmic RNA was extracted from m30.6 hybridoma cells as described (19). Complementary DNA was synthesized from RNA by reverse transcription with mouse Moloney virus reverse transcriptase (BRL, Gaithersburg, MD) using random primers. The oligonucleotide primers used for thermal cycling were based on those published by Orlandi *et al.* (20) (Table 1). Reactions for the amplification of V_H genes used the primers VH1FOR2, VH1BACK1, VH1BACK2, VH1BACK3, and VH1BACK4. For amplification of V_K gene segments the primers used were VK1FOR and VK1BACK. The reaction products were analyzed by electrophoresis through 2% (w/v) agarose gels, purified on DEAE-cellulose (NA-45 paper; Schleicher and Schuell, Keene, NH), and cloned into *Sma*I-digested pBluescript (Stratagene, La Jolla, CA). Nucleotide sequencing was performed by the dideoxy method on double-stranded plasmid templates of four (V_H) or five (V_K) independent clones using Sequenase (United States Biochemical, Cleveland, OH).

Construction of Chimeric Antibody Expression Vectors. A modification of the method used by Orlandi *et al.* (20) was used. The vectors KS.M. V_H and KS.P.MV K were constructed by substituting the pBluescript vector backbone for M13 in the original vectors, M13-VHPCR1 and M13-VKPCR1. Initially, V_H and V_K thermal cycling products were subcloned as blunt-ended fragments into pBluescript KS+. The presence of an internal *Pst*I site necessitated that the V_H segment be cloned into KS.M. V_H in two stages: (a) a 63-base pair 3' *Pst*I-*Bst*EII fragment was force-cloned into the vector, and then the remaining 262-base pair *Pst*I fragment was inserted in the correct orientation into the *Pst*I site. The V_H expression cassette, comprising the immunoglobulin promoter, leader sequence, leader intron, and 3' noncoding sequences, was recovered by digestion with *Hind*III and *Bam*HI and subcloned into the eukaryotic expression vector pSV.gpt, containing a human IgG1 constant-region gene. V_K gene sequences were removed from pBluescript by digestion with *Bgl*II and *Pvu*II and force-cloned into KS.P.MV K . The V_K expression cassette was then released with *Hind*III and *Bam*HI and subcloned into the expression vector pSV-hyg, which contains the human K-chain constant domain gene.

Transfection of Sp2/0 Cells. Plasmid DNA (20 μ g) was linearized with *Pvu*I, reprecipitated, and introduced by electroporation into Sp2/0 cells. Cells were plated into 96-well tissue culture plates with 2.5×10^4 thymocyte feeder cells/well. Selection in medium supplemented with hygromycin B (400 μ g/ml; Sigma Chemical Company, St. Louis, MO) was commenced on the next day. Wells containing transfectant colonies were screened for secretion of human immunoglobulin at 12–14 days using an enzyme-linked immunosorbent assay. Flexible polyvinyl chloride plates were coated with sheep anti-human immunoglobulin (50 μ g/ml; Silenus, Hawthorn, Australia) and nonspecific binding sites were blocked with 2% bovine serum albumin in PBS. Hybridoma culture supernatant (100 μ l) was added and incubated at 37°C for 1 h. Bound human immunoglobulin was detected with sheep anti-human immunoglobulin coupled to horseradish peroxidase (Amersham International, Sydney, Australia). The color reaction was developed using 50 μ l of 0.03% [2,2'-azino-di-(3-ethylbenzothiazoline)sulfonate] (Amersham) and read with an enzyme-linked immunosorbent assay plate reader at 405 nm. Wells expressing human immunoglobulin were cloned by limiting dilution, and the resultant clones were retested, expanded, and cryopreserved in liquid N₂.

Flow Cytometry. Cells (50 μ l at 5×10^6 cells/ml) were washed three times in PBS and incubated with antibodies on ice for 60 min. After three washings in 0.5% PBS-0.1% bovine serum albumin-sodium azide, bound

immunoglobulin was detected with rabbit anti-immunoglobulin (human or mouse) conjugated to FITC (ICN Immunobiologicals, Sydney, Australia). Fluorescence was measured by flow cytometry using a FACScan analyzer (Becton Dickinson, Mountain View, CA).

Radiolodination. c30.6 (100 μ g) was radiolabeled with ¹²⁵I using chloramine-T (21) (2 min at 4°C). Free iodine was separated from radiolabeled protein by chromatography on Sephadex G-25 (PD-10 columns; Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions of the initial (antibody) peak were easily distinguished from free ¹²⁵I denoted by a discrete second peak of radioactivity. These fractions were pooled and stored at 4°C until used. Binding affinity was determined by Scatchard analysis (22).

Competitive Binding Assay. c30.6 antibody was labeled with FITC (23). COLO 205 cells (3×10^5) were incubated with FITC-c30.6 (1.74 μ g; molar incorporation of 1.5) and various concentrations of either c30.6 or m30.6 for 1 h in PBS-2% fetal calf serum. Cells were then washed three times and fluorescence was measured on a FACScan (Becton Dickinson).

ADCC. Target cells (2×10^6) were labeled with 100 μ Ci of sodium [⁵¹Cr]chromate for 60 min at 37°C in 100 μ l of RPMI supplemented as above. The target cells were washed and incubated with antibody (25 or 5 μ g/ml) at 5×10^5 cells/ml. Effector cells were either freshly isolated human PBMC or PBMC-incubated for 36 h in medium supplemented with 100 units/ml recombinant human IL-2. Target and effector cells were mixed at various E/T ratios and incubated at 37°C for 4 h in U-bottomed wells. All assays were performed in triplicate. The percentage lysis was calculated as

$$\% \text{ of lysis} = 100 \times \frac{\text{test lysis} - \text{spontaneous lysis}}{\text{total lysis} - \text{spontaneous lysis}}$$

The spontaneous ⁵¹Cr release was calculated from target cells incubated alone, and the maximum ⁵¹Cr release was calculated from target cells incubated in medium containing 1.0% sodium dodecyl sulfate.

Biodistribution Studies. The biodistribution of ¹²⁵I-labeled mAb was analyzed in a mouse tumor model established by injecting the human COLO 205 cell line into BALB/c-scid mice. Mice were given 5×10^6 COLO 205 cells (in 0.2 ml PBS) s.c. on the anterior abdominal wall. Five days later, groups of four mice received c30.6 (5×10^5 cpm) i.v. via the tail vein. The specific activity of c30.6 was 2.99×10^5 cpm/ μ g and the immunoreactivity was 65–70%. Groups of mice were sacrificed at 24, 48, and 72 h. Samples of blood, tumor, heart, liver, spleen, lung, kidney, stomach, intestine, muscle, and skin were weighed and the radioactivity was measured in a gamma counter. Results were expressed as the percentage of injected dose/g tissue $\pm$ SE.

Inhibition of Tumor Growth in Vivo. 30.6⁺ COLO 205 tumors were established as s.c. tumors in groups of six scid mice as described above. Five days later, when tumors measured 0.3–0.45 cm², mice were treated with either c30.6 or m30.6. Control groups received PBS. Four doses of 250 μ g/dose were given by i.p. injection on days 5, 7, and 9 after tumor inoculation. Tumor size was measured daily on perpendicular axes and recorded as mean tumor size (cm²) $\pm$ SE.

RESULTS

Isolation of 30.6 V_H and V_K Gene Segments. The gene segments encoding V_H and V_K regions were amplified by thermal cycling using complementary DNA derived from the m30.6 hybridoma and gene-

Table 1 Nucleotide sequences of the synthetic oligonucleotide primers used in thermal cycling amplification^a

5' end	3' end
V_H	
VH1BACK 1	VH1FOR2
5' AGGT(C/G)AA(A/G)CTGCAG(C/G)AGTCAGG 3'	5' TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC 3'
VH1BACK2	
5' AGGT(C/G)CA(A/G)CTGCAG(C/G)AGTCAGG 3'	
VH1BACK3	
5' AGGT(C/G)CA(A/G)CTGCAG(C/G)AGTCTGG 3'	
VH1BACK4	
5' AGGT(C/G)AA(A/G)CTGCAG(C/G)ACTCTGG 3'	
V_K	
5' GACATTGACCTGACCCAGTCTCC 3'	5' GTTAGATCTCCAGCTTGGTCCC 3'

^a Restriction enzyme sites were introduced as indicated (underlined): 5' CTGCAG 3' (*Pst*I); GGTGACC (*Bst*EII); CAGCTG (*Pvu*II); GATC, (*Bgl*II).

specific primers. Agarose gel analysis of the amplified products indicated gene fragments of the predicted sizes, *i.e.*, ~350 base pairs for V_H and ~330 base pairs for V_K . Nucleotide sequencing of multiple clones confirmed the isolation of V_H and V_K sequences (Fig. 1). The predicted amino acid sequence of a representative V_H clone (Fig. 1A) was compared with sequences compiled by Kabat *et al.* (24), and it was demonstrated that the 30.6 V_H gene belonged to the subgroup IIA family. Invariant Cys residues at positions 22 and 92 that formed an intrachain disulfide bond were conserved. It was predicted that a single amino acid encoded by the consensus oligonucleotide primers would be different near the amino terminal of the

wild-type 30.6 heavy chain. Glutamine, not glutamic acid, is an invariant residue at position 6 in this subclass of IgG (24). An additional amino acid, asparagine (position 94A in Fig. 1A), was also noted. The V_H clone was found to encode an internal *Pst*I site, which necessitated subcloning into the subsequent expression plasmid in two stages (see "Materials and Methods"). Of five putative 30.6 V_K clones sequenced, one was identical to the defective NS-1 K chain (25) (data not shown). The remaining four clones encoded a novel sequence that was presumed to code for the 30.6 V_K region (Fig. 1B). Comparison with the other mouse V_K sequences demonstrated that 30.6 is a member of the Group I V_K family (24). Once again, invariant Cys residues at positions 23 and 88 were conserved. Two amino acids encoded by the consensus oligonucleotide primers are likely to differ from wild-type 30.6. Valine, not glutamine, is virtually invariant at position 3 in class I V_K chains, and methionine is found in 95% of cases at position 4 (not leucine, which is present in only 4% of sequenced light chains). Additionally, proline at position 95 in CDR3 was absent in 30.6. This site is close to the site for *V-J* recombination, and the deletion may have arisen by in-frame truncation of the *V*-region gene. Importantly, this region was not encoded by the 3' primer used for thermal cycling.

Production of c30.6. The 30.6 V_H and V_K gene segments were used to construct chimeric antibody expression vectors, pSV.VH30.6 and pSV.VK30.6. Plasmid DNA was cotransfected into the non-immunoglobulin-producing mouse hybridoma cell line, Sp2/0, and clones incorporating plasmid DNA were selected in medium supplemented with hygromycin B. Eight clones were found to secrete human immunoglobulin into the culture supernatant on the basis of binding to an anti-human immunoglobulin reagent in an enzyme-linked immunosorbent assay (data not shown). Specific binding to 30.6 antigen was demonstrated by flow cytometry using 30.6⁺ cell lines. The analysis of chimeric immunoglobulin from a representative clone is shown (Fig. 2). c30.6 bound strongly to each of the CRC cell lines COLO 205, HT29, LIM 1899, and LOVO, all of which express 30.6 antigen. The profiles observed were virtually superimposable with those obtained using m30.6. By comparison, neither c30.6 nor m30.6 bound the leukemic cell lines Daudi, Sultan, and K562 (data not shown).

Affinity. Minimal difference was found between the mouse and chimeric antibodies in their ability to bind 30.6. The affinity of binding of c30.6 and m30.6 was measured by Scatchard analysis on 30.6⁺ tumor cell lines and was identical for the two antibodies ($K_a = 1.5 \times 10^8$). In the course of these studies it was noted that a considerable fraction of m30.6 was inactivated as a result of iodination, resulting in an immunoreactive fraction of only ~10%. Therefore, the relative affinities of m30.6 and c30.6 were also compared using a competitive binding assay (Fig. 3). FITC-c30.6 was allowed to bind to 30.6 antigen in competition with varying concentrations of unlabeled c30.6 and m30.6. Fifty % inhibition of maximum binding of FITC-c30.6 was obtained with 0.59 μ g c30.6 and 1.44 μ g m30.6. Therefore, in this assay c30.6 had an apparent affinity ~2.4 times that of m30.6. This apparent mild increase in affinity of the chimeric antibody is unlikely to be functionally significant.

Antibody-dependent Cell-mediated Cytotoxicity. The c30.6 antibody was able to mediate significant lysis of ⁵¹Cr-labeled target cells (COLO 205, HT29, LIM 1899, and LOVO) using human PBMC as effector cells in an ADCC assay (Fig. 4A). Lysis (28–42% specific ⁵¹Cr release) of each of the cell lines was seen at an E/T ratio of 100/1 in the presence of 25 μ g/ml c30.6, and reduced lysis was evident over a range of E/T ratios down to 10/1. Significant lysis was not seen when the antibody was deleted from the assay. The lysis of COLO 205 by PBMC in the presence of c30.6 (25 μ g/ml) was then compared to that with m30.6 or with an isotype-matched control

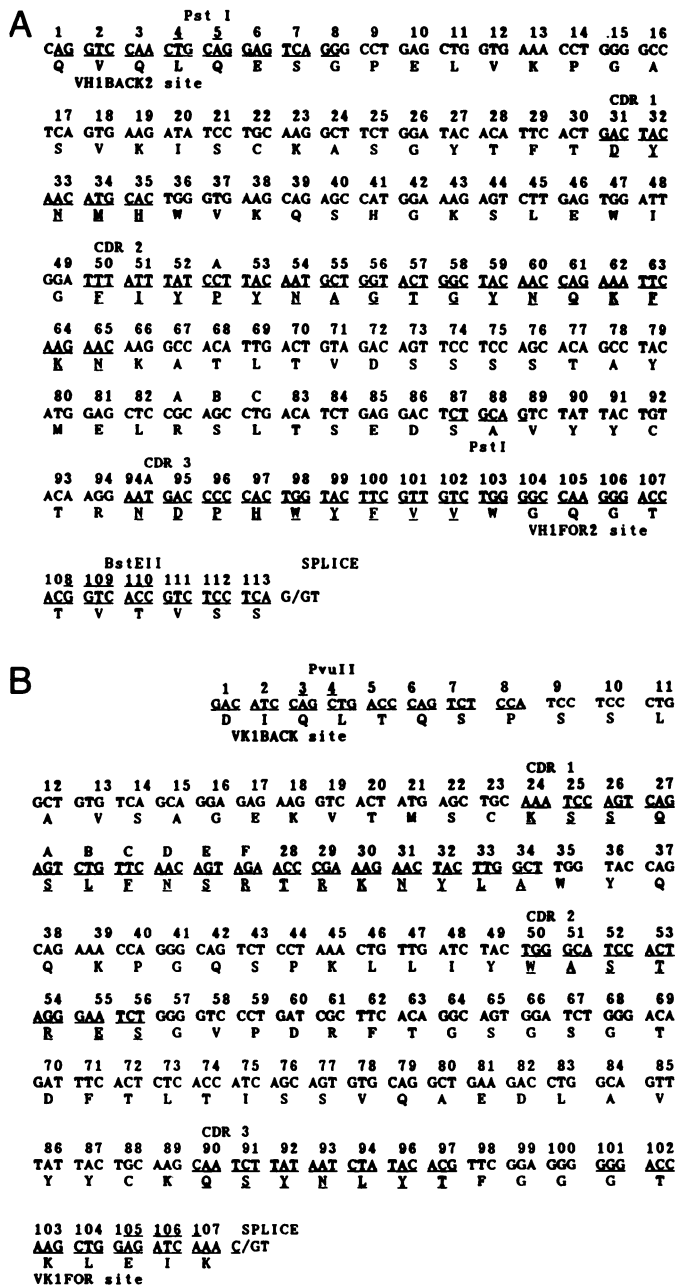


Fig. 1. Nucleotide and predicted amino acid sequences of V_H 30.6 (A) and V_K 30.6 (B). Oligonucleotide primers used in thermal cycling reactions (see also Table 1), restriction endonuclease sites used for cloning, and the predicted complementarity-determining regions of the putative heavy and light chains (underlined). Amino acid numbering and mapping of the complementarity-determining regions are predicted from the Kabat database (24). An additional *Pst*I site which necessitated a two-step subcloning strategy for the V_H clone is underlined at amino acid residues 87 to 89. "Splice" indicates the intron/exon boundary for both the heavy and light chain expression vectors.

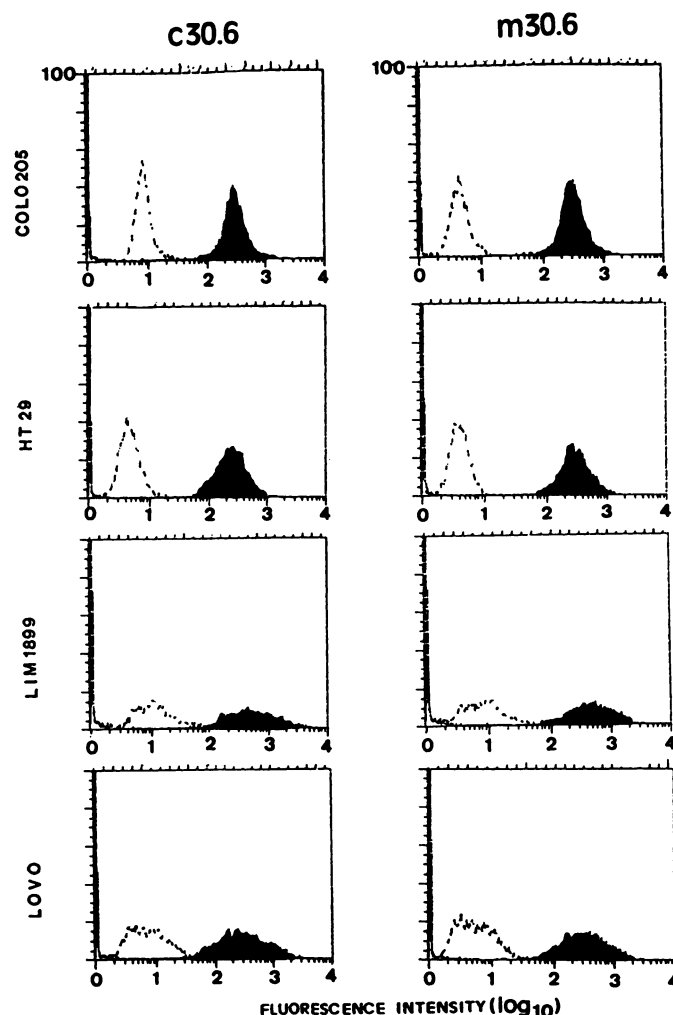


Fig. 2. Binding of c30.6 and m30.6 to human CRC cell lines. Analysis was performed on a FACScan cytofluorograph (Becton Dickinson) using purified antibodies at 25 $\mu\text{g}/\text{ml}$. Shaded traces, the binding of c30.6 and m30.6; dashed traces, negative controls: for c30.6, an isotype-matched chimeric mAb, cBC2, was used; and for m30.6, cells were preincubated in PBS instead of antibody prior to addition of fluoresceinated anti-mouse conjugate. Ordinate, relative cell numbers of each of the cell lines indicated. The human leukemic cell lines Daudi, Sultan, and K562 showed no binding of either c30.6 or m30.6 (data not shown).

chimeric antibody, cBC2 (Fig. 4B). Approximately 58% specific lysis was observed using c30.6, whereas <20% lysis was seen with m30.6 and <10% was seen with cBC2 at an E/T ratio of 100/1. This high level of lytic activity with c30.6 was maintained when lower E/T ratios were examined; however, m30.6 and cBC2 produced only background levels of lysis (<10%). When effector lymphocytes were prestimulated with IL-2 for 36 h prior to use, an increase in ADCC was noted with both 30.6 mAbs. However, this was accompanied by higher background lysis with cBC2 (Fig. 4C) and probably reflected the recruitment of antitumor lymphokine-activated killer cells.

Biodistribution of c30.6 in scid Mice Bearing COLO 205 Tumors. Initially, the circulating half-lives ($t_{1/2}$) of ^{125}I -labeled c30.6 and m30.6 were determined following i.p. administration to C57BL/6 $\times$ BALB/c F₁ mice. The $t_{1/2}\alpha$ of c30.6 was 1.01 h and that of m30.6 was 1.74 h. The $t_{1/2}\beta$ of c30.6 was 42.6 h and that of m30.6 was 135.3 h. The shorter circulating half-life of the chimeric antibody was in keeping with the findings for other mouse and chimeric antibodies (26).

^{125}I -labeled c30.6 was administered to groups of four COLO 205 tumor-bearing mice. Mice were sacrificed and tissues were counted

for radioactivity after 24, 48, or 72 h (Fig. 5A). The accumulation of c30.6 in the tumors was 15%, 13%, and 10% injected dose/g at 24, 48, and 72 h, respectively. Significant accumulation of ^{125}I other than in the tumor was seen only in the spleen. Uptake in the spleen was 16%, 13%, and 6% injected dose/g, respectively, at the same time points. Splenic accumulation was therefore higher than it was in the tumor during the first 24 h after administration but fell to near background levels by 72 h in parallel with blood levels. Because this splenic accumulation may have been due to scavenging of denatured or aggregated antibody by reticuloendothelial cells or to micrometastases, biodistribution of ^{125}I -labeled 30.6 was studied in groups of four COLO 205 tumor-bearing scid, non-tumor-bearing scid, and C57BL/6 $\times$ BALB/c F₁ mice 24 h after administration (Fig. 5B). The accumulation of ^{125}I -labeled c30.6 in scid and tumor-bearing scid was similar with 35% and 37% injected dose/g present at 24 h, respectively. This level of splenic accumulation was somewhat higher than that seen in the previous experiment (Fig. 5A). The scid mice used in the second experiment had, on the average, slightly larger spleens than those used in the first experiment, and this may have accounted for the increase in nonspecific accumulation. By contrast, the spleens of C57BL/6 $\times$ BALB/c F₁ mice (much larger than those of scid mice) accumulated only a 4% injected dose/g over the same time, and ^{125}I -labeled c30.6 accumulation in the tumor was similar in the two experiments. Accumulation in the spleen was therefore not dependent on the presence of tumor cells or due to antibody denaturation and was related to the scid phenotype in general.

Inhibition of Tumor Growth *In Vivo* by c30.6. The antitumor activity of m30.6 and c30.6 was determined in scid mice bearing COLO 205 tumors. Groups of six scid mice were given c30.6 (250 μg i.p.), m30.6 (250 μg i.p.), or PBS on days 5, 7, and 9 after tumor implantation. The tumor size was measured daily, and at the conclusion of the experiment (day 21) the mean tumor size of the c30.6-treated group was reduced 40% by comparison with the control group tumor size ($P < 0.02$, by Student's *t* test; Fig. 6). In contrast to c30.6, administration of m30.6 had no effect on tumor size.

DISCUSSION

The antigenic determinant detected by mAb 30.6 is expressed on the cell surface of the vast majority of colorectal adenocarcinomas

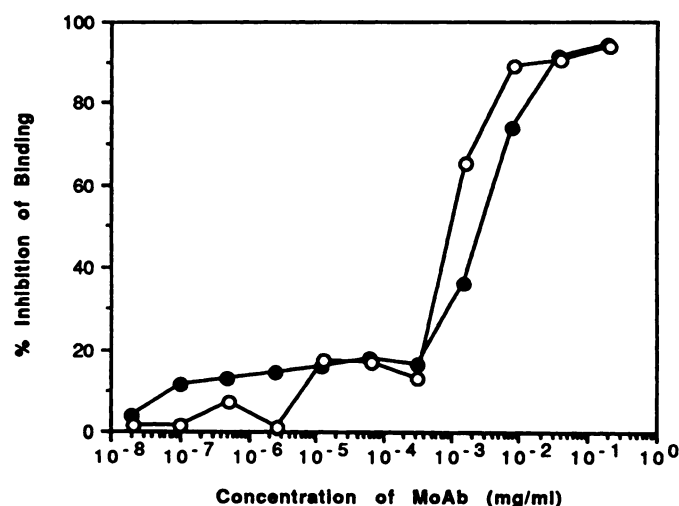


Fig. 3. Competitive binding assay of c30.6 and m30.6 on the 30.6⁺ cell line COLO 205. COLO 205 cells (3×10^5) were incubated with FITC-c30.6 and various concentrations of either c30.6 (●) or m30.6 (○). The percentage inhibition of binding was calculated relative to the FITC binding observed in the absence of competing antibody. The experiment depicted was representative of three that were performed. MoAb, monoclonal antibody.

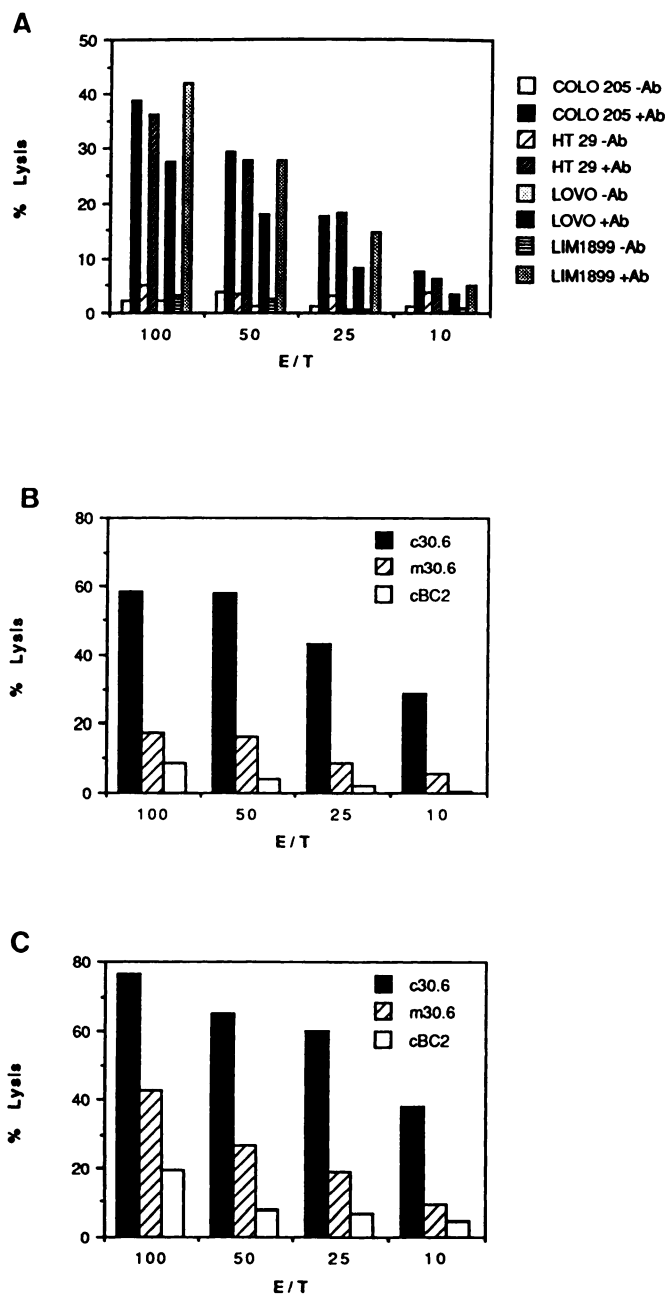


Fig. 4. Four-h ADCC assays using human CRC cell lines as targets. In A, assays were performed using freshly isolated human PBMC as effectors and four target cell lines in the presence or absence of c30.6 (25 μ g/ml). SEs were <5% for each value. In B, PBMC were used as effectors and COLO 205 cells were used as targets in the presence of either c30.6, m30.6, or cBC2 at 25 μ g/ml. SEs were <5% for each value. In C, assays were performed as in B, except that PBMC were incubated in medium supplemented with 100 units/ml recombinant human IL-2 for 36 h prior to use. In all experiments, E/T ratios varied from 100/1 to 10/1. All values were calculated as the mean of triplicate assays (see "Materials and Methods"). SEs were <5% for each value. These data are representative of four independent experiments performed using the PBMC of three unrelated donors. Ab, antibody.

(13). m30.6 was produced by conventional hybridoma technology from BALB/c mice immunized with fresh colon carcinoma cells and boosted with the colon carcinoma cell line, HT-29 (13). We have now produced a recombinant, chimeric mouse/human antibody, c30.6, that binds to the same epitope with equivalent affinity and, additionally, is able to mediate potent effector function against 30.6⁺ tumors.

The 30.6 determinant is not expressed exclusively on malignant cells, but it has been shown in immunoperoxidase and immunofluorescence studies to be expressed on benign and malignant lesions and

on secondary tumor deposits. It is expressed weakly only on normal gastrointestinal epithelial cells and on some other secretory epithelia (13). Expression is greatest on well-differentiated tumors, is less pronounced on poorly differentiated tumors, but is usually absent from the most anaplastic lesions. The biochemical nature of the 30.6 antigen has not been fully elucidated, but its expression has been shown to be confined to the luminal surface of glandular cells and it is not secreted. The antigen has been shown not to be carcinoembryonic antigen or mucin on the basis of antibody-blocking studies. Immunoprecipitation experiments detected bands corresponding to molecules with molecular weights of 22,000, 25,000, 27,000, 31,000, and 42,000; however, the reason for these multiple species is uncertain. The selective expression of the 30.6 epitope on CRC provides a possible target for anticancer immunotherapy and the possibility of an improvement in therapy as compared with existing nonspecific modes of nonsurgical treatment. Zalberg *et al.* (16) demonstrated by immunoscintigraphy that radiolabeled 30.6 localized to human CRC xenografts in nude mice and that ¹³¹I-labeled antibody strongly inhibited the growth of these tumors. Notably, however, unlabeled m30.6 was no more effective than irrelevant antibodies at inhibiting tumor growth. In a phase I/II trial, *N*-acetylmelphalan that was coupled to

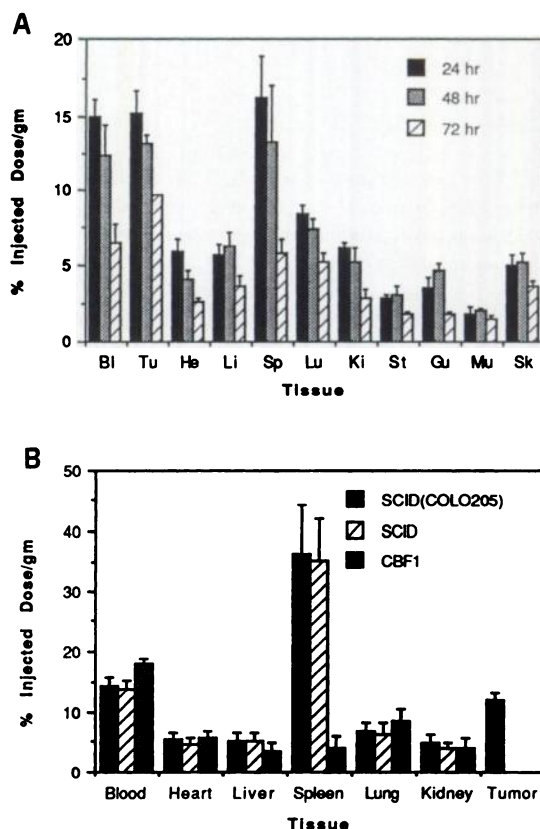


Fig. 5. Biodistribution of ¹²⁵I-c30.6 in human CRC tumor-bearing scid mice. In A, COLO 205 tumors were established s.c. on the anterior abdominal wall of scid mice, and ¹²⁵I-c30.6 administered by tail vein injection (see "Materials and Methods"). Mice were sacrificed at the times indicated and immediately dissected. Specimens were assayed for radioactivity in a gamma counter. The radioactivity detected is expressed as the mean of triplicate determinations $\pm$ SE (bars)/g tissue. Bl, blood; Tu, tumor; He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney; St, stomach; Gu, intestine; Mu, skeletal muscle; and Sk, skin. B, distribution of radioactivity in COLO 205-bearing scid mice, control scid mice, C57BL/6 $\times$ BALB/c F₁ (CBF₁) mice following administration of ¹²⁵I-c30.6. Mice were sacrificed at 24 h and organ specimens were assayed for radioactivity. The apparent increase in splenic accumulation in scid mice relative to A was due to a smaller average spleen size in the scid mice used in A. The radioactivity detected is expressed as the mean of triplicate determinations $\pm$ SE (bars).

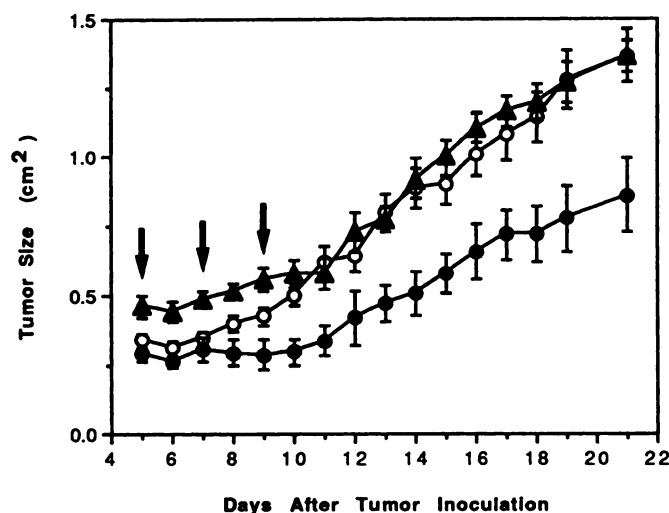


Fig. 6. Inhibition of tumor growth in COLO 205-bearing scid mice. Mice bearing s.c. tumors were treated with c30.6 (●), m30.6 (○), or PBS (▲) administered i.p. 5, 7, and 9 days after tumor inoculation (arrows). Tumor size was determined by measuring on two perpendicular axes and is expressed as the mean $\pm$ SE (bars) of six mice in each group.

30.6 was well tolerated at doses that normally produce myelosuppression; however, all patients receiving the mAb produced a HAMA response that precluded further dosage escalation (18).

The current study attempts to address the problem of HAMA by producing an antibody in which the Fc portion (~70% of the entire antibody) is replaced with a human Fc piece. The immunogenicity of chimeric antibodies compared with totally xenogeneic antibodies appears to be variable and dependent on the individual antibody. The first chimeric antibody studied for immunogenicity and pharmacokinetics was chimeric 17-1A (27), which detects a CRC-associated antigen. The results were encouraging in that the mean half-life was 6 times that of the mouse antibody and only 1 of 10 patients developed a mild HAMA response. By contrast, another antibody cB72.3 was as immunogenic as its mouse counterpart (28). Further studies are required before generalizations can be made about immunogenicity of chimeric antibodies. Indeed, CDR-grafted antibodies may not be free of immunogenicity because anti-idiotypic and anti-allotypic responses may be evoked when large doses are given. Furthermore, CDR-grafted mAbs frequently require framework remodeling to restore or increase affinity, which may lead to unexpected immunogenicity (29). Our initial results of administration of c30.6 to patients indicated that it was as well tolerated as the mouse antibody, and it evoked only weak anti-idiotypic responses.⁴

Apart from reducing the likelihood of HAMA, substitution of the mouse Fc piece (IgG2b) by the human Fc of choice (in this case human IgG1) may allow the acquisition of novel effector functions *in vitro* and *in vivo*. This was clearly the case with c30.6, which was able to mediate markedly enhanced lysis of 30.6⁺ target cells in comparison with m30.6 in *in vitro* antibody-dependent cell-mediated cytotoxicity. The same cells could not be lysed by either c30.6 or m30.6 in the presence of human or rabbit complement. Similar results have been found with a number of chimeric antibodies (30). Some such antibodies were able to bind C1q, and an inability to lyse cells was probably related to low antigen density on the target cells (31). It remains to be established whether the acquisition of effector functions such as ADCC and complement-mediated cytotoxicity *in vitro* will be of therapeutic significance *in vivo*.

A further significant difference between the biological activities of

m30.6 and c30.6 was the ability of c30.6 to significantly inhibit the growth of human CRC tumors in scid mice. While a significant overall inhibition of tumor size was noted in mice given the chimeric antibody, close examination of the data suggested that the majority of this effect occurred between days 5 and 10, *i.e.*, early in the course of tumor growth and while antibody was being administered (Fig. 6). Providing that this effect is also observed in humans, this suggests that c30.6 might be of use in an adjuvant setting, particularly in the treatment of minimal residual disease following excision of a primary lesion and mesenteric nodal metastases (Duke's Stage 3 CRC). In this regard, it is encouraging that the therapeutic effect in scid mice was observed with unconjugated c30.6. If c30.6 proves to be weakly immunogenic in humans its anticancer properties may be enhanced by conjugation with cytotoxic drugs or radioisotopes because ¹³¹I and methotrexate have been shown to confer antitumor activity on m30.6, which otherwise shows no intrinsic therapeutic benefit (16, 17).

The mechanism to explain the therapeutic effect of c30.6 in scid mice remains to be elucidated. Given the similarity of binding affinities of the two reagents it seems likely that the therapeutic effect resides in the Fc portion of the molecule. Because scid mice lack significant T and B cell responses any effector response would have to reflect either a direct toxicity of antibody for the tumor cells or the recruitment of other cells with cytotoxic capacity, such as neutrophils, monocytes/macrophages, or natural killer cells. The first possibility seems most improbable because direct cytotoxicity has not been noted with 30.6 in several previous studies. If monocytes, neutrophils, or natural killer cells are involved this would suggest binding of these cells to c30.6 via FcγRI/FcγRII (monocytes and neutrophils) or FcγRIII (natural killer cells). It is noteworthy that mouse IgG2b antibodies are generally able to efficiently bind FcγRII and FcγRIII but not FcγRI (32). By contrast, human IgG1 antibodies can generally bind to all three classes of Fc receptors. These observations point to a possible role for FcγRI in the inhibition of CRC tumor growth in scid mice. These receptors are constitutively expressed on monocyte/macrophages and are potently up-regulated by γ-interferon (33). While activated T cells are absent from scid mice, a source of γ-interferon might be natural killer cells localized to the tumor via their FcγRIII receptors. We are currently assessing the localization of these two effector populations to CRC tumors in our scid model.

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