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Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells

(immunoglobulin domain cDNA/DNA transfection/tumor antigen/complement-dependent cytolysis/antibody-dependent cellular cytotoxicity)

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ABSTRACT **A chimeric mouse-human antibody has been created that recognizes an antigen found on the surface of cells from many carcinomas. Immunoglobulin constant (C) domains of the mouse monoclonal antibody L6, $C_{\gamma 2a}$ and C_{κ}, were substituted by the human $C_{\gamma 1}$ and C_{κ} by recombining cDNA modules encoding variable or C domains. The cDNA constructs were transfected into lymphoid cells for antibody production. The chimeric antibody and mouse L6 antibody bound to carcinoma cells with equal affinity and mediated complement-dependent cytolysis. In the presence of human effector cells, the chimeric antibody gave antibody-dependent cellular cytotoxicity at 100 times lower concentration than that needed for the mouse L6 antibody. The chimeric antibody, but not the mouse L6 antibody, is effective against a melanoma line expressing small amounts of the L6 antigen. The findings point to the usefulness of the chimeric antibody approach for obtaining agents with strong antitumor activity for possible therapeutic use in man.**

The presence of tumor-associated antigens at the cell surface is a characteristic of many cancers. Since these antigens are either absent or found in much lower amounts in normal cells, it should be possible to use antibodies for targeting of tumors. A sizeable collection of relatively tumor-specific monoclonal antibodies (mAb) of mouse origin is available (1). Some of these mAb possess tumoricidal activity in the presence of human effector cells [antibody-dependent cellular cytotoxicity (ADCC)] or serum [complement-dependent cytotoxicity (CDC)] (2, 3). It has been shown (4) that partial tumor regression can be achieved when mAb possessing such functional activity are given to patients. One complication preventing repeated use of mouse mAb in man is that they are immunogenic. Furthermore, mouse mAb may interact less efficiently with human effector cells to mediate tumor destruction.

A method made possible by recombinant DNA technology was chosen to generate chimeric mouse-human antibodies. It entails the replacement of the mouse constant (C) domain regions with the corresponding human equivalents (5–7). In principle, antibody molecules obtained by this approach should retain their specificity for antigen and thus their usefulness for targeting, be much less immunogenic to man, and perhaps have increased antitumor activity.

The mouse mAb L6 [IgG2a(κ)] binds to a carbohydrate antigen found at the surface of cells from human carcinomas of the lung, breast, colon, and ovary (8). L6 can mediate CDC with human complement or ADCC with human effector cells (2). mAb L6 may thus be of use for tumor targeting, either in its native form or after conjugation of anticancer agents.

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In this study we have generated a mouse-human chimeric L6 antibody in which the mouse constant domains $C_{\gamma 2a}$ and C_{κ} are substituted by the human $C_{\gamma 1}$ and C_{κ}. First, the cDNAs encoding the immunoglobulin genes were isolated. Next, restriction enzyme recognition sites were created in the cDNA sequences at the V/C junction (where V stands for variable) (9) by *in vitro* mutagenesis using oligodeoxyribonucleotides (10). The chimeric cDNAs thus constructed were then introduced into lymphoid cells by DNA transfection. The chimeric antibody isolated from the transfectants was compared with the mouse L6 for effector functions.

MATERIALS AND METHODS

DNA Transfection of Mouse Sp2/0 Lymphoid Cells. Expression plasmid pING2114 (50 μg), linearized at a unique site (*Aat* II) in the nonessential bacterial region (see Fig. 3*A*), was transfected into 10^7 mouse Sp2/0 cells (CRL 1581, ATCC) by electroporation (11, 12). Transformants were selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (HyClone, Logan, UT) and G418 at 0.8 mg/ml (GIBCO). The transfection frequency was between 10^{-5} and 10^{-4}. Human antibody in the medium was detected by ELISA (13).

Isolation of Chimeric Antibody. Antibody-producing cells were grown to a density of 10^6 cells per ml and then shifted to serum-free DMEM 24 hr before harvest. Antibody secreted by the cells was concentrated by ultrafiltration, then chromatographed on a DEAE-cellulose column equilibrated in 40 mM NaCl/10 mM sodium phosphate, pH 8.0. The antibody in the flow-through was further purified to apparent homogeneity on protein A-Sepharose (14). For production of ascites fluid, 10^6 cells were injected into pristane-primed BALB/c mice. The chimeric antibody was purified by anti-human IgG-Sepharose chromatography (14).

Functional Tests of the Chimeric L6 Antibody. The following tests were included: (*i*) measurement of antibody binding to target cells, either positive or negative for reactivity with the mouse L6; (*ii*) competitive inhibition of binding of L6 to these cells; (*iii*) assays for CDC and ADCC. The binding tests were performed using a Coulter model EPIC-C cell sorter (8). The assays for CDC and ADCC were carried out on ^{51}Cr-labeled target cells (2, 3) that were exposed to antibodies and human serum or peripheral blood leukocytes over a 4-hr period.

Abbreviations: V, variable; C, constant; J, joining; mAb, monoclonal antibody(ies); CDC, complement-dependent cytolysis; ADCC, antibody-dependent cellular cytotoxicity; SV40, simian virus 40; H, heavy.

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A   L6 VH

                                                              met asp trp leu
     C23AGTTTGTCTTAAGGCACCACTGAGCCCAAGTCTTAGACATCATG GAT TGG CTG
BAL 31 del.          Afl II
GTCGACTCT--------------------------------------------------------------------
Sal I           leader peptide                                         |FR1
 trp asn leu leu phe leu met ala ala ala gln ser ala gln ala|gln
 TGG AAC TTG CTA TTC CTG ATG GCA GCT GCC CAA AGT GCC CAA GCA CAG

 ile gln leu val gln ser gly pro glu leu lys lys pro gly glu thr
 ATC CAG TTG GTG CAG TCT GGA CCT GAG CTG AAG AAG CCT GGA GAG ACA

                                                   FR1|CDR1
 val lys ile ser cys lys ala ser gly tyr thr phe thr|asn tyr gly
 GTC AAG ATC TCC TGC AAG GCT TCT GGG TAT ACC TTC ACA AAC TAT GGA
         Bgl II
     CDR1|FR2                                                 FR2|
 met asn|trp val lys gln ala pro gly lys gly leu lys trp met gly|
 ATG AAC TGG GTG AAG CAG GCT CCA GGA AAG GGT TTA AAG TGG ATG GGC
                                                 Aha III
CDR2
 trp ile asn thr tyr thr gly gln pro thr tyr ala asp asp phe lys
 TGG ATA AAC ACC TAC ACT GGA CAG CCA ACA TAT GCT GAT GAC TTC AAG
                                         Nde I
CDR2|FR3
 gly|arg phe ala phe ser leu glu thr ser ala tyr thr ala tyr leu
 GGA CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC TAC ACT GCC TAT TTG

 gln ile asn asn leu lys asn glu asp met ala thr tyr phe cys ala
 CAG ATC AAC AAC CTC AAA AAT GAG GAC ATG GCT ACA TAT TTC TGT GCA
                                             ---------------------
 FR3|CDR3                                        CDR3|FR4
 arg|phe ser tyr gly asn ser arg tyr ser asp tyr|trp gly gln gly
 AGA TTT AGC TAT GGT AAC TCA CGT TAC TCT GAC TAC TGG GGC CAA GGC
                  Dsp2.2
 -------------JH2----------
 thr thr leu thr val ser ser ala lys thr thr ala pro ser
 ACC ACT CTC ACA GTC TCC TCA GCC AAA ACA ACA GCC CCA TCG--------

             --------------------GC--------AG--G---------MJH2 Apa I

B   L6 Vκ
                                                  leader peptide
                 met asp phe gln val gln ile phe ser phe leu leu
  C9CCCCAAGACAAAATG GAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA
   ---GTC----------------------5'Sal I
                                         |FR1
 ile ser ala ser val ile met ser arg gly|gln ile val leu ser gln
 ATC AGT GCT TCA GTC ATA ATG TCC AGA GGA CAA ATT GTT CTC TCC CAG

 ser pro ala ile leu ser ala ser pro gly glu lys val thr leu thr
 TCT CCA GCA ATC CTG TCT GCA TCT CCA GGG GAG AAG GTC ACA TTG ACT

 FR1|CDR1                                CDR1|FR2
 cys|arg ala ser ser ser val ser phe met asn|trp tyr gln gln lys
 TGC AGG GCC AGC TCA AGT GTA AGT TTC ATG AAC TGG TAC CAG CAG AAG
                                                Kpn I
                                      FR2|CDR2
 pro gly ser ser pro lys pro trp ile tyr|ala thr ser asn leu ala
 CCA GGA TCC TCC CCC AAA CCC TGG ATT TAT GCC ACA TCC AAT TTG GCT
      Bam HI
CDR2|FR3
 ser|glu phe pro gly arg phe ser gly glu trp ser gly thr ser tyr
 TCT GAG TTC CCT GGT CGC TTC AGT GGC GAG TGG TCT GGG ACC TCT TAC

 ser leu ala ile ser arg val glu ala glu asp ala ala thr tyr tyr
 TCT CTC GCA ATC AGC AGA GTG GAG GCT GAA GAT GCT GCC ACT TAT TAC
                                    -------------------------------Jκ5-
 FR3|CDR3                            CDR3|FR4
 cys|gln gln trp asn ser asn pro leu thr|phe gly ala gly thr lys
 TGC CAG CAG TGG AAT AGT AAC CCA CTC ACG TTC GGT GCT GGG ACC AAG
 ---------------                                       ---------
 leu glu leu lys
 CTG GAG CTG AAA-------------------------------------------------
 --T----- JκHindIII
```

FIG. 1. Nucleotide sequences and predicted amino acid sequences of the L6 V_H (*A*) and V_κ (*B*). The framework (FR) and complementarity determining region (CDR) segments are indicated. The diversity (D) segment in V_H is underlined. Circles above the amino acid residues indicate that these residues matched to those obtained from peptide sequencing. The V_H sequence is present in plasmid pH3-6a. The C_{23} at the 5′ end was removed by BAL-31 nuclease digestion. The resultant DNA sequence at the 5′ end is shown below the first line. An *Apa* I site was introduced by the oligonucleotide primer MJH2ApaI. The V_κ sequence is present in plasmid pL3-12a. The C_{13} at the 5′ end was removed by oligonucleotide-mediated mutagenesis. A *Hin*dIII site was introduced by the oligonucleotide primer JκHindIII.

RESULTS

Isolation of Mouse cDNA. A cDNA library was generated from the L6 hybridoma cells by priming poly(A)$^+$ RNA with oligo(dT) as described (9, 15). The probes used to screen the library were a $J_\kappa 5$ oligonucleotide, d(GGTCCCAGCACCGAACG), for the light chain and a $J_H 2$ oligonucleotide, d(TGGCTGAGGAGACTGTGAGAG) for the heavy chain (where J stands for joining and H stands for heavy). Two methods (16, 17) were used to determine that the L6 κ mRNA contains $J_\kappa 5$ sequences and that the L6 $\gamma 2a$ mRNA contains $J_H 2$ sequences.

Preparation of Mouse V-Region cDNA Modules. Restriction enzyme sites were engineered into the immunoglobulin cDNA around the V/C border for recombining mouse V regions to human C modules. The oligonucleotide MJH2ApaI [d(ATGGGCCCTTTGTGCTGGCTGAGGAGACTGT) (with the restriction enzyme site underlined)] was used for mutagenesis of the V_H cDNA; and the oligonucleotide JκHindIII [d(CTCAAGCTTGGTCCC)] for that of the V_κ cDNA. Restriction sites on the 5′ side of the ATG codon were also created. The oligonucleotide d(GAAAATCCATTTTGTCGACGGG) was used to generate a *Sal* I site eight residues on the 5′ side of the V_κ ATG codon. By cleaving with *Sal* I the oligo[d(GC)] segment on the 5′ side of the cDNA insert was removed. To remove the oligo[d(GC)] segment on the 5′ side of the V_H cDNA, the nuclease BAL-31 (18) was used. The digested products were inserted into the vector M13mp19 (19) in such a way that the M13 *Sal* I site became a convenient site on the 5′ side of the ATG codon. The DNA sequences of V_H and V_κ are shown in Fig. 1.

Human C-Region cDNA Modules. Human C-region cDNAs were isolated from libraries generated from the mRNA from two human lymphoblastoid cell lines, GM1500 and GM2146 (Human Genetic Mutant Cell Repository). The human $C_{\gamma 1}$ module has been described (9). The cDNA clone pGMH6 contains an *Apa* I site 16 nucleotide residues on the 3′ side of the V/C border (Fig. 2).

The human C_κ module is a composite of two κ cDNAs isolated from the GM1500 and GM2146 libraries. In plasmid pGML60, the 3′-untranslated region was derived from the κ mRNA of GM2146 while the coding region was from that of GM1500. The JκHindIII oligonucleotide described above was used to engineer a *Hin*dIII recognition site at a position of the human J_κ segment analogous to that in the mouse J_κ segment.

Chimeric L6 Heavy- and Light-Chain Expression Plasmids. The cDNA constructs were inserted into the vector sequences of plasmid pING2012E (9). Directionality of insertion was achieved by using a *Sal* I–*Bam*HI bracket. pING2012E contains regulatory sequences derived from plasmid pL1 (20) that furnished the early promoter and splice donor–acceptor of simian virus 40 (SV40); and from plasmid pSV2neo (21) that furnished the transcription termination/polyadenylylation signals of SV40. We added the mouse immunoglobulin heavy-chain gene transcription enhancer, placed upstream of the SV40 promoter (9). The selectable marker is the Tn*5 neo* gene that confers resistance to the drug G418.

The heavy-chain plasmid pING2111 was constructed by first joining the mouse V_H cDNA module in a *Sal* I–*Apa* I DNA fragment with the human $C_{\gamma 1}$ cDNA module in an *Apa* I–*Bam*HI DNA fragment. The ligated fragments were then inserted into pING2012E cleaved by *Sal* I–*Bam*HI. The light-chain plasmid pING2119 was constructed by joining the mouse V_κ cDNA module in a *Sal* I–*Hin*dIII DNA fragment with the human C_κ cDNA module in a *Hin*dIII–*Bam*HI DNA fragment. The same vector fragment was used (Fig. 3*A*). In both plasmids the cDNA gene is placed 11 nucleotide residues downstream of the SV40 19S 3′-splice acceptor (9). The cDNA ends in a segment approximately $A_{70}G_{20}$, where it is joined to the SV40 transcription-termination/polyadenylylation sequences. Fig. 3*B* shows the incident nucleotide sequence changes made at the V/C junction as a result of the gene construction.

A two-gene plasmid, pING2114, was constructed from pING2111 and pING2119 in which the light- and heavy-chain gene transcription units are in tandem (Fig. 3*A*). By using this plasmid, we introduced an equal ratio of heavy- and light-chain genes into recipient cells. Unexpectedly, we observed that there was a consistently higher expression of heavy than of light chain in all transfected cell lines examined (data not shown). The two transcription units differ in that the light-chain gene is about 700 base pairs shorter than the heavy-chain gene, and the C_κ gene segment has a higher A+T content. This imbalance was reduced by introducing more light-chain gene copies carried on a second plasmid with a different selectable marker [pING2121a, an *Eco-gpt* (22) version of pING2119].

Two initial Sp2/0 transformants, D7 and 3E3, obtained by transfection with pING2114 were cultured for the isolation of chimeric antibody. D7 secretes 10% of the antibody produced by 3E3—κ (17 μg/liter) and γ (77 μg/liter) chains for D7 compared to κ (100 μg/liter) and γ (700 μg/liter) chains for 3E3.

Binding Characteristics of Chimeric Versus Mouse L6 Antibody. Table 1 shows that the chimeric L6 antibody binds to cells from a human colon carcinoma (line C-3347) that express 5×10^5 molecules per cell of the antigen defined by the mouse L6 mAb (8). In a competition assay, 50% inhibition of binding was achieved by the same amount of the chimeric and mouse L6 (Fig. 4). Cells from a T-cell line, HSB-2, did not bind either mouse L6 or the chimeric antibody. Data on the melanoma line M-2669, clone 13 (3), are also included in Table 1, since this line, which expresses a low level of the L6-defined antigen, was used for the functional studies (see below).

Chimeric L6 Antibody Mediates CDC and ADCC. Fig. 5 shows that both the chimeric and mouse L6 antibodies lysed tumor cells in the presence of human complement. The experiment further showed that the chimeric L6 gave higher CDC at all dilutions of the complement.

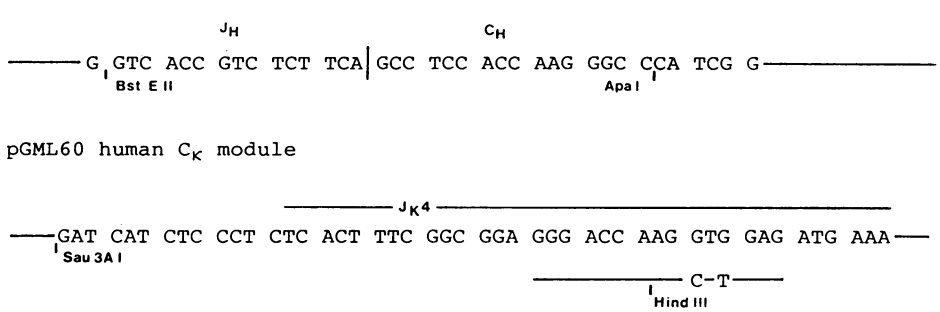

FIG. 2. Human C-domain cDNA gene modules. The relevant sequences at the V/C junction of human $C_{\gamma 1}$ and C_κ cDNA clones are shown. The $C_{\gamma 1}$ clone contains a *Bst*EII and an *Apa* I site that can be used to recombine with different V-domain cDNA genes. The C_κ clone does not contain a convenient recombination site; and an oligonucleotide containing a *Hin*dIII site was used to introduce this site into the cDNA. The C-domain cDNA modules are pGMH6 and pGML60.

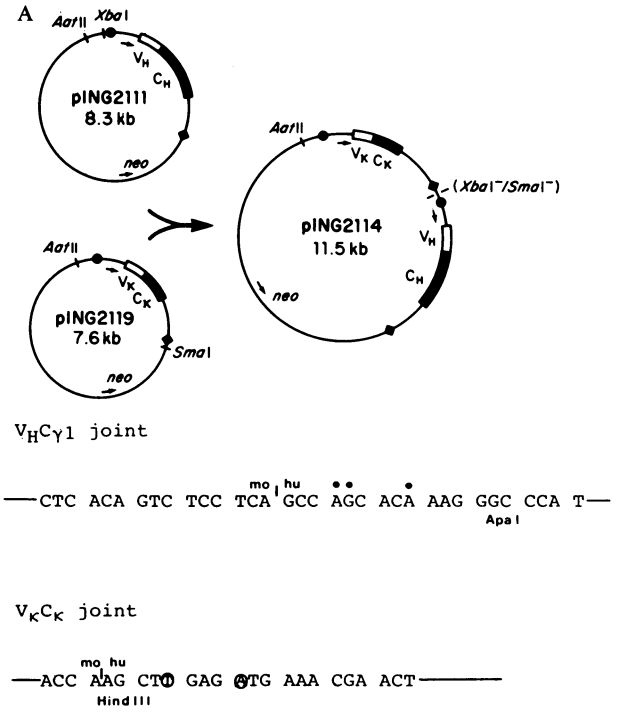

FIG. 3. (*A*) Expression plasmids. pING2111 is the heavy-chain and pING2119 is the light-chain expression plasmid. They were used to construct pING2114, a two-gene plasmid. Solid circles, mouse heavy-chain immunoglobulin gene enhancer; small arrows, SV40 early promoter; diamonds, bidirectional SV40 transcription termination/polyadenylylation signals. (*B*) Nucleotide changes made in the V/C junction. Dotted residues in the $V_HC_{\gamma 1}$ junction were introduced by oligonucleotide-mediated mutagenesis. They are silent changes. Circled residues in the $V_\kappa C_\kappa$ junction are residues contributed by the human cDNA module to the mouse V_κ gene.

Fig. 6 shows ADCC tests with cells from two cell lines. At a ratio of 100:1 human peripheral blood leukocytes to the colon carcinoma line C-3347 cells, the chimeric L6 killed a greater fraction of the target cells (a maximum of 98% versus 63%) and gave 50% ADCC at 100 times lower concentration than the mouse L6 (0.01 μg/ml versus 1 μg/ml, Fig. 6*A*). Significant ADCC of C-3347 cells (24% as compared to 3% lysis with lymphocytes alone) was observed down to a 3:1 ratio of effector cells to target cells when the chimeric L6 (at 2.5 μg/ml) was used (Fig. 6*B*). Cell killing was specific because ADCC was not observed with the following three cell lines lacking detectable L6 antigens: B-cell lines DHL-10 (Fig. 6*C*) and T51 (data not shown) and the T-cell line HSB-2

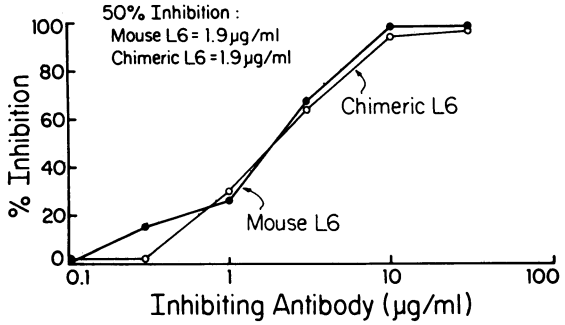

FIG. 4. Comparison between chimeric and standard mouse L6 in antibody inhibition assays, performed by fluorescence-activated cell sorting. C-3347 cells were incubated with the blocking antibodies before fluorescein isothiocyanate-conjugated mouse L6 (3 μg/ml) was added.

Table 1. Binding of chimeric L6 and mouse L6 antibodies to cell lines used as targets for functional assays

Antibody	Antibody concentration, μg/ml	Binding ratio* GAM	GAH
Human colon carcinoma line C-3347			
Mouse L6	30	38	4
	10	49	4
	3	40	3
Chimeric L6 (ascites)	30	2	108
	10	2	84
	3	1	42
Chimeric L6 (cell culture)	30	1	105
	10	1	86
	3	1	44
Human melanoma line M-2669 (clone 13)			
Mouse L6	30	7	NT
	10	3	NT
	3	1	NT
Chimeric L6 (cell culture)	30	NT	4
	10	NT	2
	3	NT	1
Human T-cell line HSB-2			
Mouse L6	10	1	1
Chimeric L6 (ascites)	10	1	1
Chimeric L6 (cell culture)	10	1	1

*The binding ratio is the number of times a test sample is brighter than a control sample when treated with GAM (fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin) or with GAH (fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin). For example, a ratio of 1 means that the test sample is as bright as the control; a ratio of 2 means that the test sample is twice as bright as the control. NT, not tested.

(data not shown). Efficacy of the chimeric L6 was further demonstrated by its ability to lyse M-2669 melanoma cells (35% at 10 μg/ml, 27% at 0.1 μg/ml); the mouse L6 had no effect on these cells (9% at 10 μg/ml, as compared to 10% lysis with lymphocytes alone, *cf.* Table 1).

DISCUSSION

The mouse mAb L6 recognizes a carbohydrate antigen present in abundance in a variety of carcinomas. Normal tissues express only trace amounts of the antigen (8). Based on this specificity there is justification in considering L6 for cancer treatment with the mAb used either alone (2) or as a carrier of anticancer agents. However, the immunogenicity of mouse L6 mAb in man is a disadvantage for its sustained use in patients, and its functional activity (ADCC and CDC) may be insufficient to effect optimal tumor destruction at the

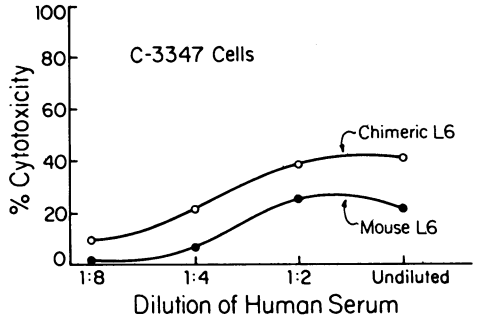

FIG. 5. Titration of human serum (as a source of complement) in the presence of chimeric or mouse L6 at 2.5 μg/ml.

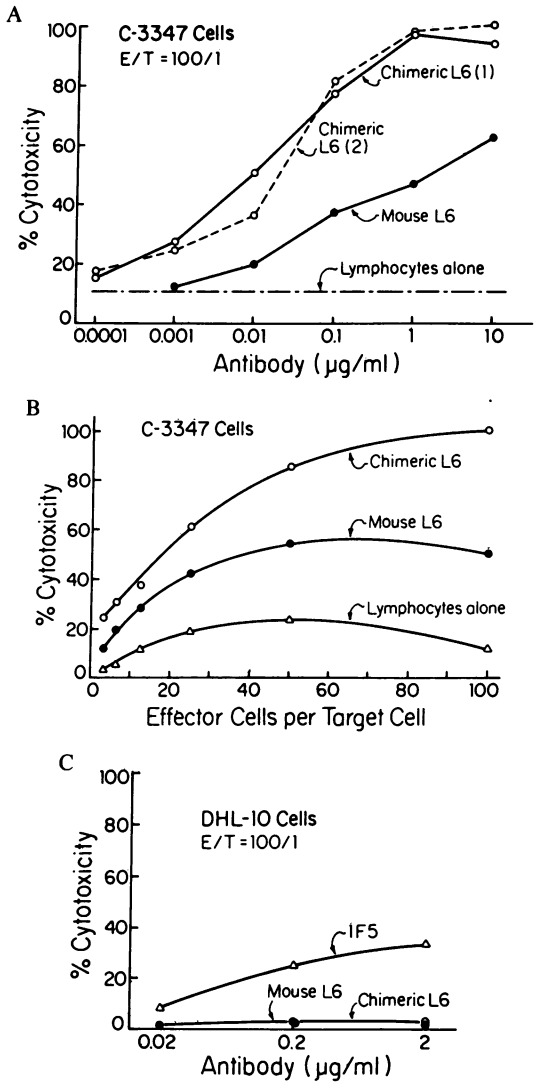

FIG. 6. (*A*) Titration of chimeric and mouse L6 antibodies in ADCC assays with human peripheral blood leukocytes. E/T, effector–target cell ratio. Two preparations of chimeric L6 were used. (*B*) Titration of human peripheral blood leukocyte effector cells mediating ADCC in the presence of antibodies (2.5 μg/ml). (*C*) Titration of L6 (chimeric and mouse) in ADCC assays on the DHL-10 T-cell line. 1F5 is a mouse mAb that recognizes the DHL-10 cells.

concentrations that can be attained in tumors after intravenous administration.

It is estimated that a major immunogenic site resides in the C_H2 region (23) of the IgG molecule. As one approach to decrease this problem, one could generate chimeric mouse-human antibodies thereby replacing the immunogenic C domains of the mouse immunoglobulins with those of human immunoglobulins. We did this using cDNA rather than cloned genomic DNA (24, 25). We show here that this is a useful approach for producing chimeric antibody. In cell line 3E3 and its subclones close to 1 μg/ml of IgG1 protein was detected.

The chimeric antibody was found to bind to tumor cells as well as the mouse L6 antibody. The chimeric antibody was much more efficient than L6 in ADCC assays, killing a greater fraction of target cells at a concentration lower by a factor of 100. Furthermore, the chimeric L6 killed cells from a melanoma line that was refractory to ADCC by the mouse L6. In patients one may speculate that the chimeric L6 would remain longer in the circulation. This, in combination with the functional attributes of chimeric L6, should make it a strong candidate for therapeutic trials. Some of the antibodies induced in man to mouse mAb were directed to idiotypic determinants (26, 27). It remains to be seen whether the immunogenicity of those determinants of the chimeric L6 will be different from that of the mouse L6.

The advantage of the cDNA approach lies in the ease with which immunoglobulin gene cDNAs can be isolated. The technology used for the present work should make it possible to convert many other mouse mAb to chimeric antibodies with improved antitumor activity via ADCC and CDC mechanisms. The chimeric antibodies will augment the relatively few human mAb currently used in the treatment of cancer (28).

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