

A humanized antibody that binds to the interleukin 2 receptor

(chimeric antibody/antibody affinity/autoimmune disease)

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ABSTRACT The anti-Tac monoclonal antibody is known to bind to the p55 chain of the human interleukin 2 receptor and to inhibit proliferation of T cells by blocking interleukin 2 binding. However, use of anti-Tac as an immunosuppressant drug would be impaired by the human immune response against this murine antibody. We have therefore constructed a "humanized" antibody by combining the complementarity-determining regions (CDRs) of the anti-Tac antibody with human framework and constant regions. The human framework regions were chosen to maximize homology with the anti-Tac antibody sequence. In addition, a computer model of murine anti-Tac was used to identify several amino acids which, while outside the CDRs, are likely to interact with the CDRs or antigen. These mouse amino acids were also retained in the humanized antibody. The humanized anti-Tac antibody has an affinity for p55 of $3 \times 10^9 \text{ M}^{-1}$, about 1/3 that of murine anti-Tac.

The cellular receptor for the lymphokine interleukin 2 (IL-2) plays an important role in regulation of the immune response (reviewed in ref. 1). The complete IL-2 receptor (IL-2R) consists of at least two IL-2-binding peptide chains: the p55 or Tac peptide (2, 3), and the recently discovered p75 peptide (4, 5). Identification and characterization of the p55 peptide were facilitated by the development of a monoclonal antibody, anti-Tac, which binds to human p55 (2). The p55 peptide was found to be expressed on the surface of T cells activated by an antigen or mitogen but not on resting T cells. Treatment of human T cells with anti-Tac antibody strongly inhibits their proliferative response to antigen or to IL-2 by preventing IL-2 binding (3, 6).

These results suggested that anti-IL-2R antibodies would be immunosuppressive when administered *in vivo*. Indeed, injection of an anti-IL-2R antibody into mice and rats greatly prolonged survival of heart allografts (7, 8). Anti-IL-2R was also effective in rats against experimental graft-versus-host disease (9). In animal models of autoimmune disease, an anti-IL-2R antibody alleviated insulinitis in nonobese diabetic mice and lupus nephritis in NZB \times NZW mice (10). Anti-Tac itself was highly effective in prolonging survival of kidney allografts in cynomolgus monkeys (11).

In human patients, the specificity of anti-Tac for activated T cells might give it an advantage as an immunosuppressive agent over OKT3 (monoclonal anti-CD3), which is effective in treating kidney transplant rejection (12), but which suppresses the entire peripheral T-cell population. In fact, in phase I clinical trials for kidney transplantation, prophylactic administration of anti-Tac significantly reduced the incidence of early rejection episodes, without associated toxicity (13). Furthermore, treatment with anti-Tac induced temporary

partial or complete remission in three of nine patients with Tac-expressing adult T-cell leukemia (14). However, as a murine monoclonal antibody, anti-Tac elicits a strong human antibody response against itself, as does OKT3 (15). This response would prevent its long-term use in treating autoimmune conditions or suppressing organ transplant rejection.

The immune response against a murine monoclonal antibody may potentially be reduced by transforming it into a chimeric antibody. Such antibodies, produced by methods of genetic engineering, combine the variable (V) region binding domain of a mouse (or rat) antibody with human antibody constant (C) regions (16–18). Hence, a chimeric antibody retains the binding specificity of the original mouse antibody but contains less amino acid sequence foreign to the human immune system. Chimeric antibodies have been produced against a number of tumor-associated antigens (19–21). In some but not all cases, the chimeric antibodies have mediated human complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) more efficiently than the mouse antibodies (21).

When the murine antibody OKT3 is used in human patients, much of the resulting antibody response is directed against the V region of OKT3 rather than the C region (15). Hence, chimeric antibodies in which the V region is still nonhuman may not have sufficient therapeutic advantages over mouse antibodies. To further reduce the immunogenicity of murine antibodies, Winter and colleagues constructed "humanized" antibodies in which only the minimum necessary parts of the mouse antibody, the complementarity-determining regions (CDRs), were combined with human V region frameworks and human C regions (22–25). We report here the construction of chimeric and humanized anti-Tac antibodies.¶ For the humanized antibody, sequence homology and molecular modeling were used to select a combination of mouse and human sequence elements that would reduce immunogenicity while retaining high binding affinity.

MATERIALS AND METHODS

Construction of Plasmids. cDNA cloning was by the method of Gubler and Hoffman (26), and sequencing was by the dideoxy method (27). The plasmid pV κ 1 (Fig. 1A) was constructed from the following fragments: an approximately 4550-base-pair (bp) *Bam*HI–*Eco*RI fragment from the plas-

Abbreviations: IL-2R, interleukin 2 receptor; CDR, complementarity-determining region; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; V, variable; J, joining; C, constant.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M28250 and M28251).

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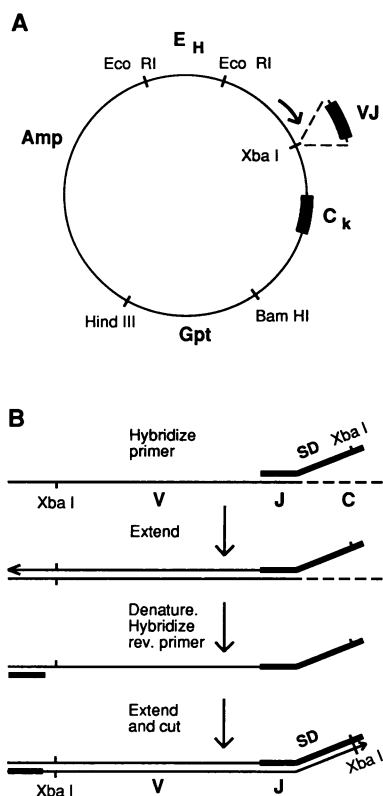


FIG. 1. (A) Schematic diagram of the plasmids pV κ 1 and pLTac. Light chain exons are shown as boxes. An arrow indicates the direction of transcription from the κ promoter. E_H , heavy chain enhancer. Not drawn to scale. (B) Schematic diagram of the method used to excise the V-J region. SD, splice donor sequence; rev. primer, reverse primer.

mid pSV2gpt (28) containing the *amp* and *gpt* genes; an 1800-bp *EcoRI*-*Bgl* II fragment from pKcatH (29) containing the heavy chain enhancer and κ promoter; and a 1500-bp *EcoRI*-*Xba* I fragment containing the human C_κ region (30). Similarly, pV γ 1 was constructed starting from a 4850-bp *Bam*HI-*EcoRI* fragment of the plasmid pSV2hph (a gift of A. Smith, A. Miyajima, and D. Strehlow, Stanford University), which is analogous to pSV2gpt except that the *gpt* gene is replaced by the *hyg* gene (31). This fragment was combined with the *EcoRI*-*Bgl* II fragment from pKcatH and a 2800-bp *Hind*III-*Pvu* II fragment containing the human γ 1 constant region, isolated from a phage kindly provided by L. Hood (32). In each case, the fragments were combined by standard methods (ref. 33, pp. 390-401), with an *Xba* I linker inserted between the κ promoter fragment and the 5' end of the C region fragment.

Construction of Chimeric Genes. *EcoRI* fragments containing the anti-Tac light and heavy chain cDNAs were separately inserted into the *EcoRI* site of the phage M13mp11D, a variant of M13mp11 (34) in which the *EcoRI* and *Xba* I sites of the polylinker were filled in and joined. The resulting phage, in which the 5' ends of the cDNAs abutted the *Xba* I site, were respectively denoted M13L and M13H. The V-J (J, joining) segments of the cDNAs, followed by splice donor signals, were precisely excised from these phage, using a double-priming scheme (Fig. 1B). For the light chain, the following primer was synthesized (Applied Biosystems model 380B DNA synthesizer) and purified by gel electrophoresis: 5'-CCAGAATTCTAGAAAAGTGTACTTACGTTTCAGCTCCAGCTTGGTCCC-3'. From the 3' end, the first 22 residues of the primer are the same as the last 22 residues of the $J_{\kappa 5}$ segment (noncoding strand). The next 16 nucleotides are the same as the sequence that follows $J_{\kappa 5}$ in

mouse genomic DNA and therefore includes a splice donor signal. The final 10 nucleotides of the oligonucleotide include an *Xba* I site.

We hybridized this oligonucleotide to M13L and extended it with the Klenow fragment of DNA polymerase. The DNA was heat-denatured, hybridized with an excess of the "reverse primer" 5'-AACAGCTATGACCATG-3', again extended with Klenow DNA polymerase, and digested with *Xba* I. The digested DNA was run on a gel, and an approximately 400-bp fragment was excised and inserted into the *Xba* I site of pV κ 1. Sequencing showed that the fragment consisted of the V-J region of the light chain cDNA followed by the splice donor "tail," as expected (Fig. 1B), and pLTac, a clone with the appropriate orientation, was chosen. In an analogous fashion, the heavy chain V-J segment, followed by the mouse J_H2 splice donor sequence, was excised from M13H and inserted into the *Xba* I site of pV γ 1 to yield pGTac.

Computer Analysis. Sequences were manipulated and homology searches were performed with the MicroGenie Sequence Analysis Software (Beckman). The molecular model of the anti-Tac V region was constructed with the ENCAD program (35) and examined with the MIDAS program (36) on an IRIS 4D-120 graphics workstation (Silicon Graphics).

Construction of Genes for Humanized Antibody. Nucleotide sequences were selected that encoded the protein sequences of the humanized light and heavy chain V regions including signal peptides (*Results*), generally utilizing codons found in the mouse anti-Tac sequence. These nucleotide sequences also included the same splice donor signals used in the chimeric genes and an *Xba* I site at each end. For the heavy chain V region, four overlapping 120- to 130-nucleotide-long oligonucleotides were synthesized that encompassed the entire sequence on alternating strands. The oligonucleotides were phosphorylated with polynucleotide kinase, annealed, extended with T4 DNA polymerase, cut with *Xba* I, and ligated into the *Xba* I site of pUC19 (34), using standard reaction conditions. An insert with the correct sequence was recloned in pV γ 1. The humanized light chain V region was constructed similarly.

Transfections. For each antibody constructed, the light chain plasmid was first transfected into Sp2/0 mouse myeloma cells (ATTC CRL 1581) by electroporation (Bio-Rad Gene Pulser) and cells were selected for *gpt* expression (28). Clones secreting a maximal amount of light chain, as determined by ELISA, were transfected with the heavy chain plasmid and cells were selected for hygromycin B resistance (31). Clones secreting a maximal amount of complete antibody were detected by ELISA. The clones were used for preparation of chimeric and humanized antibodies.

Antibody Purification. Medium from confluent cells was passed over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia), and antibody was eluted with 3 M MgCl₂. Antibody was further purified by ion-exchange chromatography on BakerBond ABx (J. T. Baker). Final antibody concentration was determined, assuming that 1 mg/ml has an A_{280} of 1.4. Anti-Tac antibody itself was purified as described (2).

Affinity Measurements. Affinities were determined by competition binding. HuT-102 human T-lymphoma cells (ATTC TIB 162) were used as source of p55 Tac antigen. Increasing amounts of competitor antibody (anti-Tac, chimeric, or humanized) were added to 1.5 ng of radioiodinated (Pierce Iodo-Beads) tracer anti-Tac antibody (2 μ Ci/ μ g; 1 Ci = 37 GBq) and incubated with 4×10^5 HuT cells in 0.2 ml of binding buffer (RPMI 1040 medium with 10% fetal calf serum, human IgG at 100 μ g/ml, 0.1% sodium azide) for 3 hr at room temperature. Cells were washed and pelleted, and their radioactivities were measured, and the concentrations of bound and free tracer antibody were calculated. The affinity of mouse anti-Tac was determined by Scatchard plot analy-

sis, using anti-Tac itself as the competitor. Then the affinities of the chimeric and humanized antibodies were each calculated according to the formula $[X] - [\text{anti-Tac}] = (1/K_x) - (1/K_a)$, where K_a is the affinity of anti-Tac ($9 \times 10^9 \text{ M}^{-1}$), K_x is the affinity of the competitor X, [] indicates the concentration of competitor antibody at which bound/free tracer binding is $R_0/2$, and R_0 is maximal bound/free tracer binding (37).

RESULTS

Cloning of Light and Heavy Chain cDNA. A cDNA library in λ gt10 was prepared from anti-Tac hybridoma cells and screened with oligonucleotide probes for the mouse κ and γ 2a constant regions. The cDNA inserts from four κ -positive and four γ 2a-positive phage were subcloned in M13mp19. Partial sequencing showed that two of the κ isolates had one sequence, and the other two had another sequence. In one pair, a V_κ gene segment was joined to the $J_{\kappa 2}$ segment out of its reading frame. In addition, the conserved cysteine at position 23 was absent from this V segment, and the sequences of the two isolates differed slightly. Presumably, these clones were the result of an aberrant joining event in one κ allele, which continued to undergo somatic mutation after the formation of the hybridoma.

The V-J segments of the other pair of κ clones were sequenced completely and were identical. This light chain uses the $J_{\kappa 5}$ segment. Partial sequencing of the four γ 2a clones showed they were all from the same gene. The V-J segments of two were sequenced completely and were identical. This heavy chain uses the J_{H2} segment and is of subgroup II (38). The DNA sequences have been deposited with GenBank; the deduced protein sequences are shown in Fig. 2. As both alleles of the κ light chain were accounted for and only one heavy chain sequence was detected, we tentatively assigned these sequences to the anti-Tac antibody genes.

Construction of Chimeric Genes. Plasmid vectors were prepared for the construction and expression of chimeric light and heavy chain genes. The plasmid pV κ 1 (Fig. 1A) contains the human genomic C_κ segment, including 336 bp of the preceding intron and the poly(A) signal. It also contains the promoter sequence from the MOPC 41 κ gene and the heavy chain enhancer sequence, which synergize to form a very strong transcriptional unit (29). There is a unique *Xba* I site between the promoter and the intron. A similar plasmid, pV γ 1, was prepared by using the human $C_{\gamma 1}$ region in place of the C_κ region. In that case, the region inserted between the *Xba* I and *Bam*HI sites extended from about 210 bp 5' of the C_{H1} exon to beyond the C_{H3} exon.

Our strategy was to insert the V-J region from the anti-Tac κ cDNA, followed by a splice donor signal, at the *Xba* I site

of pV κ 1 to construct the plasmid pLTac. Doing so created a chimeric κ gene with a short synthetic intron between the mouse V-J and human C_κ segments (Fig. 1A). For this purpose, we used a form of double primer-directed mutagenesis (*Materials and Methods*; Fig. 1B). Similarly, the V-J region from the anti-Tac γ 2a heavy chain cDNA, followed by a splice donor signal, was inserted into the *Xba* I site of pV γ 1. The resulting plasmid, pGTac, contained a chimeric heavy chain gene, with a synthetic intron between the mouse V-J and human $C_{\gamma 1}$ segments.

Construction of a Humanized Anti-Tac Antibody. In selecting a human antibody to provide the variable region framework for the humanized anti-Tac antibody, we reasoned that the more homologous the human antibody was to the original anti-Tac antibody, the less likely would combining the anti-Tac CDRs with the human framework be to introduce distortions into the CDRs. The anti-Tac heavy chain sequence was therefore compared by computer with all the human heavy chain sequences in the National Biomedical Research Foundation Protein Identification Resource (release 15). The heavy chain V region of the Eu antibody (of human heavy chain subgroup I; ref. 38) was 57% identical to the anti-Tac heavy chain V region (Fig. 2B); all other complete V_H regions in the data bank were 30–52% identical. However, no one human light chain V region was especially homologous to the anti-Tac light chain. We therefore chose to use the Eu light chain (of human light chain subgroup I; ref. 38) together with the Eu heavy chain to supply the framework sequences for the humanized antibody. The CDRs in the humanized antibody were of course chosen to be identical to the anti-Tac CDRs (Fig. 2).

A computer program was used to construct a plausible molecular model of the anti-Tac V domain (Fig. 3), based on homology to other antibody V domains with known crystal structure and on energy minimization. Graphic manipulation shows that a number of amino acid residues outside of the CDRs are in fact close enough to them to either influence their conformation or interact directly with antigen. When these residues differ between the anti-Tac and Eu antibodies, the residue in the humanized antibody was chosen to be the anti-Tac residue rather than the Eu residue. This choice was made for residues 27, 30, 48, 67, 68, 98, and 106 in the humanized heavy chain, and for 47 and 59 in the humanized light chain (Figs. 2 and 3; amino acids shown in blue in Fig. 3), although we now consider the light chain residue 59, which was chosen on the basis of an earlier model, to be doubtful. In this way, we hoped to better preserve the precise structure of the CDRs at the cost of possibly making the humanized antibody slightly less "human."

Different human light or heavy chain V regions exhibit strong amino acid homology outside of the CDRs, within the framework regions. However, a given V region will usually

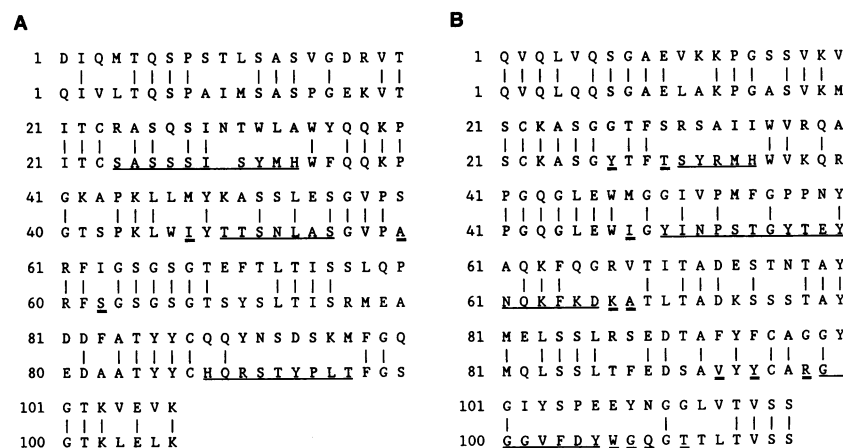


FIG. 2. Amino acid sequences of the humanized anti-Tac light (A) and heavy (B) chains. The sequences of the Eu antibody light and heavy chains (upper lines) are shown aligned above the mouse anti-Tac light and heavy chain sequences (lower lines), with a | indicating identity of amino acids. The three CDRs in each chain are underlined, and the other mouse amino acids used in the humanized antibody are double underlined. Hence, the humanized sequences are the same as the upper (Eu) sequences, except where the amino acid is underlined or double underlined.

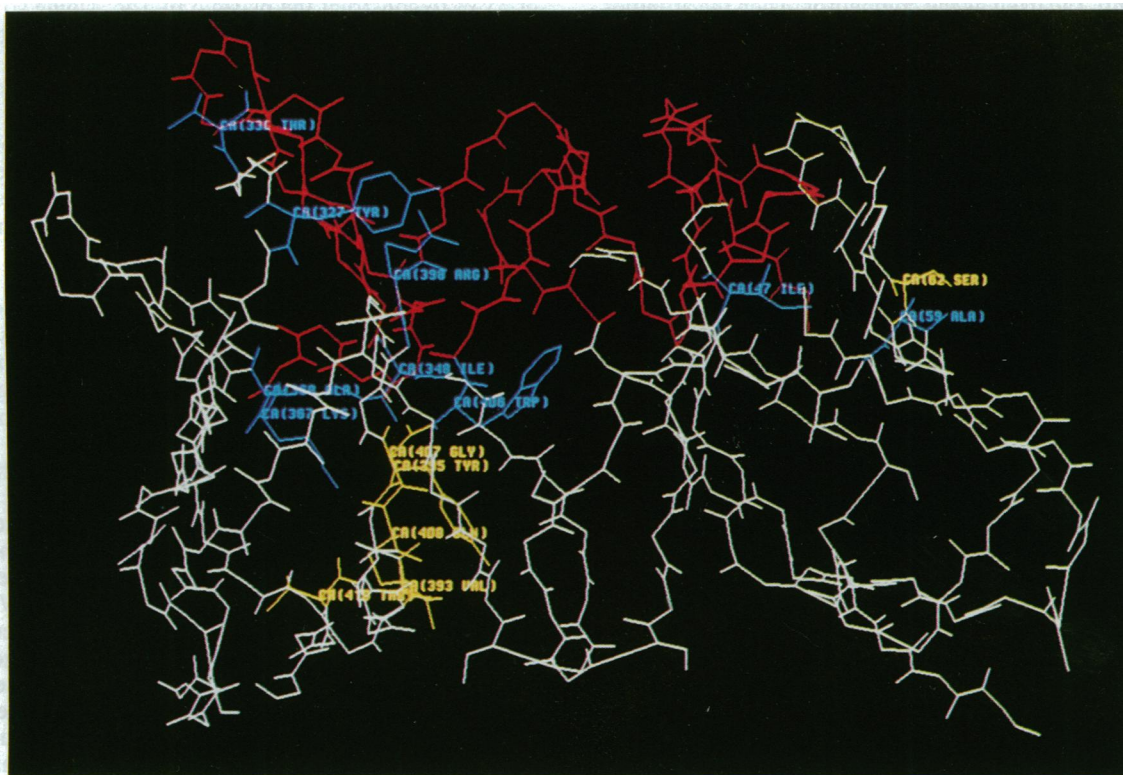


FIG. 3. Model of the mouse anti-Tac antibody V region, generated with the ENCAD program and displayed with the MIDAS program. Amino acids in the CDRs are shown in red; amino acids potentially interacting with the CDRs are shown in blue; other mouse amino acids used in the humanized antibody are shown in yellow, as described in the text. Thus, all amino acids transferred from the anti-Tac sequence to the humanized antibody are shown in red, blue, or yellow. Residue 1 is the first amino acid of V_{κ} ; residue 301 is the first amino acid of V_H .

contain exceptional amino acids, atypical of other human V regions, at several framework positions. The Eu antibody contains such unusual residues at positions corresponding to 93, 95, 98, 106, 107, 108, and 110 of the humanized heavy chain and 47 and 62 of the light chain (Fig. 2), as determined by visual comparison of the Eu heavy and light chain V regions with other human V regions of subgroup I (38). The Eu antibody contains several other unusual residues, but at the listed positions, the murine anti-Tac antibody actually has a residue much more typical of human sequences than does Eu. At these positions, we therefore chose to use the anti-Tac residue rather than the Eu residue in the humanized antibody, to make the antibody more generically human. Some of these residues had already been selected because of their proximity to the CDRs, as described above (the remaining ones are shown in yellow in Fig. 3).

These criteria allowed the selection of all amino acids in the humanized antibody V regions as coming from either anti-Tac or Eu (Fig. 2). DNA segments encoding the desired heavy and light chain amino acid sequences were synthesized. These DNA segments also encoded typical immunoglobulin signal sequences for processing and secretion, and they contained splice donor signals at their 3' end. The light and heavy chain segments were cloned, respectively, in pV κ 1 and pV γ 1 to form the plasmids pHuLTac and pHuGTac.

Properties of Chimeric and Humanized Antibodies. Sp2/0 cells, a nonproducing mouse myeloma line, were transfected sequentially with pLTac and pGTac (chimeric genes) or with pHuLTac and pHuGTac (humanized genes). Cell clones were selected first for antibiotic resistance and then for maximal antibody secretion, which reached $3 \mu\text{g}/10^6$ cells per 24 hr. S1 nuclease mapping of RNA extracted from the cells transfected with pLTac and pGTac showed that the synthetic introns between the V and C regions (Fig. 1A) were correctly spliced (data not shown). Antibody was purified from the

culture medium of cells producing the chimeric or humanized antibody. When analyzed by reducing SDS/polyacrylamide gel electrophoresis, the antibodies showed only two bands, having the expected molecular weights 50,000 and 25,000.

Flow cytometry showed that the chimeric and humanized antibodies bound to Hut-102 and CRII.2 cells, two human T-cell lines that express the p55 chain of the IL-2R, but not to CEM and other cell lines that do not express the IL-2R. To determine the binding affinity of the chimeric and humanized antibodies, their ability to compete with labeled mouse anti-Tac for binding to Hut-102 cells was determined. The affinity of chimeric anti-Tac was indistinguishable from that of anti-Tac (data not shown), as expected from the fact that their entire V regions are identical. The affinity of humanized anti-Tac for membrane-bound p55 was $3 \times 10^9 \text{ M}^{-1}$, about 1/3 the measured affinity of $9 \times 10^9 \text{ M}^{-1}$ of anti-Tac itself (Fig. 4).

DISCUSSION

Because monoclonal antibodies can be produced that are highly specific for a wide variety of cellular targets, antibody therapy holds great promise for the treatment of cancer, autoimmune conditions, and other diseases. However, this promise has not been widely realized, largely because most monoclonal antibodies, which are of mouse origin, are immunogenic when used in human patients and are ineffective at recruiting human immune effector functions such as CDC and ADCC. A partial solution to this problem is the use of chimeric antibodies (16), which combine the V region binding domains of mouse antibodies with human antibody C regions. Initially, chimeric antibodies were constructed by combining genomic clones of the V and C region genes. However, this method is very time consuming because of the difficulty of genomic cloning, especially from tetraploid hybridomas.

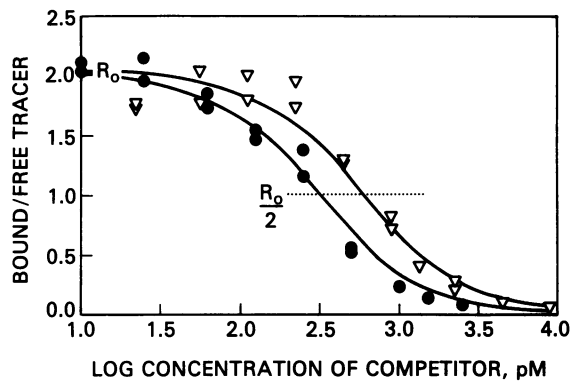


FIG. 4. Competitive binding of labeled anti-Tac tracer to Hut-102 cells. Duplicate samples are shown. ●, Mouse anti-Tac competitor; ▽, humanized anti-Tac competitor.

More recently, cDNA clones of the V and C regions have been combined, but this method is also tedious because of the need to join the V and C regions precisely (20, 21). Here we show that the V region from a readily obtainable cDNA clone can be easily joined to a human genomic C region, which need only be cloned once, by leaving a synthetic intron between the V and C regions. When linked to suitable transcriptional regulatory elements and transfected into an appropriate host cell, such chimeric genes produce antibody at a high level.

Chimeric antibodies represent an improvement over mouse antibodies for use in human patients, because they are presumably less immunogenic and sometimes mediate CDC or ADCC more effectively (21). For example, chimeric anti-Tac mediates ADCC with activated human effector cells, whereas murine anti-Tac does not (unpublished data). However, the mouse V region can itself be highly immunogenic (15). Winter and colleagues therefore took the further, innovative, step of combining the CDRs from a mouse (or rat) antibody with the framework region from a human antibody (22–25), thus reducing the xenogeneic elements in the humanized antibody to a minimum. Unfortunately, in some cases the humanized antibody had significantly less binding affinity for antigen than did the original mouse antibody. This is not surprising, because transferring the mouse CDRs from the mouse framework to the human framework could easily deform them.

In humanizing the anti-Tac antibody, which binds to the p55 chain of the human IL-2R, we have introduced two ideas that may have wider applicability. First, the human framework was chosen to be as homologous as possible to the original mouse antibody to reduce any deformation of the mouse CDRs. Second, computer modeling was used to identify several framework amino acids in the mouse antibody that might interact with the CDRs or directly with antigen, and these amino acids were transferred to the human framework along with the CDRs. The resulting humanized antibody has a high affinity, $3 \times 10^9 \text{ M}^{-1}$, for its antigen. Further work is needed to determine to what extent the choice of human framework and the preservation of particular mouse amino acids in fact contributed to the affinity of the humanized antibody. The extent to which humanization eliminates immunogenicity will need to be addressed in clinical trials, where humanized anti-Tac will be administered to patients with Tac-expressing lymphomas or selected autoimmune diseases or to patients receiving organ transplants.

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