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 × 10^13 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 insulitis in nonobese diabetic mice and lupus nephritis in NZB × 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 pVc1 (Fig. 1A) was constructed from the following fragments: an approximately 4550-base-pair (bp) BamHI–EcoRI fragment from the plasmid.

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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3The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M28250 and M28251).
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were transfected with the heavy chain

Antibody Purification. Medium from confluent cells was

passsed 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 chro-

mography on BakerBond ABx (J. T. Baker). Final antibody

concentration was determined, assuming that 1 mg/ml

has an A320 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 (ATCC

TIB 162) were used as source of pS5 Tac antigen. Increasing

amounts of competitor antibody (anti-Tac, chimeric, or hu-

manized) were added to 1.5 ng of radioiodinated (Pierce

Iodo-Beads) tracer anti-Tac antibody (2 μCi/μg; 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 μg/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-
RESULTS

Cloning of Light and Heavy Chain cDNA. A cDNA library in Agt10 was prepared from anti-Tac hybridoma cells and screened with oligonucleotide probes for the mouse κ and γ2a constant regions. The cDNA inserts from four κ-positive and four γ2a-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 Vκ gene segment was joined to the Jκ2 segment out of its reading frame. In addition, the conserved cysteine at position 23 was absent from this Vκ 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 Jκ5 segment. Partial sequencing of the four γ2a 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 Jκ2 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 pcV1 (Fig. 1A) contains the human genomic Cκ segment, including 336 bp of the preceding intron and the poly(A) signal. It also contains the promoter sequence from the MOPC41 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, pcVγ1, was prepared by using the human Cγ1 region in place of the Cκ region. In that case, the region inserted between the Xba I and BamHI sites extended from about 210 bp 5' of the Cγ1 exon to beyond the Cγ3 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 pcVκ1 to construct the plasmid pLTac. Doing so created a chimeric κ gene with a short synthetic intron between the mouse V-J and human Cκ 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 γ2a heavy chain cDNA, followed by a splice donor signal, was inserted into the Xba I site of pcVγ1. The resulting plasmid, pGTac, contained a chimeric heavy chain gene, with a synthetic intron between the mouse V-J and human Cγ1 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 with 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 Vγ 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. Graphically 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
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 pVx1 and pVyl 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 µg/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 x 10^6 M^-1, about 1/3 the measured affinity of 9 x 10^6 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.
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 xenogenic 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 × 10^9 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.