

Anti-Tac-H, a Humanized Antibody to the Interleukin 2 Receptor with New Features for Immunotherapy in Malignant and Immune Disorders

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ABSTRACT

The *M*_r 55,000 interleukin 2 receptor peptide (Tac; CD25) is not expressed by normal resting T-cells but is markedly up-regulated in adult T-cell leukemia and other malignancies, as well as on T-cells activated in normal immune, autoimmune, allograft, and graft-versus-host settings. Anti-Tac is a mouse monoclonal antibody directed against the Tac peptide. Our prior attempts to use this antibody in humans for antitumor therapy and immune regulation have been limited by weak recruitment of effector functions and neutralization by antibodies to mouse immunoglobulins. To circumvent these difficulties, we prepared several chimeric "humanized" anti-Tac antibodies by genetic engineering, including one "hyperchimeric" antibody (anti-Tac-H) in which the molecule is human except for the small hypervariable segments of the complementarity-determining regions retained from the mouse antibody. These constructs maintain high affinities for antigen and abilities to block T-cell activation and demonstrate new capabilities to perform antibody-dependent cell-mediated cytotoxicity, absent in the mouse anti-Tac. Hence, humanized antibodies have been developed to a tumor-associated antigen and activated T-cell marker with significant features that offer new therapeutic possibilities for select neoplastic and immune disorders.

INTRODUCTION

Therapy with monoclonal antibodies has been hampered by two major problems. First, most monoclonal antibodies are foreign proteins and are neutralized when patients develop antibodies to the foreign antigens, usually within the first month of treatment. Second, most monoclonal antibodies are of mouse origin and are often less effective than human antibodies at recruiting human immune effector functions. Consequently, the record of monoclonal antibodies in therapy has been unimpressive overall, and the search for effective alternatives has focused on overcoming these two difficulties (1, 2).

Most interventions to control T-cell malignancies or immune function with immunotherapy have used antibodies against molecules distributed on all T-cells, with normal T-cells being eliminated along with those involved in the disease process (1, 2). To develop a more directed strategy, we have focused on the p55 peptide of the IL2² receptor system (Tac; CD25). Resting T-cells do not display this peptide on their surface but, when exposed to appropriate antigen, they express the p55 Tac peptide and secrete IL2. The newly expressed Tac cooperates with a second *M*_r 70,000/75,000 IL2-binding peptide (p75) to form the high affinity IL2-R and augments the activation response through improved IL2 binding (3, 4).

The p55 IL2-R peptide is a potentially versatile therapeutic target. High levels may be expressed on malignant cells in patients with certain lymphoid cancers (e.g., adult T-cell leu-

kemia, cutaneous T-cell lymphoma, hairy cell leukemia, Hodgkin's disease) and on activated T-cells in select autoimmune diseases (e.g., scleroderma, tropical spastic paraparesis, rheumatoid arthritis), as well as in individuals rejecting allografts or experiencing graft-versus-host responses (4). Agents that eliminate Tac-expressing malignant cells or activated T-cells could be effective in these disorders yet should not harm the normal Tac-negative mature T-cells and their precursors, thus preserving the full repertoire of cells and antigen receptors required for subsequent normal T-cell immune responses.

Anti-Tac is a mouse IgG2a,_κ antibody that recognizes the p55 Tac peptide. Anti-Tac competes with IL2 for Tac binding and blocks the activation of T-cells in antigen-specific assays, preventing the generation of cytotoxic T-lymphocytes. Unmodified anti-Tac has been used in renal allograft patients and successfully delays graft rejection but is limited by an early antiglobulin response. Although inactive in ADCC with human effectors, anti-Tac has also been used with modest effect in human T-cell leukemia virus-1-associated adult T cell leukemia, with responses of 1 to 8 months in 6 of 16 patients. Our experience with anti-Tac thus recapitulates the difficulties with monoclonal antibodies stated above: immunogenicity and ineffective recruitment of host effector functions (4, 5).

As one approach to these problems, we have produced humanized chimeric and "hyperchimeric" forms of anti-Tac by genetic engineering (6). In the chimeric versions, human constant regions are joined to mouse variable regions from the light and heavy chains of anti-Tac. In the more extensively reconstructed hyperchimeric antibody, the variable framework regions are replaced with human sequences as well, retaining the small CDRs from the original mouse anti-Tac antibody that are essential to epitope recognition. We propose to call an antibody prepared in this manner (7-9) a "hyperchimeric" antibody, and our particular construct anti-Tac-H, following the convention of Hale *et al.* (10). In other systems, this strategy has reduced immunogenicity of antibodies (10, 11) and, by exchanging human for mouse constant regions, may improve effector function as well (12-15).

In the present report, we describe the functional properties of these constructs. We demonstrate that humanized versions of anti-Tac retain high affinity for the Tac peptide and block proliferation of T-cells activated by foreign or alloantigens. Moreover, these antibodies add the new functional activity of ADCC against human tumor targets that is enhanced by lymphokine activation of effectors. This activity, absent in the original mouse anti-Tac, may be essential to the success of these products as antitumor agents.

MATERIALS AND METHODS

Cell Lines. The human adult T-cell leukemia line HuT-102-B2 was obtained from American Type Culture Collection. This line is phenotypically an activated T-cell and expresses Tac at high levels (2×10^5 molecules/cell).

Antibodies. Anti-Tac was purified from murine ascites as described previously (16). 7G7/B6 was a gift of Dr. D. Nelson. Human anti-B17

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² The abbreviations used are: IL2, interleukin 2; IL2-R, IL2 receptor; ADCC, antibody-dependent cell-mediated cytotoxicity; CDR, complementarity-determining region; PBMC, peripheral blood mononuclear cell; CDC, complement-dependent cytotoxicity; MLR, mixed leukocyte reaction; E/T ratio, effector-to-target ratio; HLA, human leukocyte antigen; p55, the *M*_r 55,000 peptide of the IL2-R; p75, the *M*_r 70,000/75,000 peptide of the IL2-R.

anti-HLA antiserum was obtained from the NIH tissue-typing laboratory. Other reagents were obtained from commercial sources: rabbit anti-mouse immunoglobulin antiserum (Behring, Somerville, NJ), rabbit anti-human immunoglobulin antiserum (Cappel, Malvern, PA), and IgG2a, κ mouse myeloma proteins UPC-10 (Litton, Charleston, SC) and RPC-5 (Cappel). Antibody concentrations were measured by the Bradford microassay (17).

Preparation of Chimeric and Hyperchimeric Antibodies. Details of the construction of these antibodies are presented elsewhere (6). In brief, complementary DNA clones of the anti-Tac light and heavy chain variable regions were combined with genomic clones of the human κ , G1, and G3 constant regions supplied by L. Hood (California Institute of Technology, Pasadena, CA) and I. Kirsch (National Cancer Institute, Bethesda, MD) and with mouse G2a and G3 constant regions supplied by P. Tucker (University of Texas, Dallas, TX). The light and heavy chain constructs were placed on mammalian expression vectors, which respectively contained *gpt*- and *hyg*-selectable markers, and were transfected into mouse myeloma Sp2/0 cells by electroporation. Producer clones were selected first by appropriate antibiotic resistance and then by enzyme-linked immunosorbent assay for secretion of human immunoglobulin. Antibodies were purified from culture supernatant using Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) and a BakerBond ABx column (Baker, Phillipsburg, NJ).

For construction of the hyperchimeric antibody, variable region sequences were prepared that combined the framework of the human EU antibody (18) with the six CDRs of the anti-Tac light and heavy chains. Several other amino acids were also retained from the anti-Tac sequences to help maintain conformational integrity of the transplanted CDRs, as described (6). The hyperchimeric variable regions were constructed by total gene synthesis and combined with human constant G1 and κ regions as outlined above.

Bindability. Activity was determined as the fraction of antibody that could be bound by excess antigen. Antibody was labeled with ^{125}I with Iodobeads (Pierce, Rockford, IL) to 0.3–1 $\mu\text{Ci}/\mu\text{g}$ and stored in 1 mg/ml ovalbumin. Radiolabeled antibody (1–5 ng) was incubated with $10\text{--}20 \times 10^6$ Tac⁺ HuT-102 cells in 0.2–0.4 ml of binding medium [RPMI plus 10% fetal calf serum, 100 $\mu\text{g}/\text{ml}$ human IgG (Gammagard, Highland, Glendale, CA), 0.1% sodium azide] for 1–2 h at 22°C under constant agitation. These conditions yield 17 nM Tac peptide with a binding capacity for anti-Tac of 500–1000 ng. Cells were washed and centrifuged and pellets were counted. Assays at 4°C proceeded to the same endpoint of functional binding but with slower kinetics (not shown).

Scatchard Analysis. Antigen-antibody affinity was assessed by binding of radiolabeled antibody to HuT-102 targets. Increasing amounts of unlabeled anti-Tac were mixed with 1.5 ng of ^{125}I -radiolabeled tracer anti-Tac (2 $\mu\text{Ci}/\mu\text{g}$), incubated with 4×10^5 HuT-102 cells in 0.2 ml binding buffer for 3 h at room temperature, and processed as in the bindability tests above. After counting, the concentrations of bound and free antibody were calculated and the antibody affinity was derived according to the method of Scatchard.

Competition Binding. Parallel competition binding assays were performed with anti-Tac and chimeric antibodies against radiolabeled tracer anti-Tac. Assays were performed as for the Scatchard analysis above. The affinity constant K_x for each competitor X was derived from the formula $([X]_{1/2} - [\text{anti-Tac}]_{1/2}) = (1/K_x - 1/K_0)$, where K_0 is the affinity constant for anti-Tac ($9 \times 10^9 \text{ M}^{-1}$) and $[]_{1/2}$ indicates the concentration of unlabeled competitor at which tracer binding is $R_0/2$ (19), where R_0 is the bound/free tracer ratio at maximal binding. Competitor concentrations were normalized on the basis of antibody bindability determined above. Competition data corresponded closely with theoretical curves (not shown).

T-Cell Proliferation. PBMCs were harvested on Ficoll density medium from human whole blood and aliquoted in triplicate into 96-well plates at 10^5 cells/0.2 ml of medium (RPMI plus 10% human AB serum), with antigen and varying concentrations of antibody. The quantities of influenza A virus and tetanus toxoid antigens were determined by prior titration. Plates were maintained in a 5% CO_2 incubator at 37°C for 6 days, when 1 μCi [^3H]thymidine was added to each well. Incubation continued overnight, and cells were harvested on day 7 with

a Skatron cell harvester and counted. MLR assays were performed as above with the exception that 10^5 X-irradiated (2000 rad) heterologous human PBMCs/well were used as the source of antigen.

CDC. Antibody was added at 1 $\mu\text{g}/\text{ml}$ to 2000–5000 ^{51}Cr -labeled HuT-102 cells, in 0.15 ml growth medium in triplicate in 96-well U-bottomed plates, and neonatal rabbit serum (Pelfreez, Brown Deer, WI) was added to 1:30 or 1:15 final concentration and maintained at 37°C in a CO_2 incubator for 1 h. Supernatants were harvested with a Skatron tampon harvester and counted. Percent specific release was calculated as the fraction [(experimental release – release without antibody)/(Triton release – release without antibody)] $\times 100$.

Complement Fixation. HuT-102 cells (10^6 /assay) were precoated with 1 $\mu\text{g}/\text{ml}$ antibody, at 0°C for 1 h in serum-free AIM5 growth medium (GIBCO, Grand Island, NY) in triplicate, and washed once with cold phosphate-buffered saline plus 0.1% sodium azide. ^{125}I -labeled purified human C1q (2 $\mu\text{Ci}/\mu\text{g}$) (gift of Dr. A. Tenner, American Red Cross, Rockville, MD) was added to 0.5 $\mu\text{g}/\text{ml}$ in 0.2 ml cold phosphate-buffered saline plus 0.1% azide and incubated on ice for 15 min. Cells were spun through oil (80:20 dibutylphthalate:olive oil) and pellets were counted. Backgrounds with no antibody were the same as with nonspecific mouse antibody UPC-10. Backgrounds were subtracted from all samples to obtain specific bound counts, and molecules bound per cell were calculated.

ADCC. ADCC was measured as for CDC with the exception that, instead of complement, PBMCs were added as effector cells at specified ratios and incubations were for 4 h. PBMCs were harvested during the day preceding the assay and maintained overnight in complete medium in 37°C in a CO_2 incubator. PBMCs treated with IL2 were incubated for 14–16 h in the presence of 1 nM (300 units/ml) human recombinant IL2 (20×10^6 Cetus units/mg, 100% bioactive; Hoffmann-La Roche, Nutley, NJ), washed twice in complete medium, and stored at room temperature until used. PBMCs from three different individuals were tested in the ADCC assay with comparable activities; all results reported are from a single individual.

RESULTS

Construction of Chimeric Antibodies. Humanized anti-Tac antibodies were prepared in two stages, first as chimeric then as hyperchimeric constructs (Fig. 1). Chimeric antibodies were prepared by combining the cloned light and heavy chain variable region genes of anti-Tac, a mouse IgG2a, κ antibody, with human constant region genes from available genomic libraries. The details of the cloning and the molecular constructions are described elsewhere (6). The sequences of the mouse κ constant region and part of the mouse G2a constant region were determined and agree with published results (18). The human κ and G1 constant regions used in the chimeric and hyperchimeric antibodies were fully sequenced and had allotypes Km(inv3) (20) and G1m(1,17) (21). A second G1 and a G3 constant region were also used to prepare chimeric antibodies, which were not fully sequenced and are of unknown allotypes. The completed heavy and light chain genes were transfected into Sp2/0 myeloma cells, which secreted the desired antibody.

The hyperchimeric antibody (Fig. 1) was itself constructed in

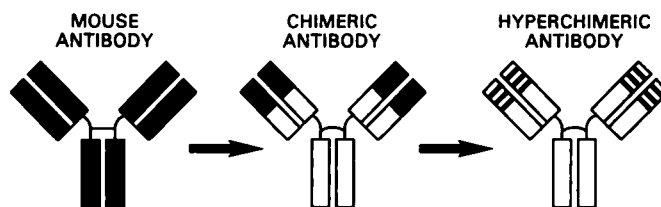


Fig. 1. Strategy for preparing humanized antibodies. The chimeric antibody has human heavy and light chain constant regions and mouse heavy and light chain variable regions, whereas the hyperchimeric antibody is human except for the CDRs, which are retained from the mouse antibody. ■, Sequences of mouse origin; □, sequences of human origin.

two stages. First, the six CDRs of the anti-Tac light and heavy chain variable domains were synthetically combined with the variable framework regions of the human EU antibody (18). Several additional amino acids were retained from the murine anti-Tac sequence to maintain the conformation of the CDRs within the binding domain (6). Next, the reconstructed light and heavy chain variable regions were combined with human κ and G1 constant regions, respectively. The completed genes were expressed in host cells as for the chimeric antibodies above.

Bindability and Affinity for Antigen. The active fractions of the chimeric and hyperchimeric antibodies were assessed by their ability to bind to the Tac-expressing human T-cell line HuT-102 (see Materials and Methods). All antibody constructs showed binding in the range of 60–80% with this cell line and were comparable to the native mouse antibody (Table 1). Scatchard analysis of mouse anti-Tac binding to HuT-102 cells gave linear graphics and a K_a of $9 \times 10^9 \text{ M}^{-1}$ (not shown). Competition binding demonstrated similar antigen affinities for the mouse anti-Tac and the human G1 and G3 chimeras, while the hyperchimeric antibody binding showed a 3-fold reduction in affinity (Table 1).

Inhibition of IL2-dependent Antigen-induced T-Cell Proliferation. One functional activity of the anti-Tac monoclonal antibody is its ability to block antigen-induced T-cell proliferation. In assays that measure the response of sensitized T-lymphocytes to antigen, the anti-Tac antibody blocks thymidine incorporation in a dose-dependent manner for both tetanus toxoid (Fig. 2) and influenza virus (not shown) stimulants. This capability is fully preserved in the chimeras as well as in the hyperchimeric antibody but is not observed with a control nonspecific mouse IgG2a, κ antibody. MLR assays were also performed to measure suppression of response to antigen on allogeneic cells, which yielded similar results (not shown). For both types of assay, the mean antibody concentrations required for 50% inhibition of T-cell proliferation were in the range of 0.5–1 $\mu\text{g}/\text{ml}$ (3–7 nM).

CDC. The parental mouse anti-Tac kills ^{51}Cr -labeled HuT-102 cells in the presence of neonatal rabbit serum (Fig. 3A). Although anti-Tac fixes human Clq, HuT-102 cells are not killed with this antibody when human serum is the complement source (not shown). None of the human chimeric or hyperchimeric antibodies was effective in CDC with HuT-102 cells with either rabbit (Fig. 3A) or human (not shown) complement. This was also true when an alternative chimeric G1 of unknown but presumably different allotype was prepared using a G1 constant region from another human genomic library. For each chimeric or hyperchimeric antibody, several transfectant clones were independently tested, and CDC was never detected. However, the addition of a rabbit anti-human immunoglobulin antiserum to chimeric or hyperchimeric antibody-coated cells led to cell killing (Fig. 3B). The addition of chimeric antibody (G1 or G3) to cells before mouse anti-Tac blocked cell lysis by the mouse antibody, whereas addition of chimeric antibody after the mouse anti-Tac had no inhibitory effect (not shown). These results demonstrate that the loss of CDC is neither due to the failure

Table 1 Binding characteristics of chimeric and hyperchimeric antibodies with Tac antigen

Binding assays and affinity measurements were performed as described in "Materials and Methods."

	Fraction bindable		Affinity, K_a (M^{-1})
	Expt. 1	Expt. 2	
Anti-Tac	0.74	0.67	9×10^9
Chimeric G1	0.64		9×10^9
Chimeric G3	0.80		9×10^9
Hyperchimeric G1		0.60	3×10^9

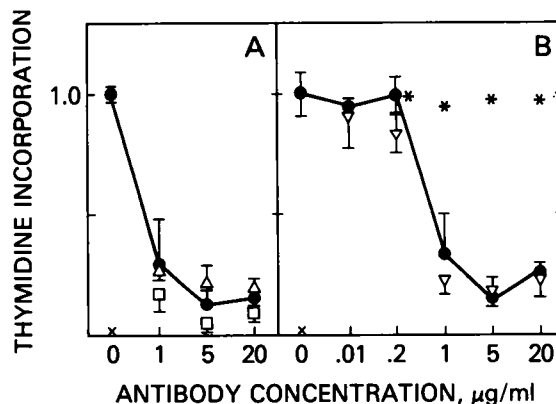


Fig. 2. Suppression of T-cell proliferation to the soluble antigen tetanus toxoid. Seven-day antigen stimulation assays were performed as described in "Materials and Methods," with varying concentrations of antibody included in the mixture. A, comparison of chimeric G1 and chimeric G3 with anti-Tac; B, comparison of hyperchimeric G1 with anti-Tac. ●, anti-Tac; □, chimeric G1; △, chimeric G3; ▽, hyperchimeric G1; ×, cells without added antigen; *, nonspecific mouse IgG2a, κ monoclonal antibody RPC-5. Values were normalized to incorporation in the absence of added antibody. Error bars represent 1 SD.

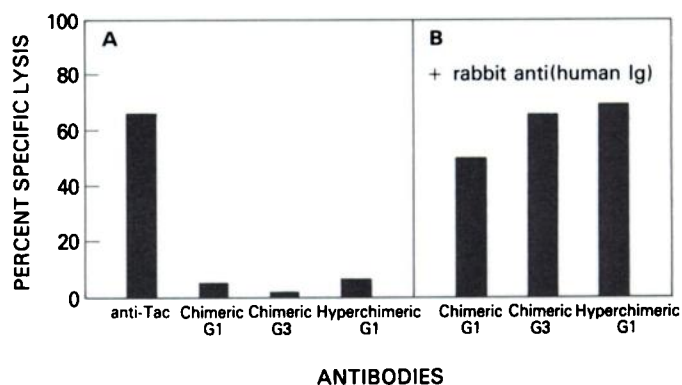


Fig. 3. CDC with chimeric antibodies. A, comparison of chimeric antibodies with anti-Tac; B, activity of chimeric antibodies with subsequent addition of second stage rabbit antibody. Chimeric antibody was incubated with targets for 30 min followed by washing to remove excess antibody. Rabbit anti-human immunoglobulin antiserum and rabbit complement were then added and incubated as in the standard assay. Rabbit anti-human immunoglobulin antiserum with control nonreactive human antibody gave no lysis.

of chimeric antibodies to bind to the targets nor due to the presence of an inhibitor of complement activity contaminating the chimeric antibody preparations but reflects instead a property intrinsic to the chimeric antibodies themselves.

A series of genetically engineered anti-Tac variants were prepared to define the cause of CDC failure with the chimeric antibodies. To examine whether there was some loss of a critical region of the anti-Tac variable domain during the complementary DNA cloning and transfection, genomically cloned mouse G2a and κ constant regions of known sequence were joined with the anti-Tac variable regions to reconstruct a "native" mouse anti-Tac. This construction retrieved CDC activity (mouse G2a, κ in Fig. 4A) and established that the cloned variable domain retained this function expressed by the parental mouse antibody. Another mouse antibody class-switch variant using the mouse G3 in place of the mouse G2a constant region was inactive (mouse G3, κ in Fig. 4A). To examine separately the role of heavy and light chains in this loss of CDC activity, two hybrid antibodies were prepared by transfection of appropriate genes into host cells. A hybrid antibody consisting of the mouse anti-Tac light chain and a human-mouse chimeric heavy chain lacked CDC activity, while an antibody consisting of the mouse heavy chain and the chimeric light chain retained activity (Fig.

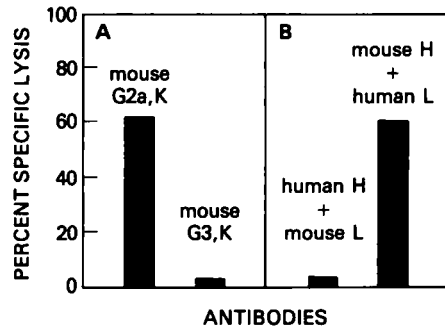


Fig. 4. CDC with reconstructed antibodies. *A*, comparison of mouse antibodies constructed by genetic engineering. *mouse G2a, κ* , mouse G2a heavy chain and mouse κ light chain constant regions joined with anti-Tac variable regions; *mouse G3, κ* , mouse G3 heavy chain and mouse κ light chain constant regions joined with anti-Tac variable regions. *B*, comparison of hybrid chimeric antibodies. *human H + mouse L*, chimeric G1 heavy chain with mouse anti-Tac κ light chain; *mouse H + human L*, mouse anti-Tac G2a heavy chain with chimeric κ light chain.

Table 2 Complement fixation by chimeric antibodies

Complement fixation was determined with antibody-coated HuT-102 cells and 125 I-labeled C1q as described in "Materials and Methods." Nonreactive mouse monoclonal antibody UPC-10 is an isotype-matched control for anti-Tac. Anti-B17, a control positive human anti-HLA antiserum reactive with HuT-102, was used in subsaturating quantities.

Antibody	Specific binding of human C1q (molecules/cell)
Cells + anti-Tac	15,600 \pm 1,000
Cells + UPC-10	<100
Cells + chimeric G1	<100
Cells + chimeric G3	<100
Cells + NHS ^a	100
Cells + anti-B17 (limiting)	3,100

^a NHS, normal human serum.

4B). Hence, the abrogation of CDC activity is associated with alterations in the heavy chain component of the chimeric antibodies.

The fixation of C1q by antibodies precedes the steps where any of the known inhibitory factors (*i.e.*, decay-accelerating factor and homologous restriction factor) have actions that protect human hematopoietic and other cells from lysis by autologous complement (22–24). Although anti-Tac did not promote lysis of HuT-102 with human serum as the complement source, it can fix rabbit and human C1q on sensitized target cells. In the case of the G1 and G3 chimeras, however, the loss of activity in the CDC assay with rabbit serum correlated with a loss of ability to fix human C1q (Table 2).

ADCC. This assay measures the ability of an antibody to recruit cellular CD16 (Fc receptor)-positive effectors of the large granular lymphocyte class to perform killing of sensitized targets (25). Native mouse anti-Tac is inactive in ADCC. Therefore, a different positive control was needed for these assays. For this purpose, we wanted an antibody with human constant regions that could be a model for activity or lack thereof in the chimeric antibodies. The HuT-102 cell line was shown to express HLA phenotype A3, A30, B17, B18, DR2, DQw1. Of several human anti-HLA antisera evaluated, an anti-B17 serum was selected as the most active in ADCC and was utilized as positive control throughout (Fig. 5A).

Neither anti-Tac nor 7G7/B6, a mouse IgG2a, κ monoclonal antibody directed toward another epitope of the p55 IL2-R peptide (26), was able to promote ADCC activity, even when used in combination (Fig. 5B). Mouse antibodies are often less active than human, rat, or rabbit antibodies in ADCC with human effector cells and targets (2, 27). Hence, when a second stage rabbit anti-mouse immunoglobulin antiserum was added

after incubation of the HuT-102 with mouse anti-Tac plus 7G7/B6, substantial ADCC was obtained (Fig. 5B), comparable to that observed with the control anti-HLA B17 antiserum (Fig. 5A). In contrast to the mouse anti-Tac, the chimeric G1 and hyperchimeric G1 expressed ADCC activity, which was modestly augmented with higher E/T ratios and was stimulated when effector cells were activated with IL2 (Fig. 5C). While ADCC with resting (nonactivated) effectors was significantly improved with a second stage rabbit anti-human immunoglobulin antibody, no improvement in ADCC with IL2-stimulated effectors was obtained with this treatment. This suggests that target engagement by resting PBMCs is enhanced with the denser antibody "halo" afforded by the addition of a second stage antibody but that target engagement is already maximal with chimeric antibodies alone when activated effector cells are employed.

The influence of the E/T ratio on the expression of ADCC was assessed with each antibody at 1 μ g/ml, without (–IL2) and with (+IL2) activation of effectors (Fig. 6). An anti-B17 control was run in parallel. From 5:1 to 200:1, increases in E/T ratio promoted a steady increase in target lysis with the G1 chimeric and hyperchimeric antibodies, which was further stimulated by IL2 activation of the effectors. The mouse anti-Tac antibody and G3 chimera were negative in the assay (Fig. 6), as was the class-switch construct of mouse G3 isotype (not shown), reported to have improved effector cell recruitment (25). Killing is proportional to the E/T ratio only up to 10:1 or 30:1 for the active constructs. (Note that abscissa is not a linear scale.) Above this level, the increment in killing grew progressively smaller but never showed a true plateau over the range of the assay.

The concentration dependence of the different mouse and humanized anti-Tac antibodies in ADCC was studied with a fixed 100:1 E/T ratio, without (–IL2) and with (+IL2) activation of effectors (Fig. 7). Only the G1 chimera and hyperchimeras expressed activity. The parental mouse anti-Tac antibody and the G3 chimera were again essentially inactive even at the highest concentrations. The activity with the chimeric G1 plateaued at 0.1 μ g/ml, although this concentration is suboptimal for the hyperchimeras in the +IL2 ADCC, for which the activity curve is shifted to the right. At concentrations that are clearly saturating (1 μ g/ml = 7 nM), however, there is no consistent difference in ADCC between the two G1 constructs in this or other tests (*e.g.*, Fig. 5).

One possible explanation for the slight shift in the concentration-activity curve for the hyperchimeras to higher antibody concentrations (Fig. 7) is a less efficient coating of target cells as a consequence of its lower binding affinity (Table 1). This hypothesis is examined in Fig. 8, where calculated binding curves for the G1 chimeric and hyperchimeric antibodies show remarkable concordance with observed ADCC data replotted from Fig. 7B. We therefore conclude that the difference in ADCC activities at lower concentrations of antibody is compatible with the difference in antibody affinities for the target; that is, for equivalent antibody bound, the hyperchimeric G1 was as active in ADCC as the chimeric G1.

The minimum concentration of antibody for detecting ADCC is somewhere between 1 and 10 ng/ml (7 and 70 pM) for the +IL2 ADCC and near 10 ng/ml for the –IL2 ADCC (Fig. 7). The concentration of cellular Tac peptide in the ADCC assay is of the order of 7 pM. On the basis of the degree of receptor saturation expected with different concentrations of antibody (Fig. 8), we can predict that ADCC is first measurable between 10,000 and 30,000 antibody molecules bound/cell and sets the

Fig. 5. ADCC with chimeric and hyperchimeric antibodies. *A*, ADCC with control anti-B17 human antiserum. *B*, ADCC with mouse antibodies. For the + rabbit anti-(mouse Ig) tests, anti-Tac plus 7G7B6 were followed by a second stage rabbit anti-mouse immunoglobulin antiserum, with an intervening cell wash to remove excess mouse antibody. *C*, ADCC with chimeric antibodies. For the + rabbit anti-(human Ig) tests, hyperchimeric antibody was followed by a second stage rabbit anti-human immunoglobulin antiserum, with an intervening cell wash to remove excess hyperchimeric antibody. All antibodies were at 1 $\mu\text{g}/\text{ml}$ and second stage antisera and anti-B17 were at 1:100 dilution. Rabbit antisera with control nonreactive mouse or human antibody gave no lysis. The first pair of bars for each experiment is prepared with an E/T ratio of 30:1 and 100:1 without IL2 activation (-IL2) and the second pair of bars is prepared with an E/T ratio of 30:1 and 100:1 with IL2 activation (+IL2).

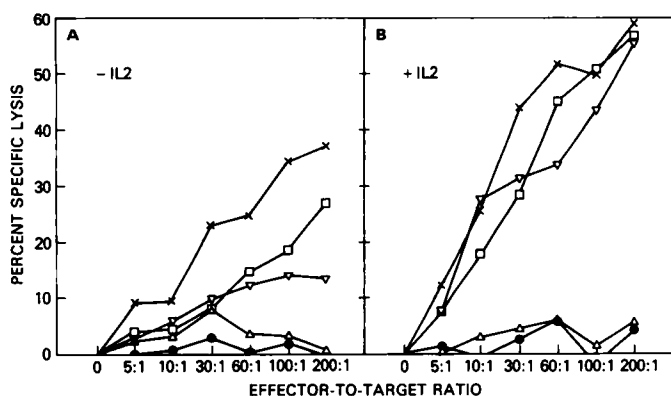
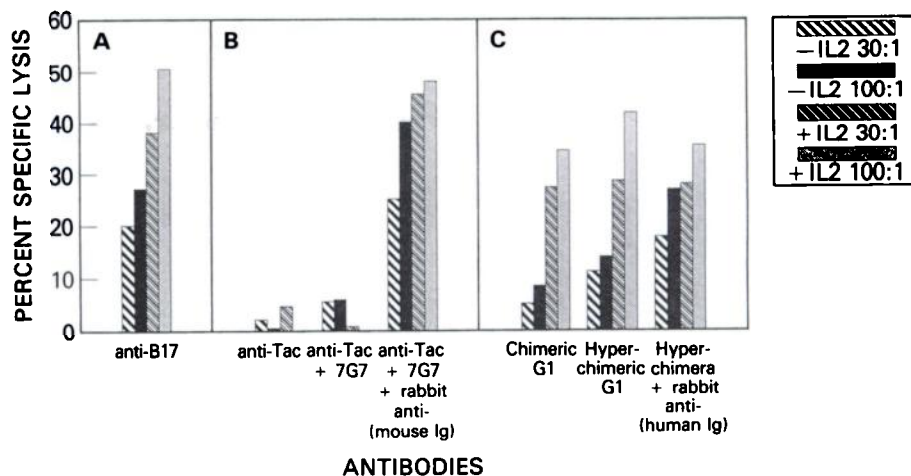


Fig. 6. ADCC of chimeric antibodies as a function of E/T ratio and IL2 activation. *A*, without IL2 activation of effectors (-IL2). *B*, with IL2 activation of effectors (+IL2). All antibodies were at 1 $\mu\text{g}/\text{ml}$ and control anti-B17 antiserum at 1:50 dilution. ●, anti-Tac; □, chimeric G1; △, chimeric G3; ▽, hyperchimeric G1; ×, anti-B17.

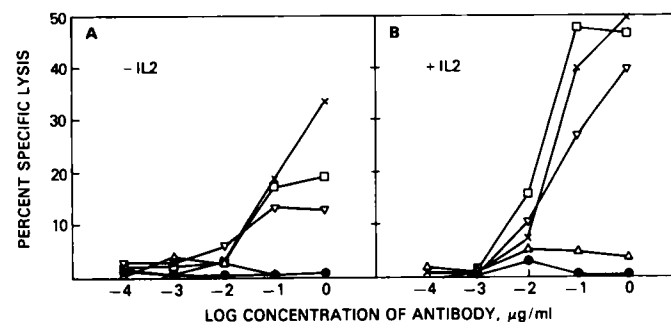


Fig. 7. ADCC of chimeric antibodies as a function of concentration and IL2 activation. ADCC was measured with an E/T ratio of 100:1 and antibodies at the concentrations indicated on the abscissa, except for the anti-B17 antiserum, which is successively 10-fold diluted from a maximum of 1:50. *A*, without IL2 activation of effectors (-IL2). *B*, with IL2 activation of effectors (+IL2). ●, anti-Tac; □, chimeric G1; △, chimeric G3; ▽, hyperchimeric G1; ×, anti-B17.

minimum limits for antigen density for a target cell if it is to be useful in this type of assay.

DISCUSSION

We have applied the technology for production of humanized antibodies to the mouse monoclonal antibody anti-Tac, which identifies an antigen that plays a pivotal role in T-cell activation, proliferation, and function and that is present as a tumor marker in certain malignancies. Chimeric antibodies have been

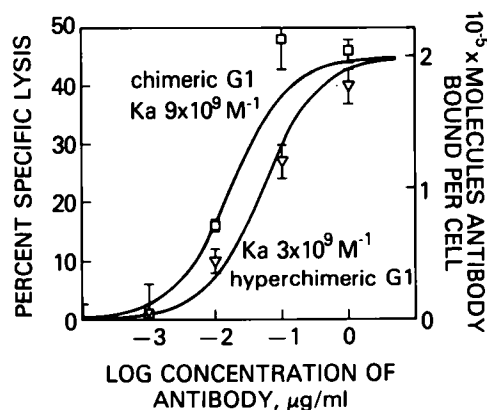


Fig. 8. Comparison of antibody binding with ADCC activity. The data for the G1 chimera and hyperchimeric G1 from Fig. 7B are replotted with superposition of binding curves (solid lines) showing the number of molecules of antibody bound per cell for each concentration of input antibody. Binding curves were calculated from mass action equations with a computer program written for that purpose, using a measured value of 200,000 Tac receptors/cell. The K_a for the left-most curve is shown as $9 \times 10^9 \text{ M}^{-1}$, corresponding to that of the G1 chimera, and the K_a for the right-most curve is shown as $3 \times 10^9 \text{ M}^{-1}$, corresponding to the lower affinity of the hyperchimeric antibody. □, chimeric G1; ▽, hyperchimeric G1. Error bars represent 1 SD.

prepared to yield molecules with human constant regions and mouse variable regions. Furthermore, a hyperchimeric construct, termed anti-Tac-H, has been generated, in which even the variable framework regions have been replaced by human equivalents, i.e., everything has been made "human" except the mouse hypervariable segments critical to the specificity of epitope binding. The retained mouse segments vary in size from 6 to 19 amino acids and account for less than 10% of the protein mass, making the hyperchimeric antibody greater than 90% human. Whether these mouse segments are small enough or lack essential structural characteristics to avoid recognition as foreign antigens by human helper T-cells (28) will be determined in clinical trials, but results in animal (29, 30) and human (10, 11) systems suggest that an antglobulin response can be avoided. Our *in vitro* assessments of biological activities of these chimeric antibodies suggest that they may be superior to the native mouse antibody in recruiting the effector functions necessary to kill targeted cells in therapeutic applications. Table 3 is a summary of the results of these studies categorized by binding characteristics, blockade of T-cell activation, and immune effector functions.

Antigen Binding. All constructs showed bioactive (bindable) fractions comparable to that of the native mouse antibody

Table 3 *In vitro* activities of antibodies

Antibody construct ^a	Binding		Block T-cell proliferation		Effector functions		
	ELISA ^b or competition	K_a (M^{-1})	Soluble antigen-induced	MLR	Humoral		Cellular
					CDC	Human C1q fixation	ADCC
Mouse							
Anti-Tac mouse G2a	+	9×10^9	+	+	+ ^c	+	-
Anti-Tac mouse G3	+				-		-
Chimeras							
Chimeric human G1	+	9×10^9	+	+	-	-	+
Chimeric human G3	+	9×10^9	+	+	-	-	-
Hyperchimeric human G1	+	3×10^9	+	+	-	-	+
Mouse-chimera hybrids							
Mouse light/chimeric G1 heavy	+				-		
Chimeric light/mouse G2a heavy	+				+ ^c		

^a Mouse and chimeric constructs use mouse and human κ light chain constant domains, respectively.

^b ELISA, enzyme-linked immunosorbent assay.

^c Result is for rabbit serum only; does not perform CDC with human serum.

(Table 1). There was no evidence for disruption of the mechanisms of antibody assembly in the transfected cells by binding assay or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reduced and nonreduced proteins (not shown). We have also shown preservation of binding affinities with the G1 and G3 chimeras at $9 \times 10^9 M^{-1}$. This was anticipated because no change was made in the variable regions of the constructs and accords with the results of others (12, 13, 31–33). On the other hand, the hyperchimeric antibody showed a factor of 3 loss of affinity to $3 \times 10^9 M^{-1}$, in itself not surprising given the extent of manipulation required to prepare the engineered protein. This compares favorably with other hapten (7) or protein (8, 9) antigen systems in which antibody framework regions have been manipulated, with losses of affinity of 1.6- to 10-fold. Because of the exceptional avidity of the parental mouse anti-Tac antibody, this 3-fold reduction nonetheless results in high affinity binding by the hyperchimeric product.

Inhibition of T-Cell Activation and Proliferation. Activation of T-lymphocytes is a complex process. After antigen presentation in the context of major histocompatibility complex class II on presenting cells, appropriate T-cells are stimulated to produce IL2 and express Tac. The initial phase of activation is antigen specific, but the magnitude of T-cell proliferation is determined by IL2 and the IL2-R response. While the p75 peptide of the IL2-R is sufficient by itself to initiate proliferation in high, supraphysiological concentrations of IL2 (3, 34–37), the presence of the p55 (Tac) peptide greatly amplifies this response under physiological conditions by increasing the affinity for IL2 in forming the so-called high affinity p55-p75 IL2-R (38–42). Anti-Tac competes with IL2 for p55 protein binding and blocks T-cell activation by preventing formation of the high affinity IL2-R complex. Accordingly, for a chimeric anti-Tac antibody to inhibit T-cell activation and proliferation, it would seem only to require that antibody binding be preserved. Indeed, our data show that the G1 and G3 chimeras and the G1 hyperchimera are indistinguishable from mouse anti-Tac in both soluble antigen and MLR assays that measure suppression of T-cell proliferation.

CDC. Complement-dependent killing is a feature expressed by many γ -globulins of both mouse and human origin. Human G1, G2, and G3 (but not G4) and murine G2a, G2b, and G3 (but not G1) antibodies all fix complement and promote lysis of coated targets (43). Anti-Tac efficiently lyses cells in the presence of rabbit complement. Yet several constructs involving manipulation of the heavy chain, including two human G1

constructs of (presumably) different allotypes, a human G3 construct, and a mouse G3 class-switch variant, were curiously ineffective in complement fixation and lysis. When the cloned anti-Tac heavy chain variable region was genetically reassembled with a mouse G2a constant region, complement-dependent lysis was recovered.

This result was unanticipated. With intact CH2 domains, it has been considered that the sole requirement for C1 fixation and complement activation was the formation of IgG antibody doublets on the cell surface (44, 45). Yet this finding suggests that such doublets are not by themselves sufficient to fix and activate complement and revives the dormant notion that some antigen-induced conformation change must also occur to initiate the process (46, 47). Inasmuch as the simple chimeras and the hyperchimera in the present study were equally affected, we must consider that there is something unusual about the anti-Tac heavy chain variable domain that makes it unable to transmit such an antigen-induced signal through any but a mouse G2a constant region.

While of theoretical concern to the construction of chimeric antibodies, the loss of complement fixation is probably inconsequential for therapy. Cells of the human hematopoietic system (especially activated T-cells, such as HuT-102) are richly endowed with potent phosphoinositol-linked membrane activities, decay-accelerating factor and homologous restriction factor, which act at steps subsequent to C1 fixation to inactivate the human complement cascade (22–24). Rabbit serum kills because it bypasses the human species restriction of these activities (22, 23). Hence, although mouse anti-Tac performs this early step unimpeded and fixes human C1 efficiently, it is unable to lyse HuT-102 with human serum; rabbit antilymphocyte globulin and several human anti-HLA antisera (A3, B17, B18, DR2) also failed to kill with human serum (not shown). Similarly, it is probable that the targets for anti-Tac *in vivo* would also be resistant to complement killing even if the chimeric antibodies were capable of C1 fixation.

ADCC. Mouse antibodies are frequently unable to promote ADCC in assays with human effectors and nucleated target cells displaying natural (nonhapten) antigens (12–15, 27). Yet the ability to recruit cellular effector mechanisms is presumed to be important to the therapeutic efficacy of any antibody, whether its target be tumor or normal activated T-cells. These studies accordingly demonstrate the accomplishment of one of the major premises of the effort to prepare chimeric antibodies: that exchange of human for mouse constant regions can im-

prove effector function of the antibody.

Of the antibodies studied, only those of the human G1 isotype promoted ADCC (Table 3). This conforms with the results of others showing IgG1 to be the best of the human isotypes in ADCC (48, 49). Studies of antibody concentration and ADCC demonstrated activity in the assay below 10 ng/ml that plateaued near 1 µg/ml, information that is important in the design of patient trials to ensure continued therapeutic levels. The concentration-activity curve for the hyperchimeric G1 was displaced relative to the chimeric G1, but the shift was entirely accounted for by the 3-fold lower affinity of the hyperchimeric antibody. The implication of this result is that, per molecule of antibody bound, the G1 chimera and hyperchimeric are equally potent in ADCC. This accords with expectation inasmuch as both antibodies have identical Fc regions.

ADCC with these antibodies was also markedly (2- to 5-fold) stimulated with activation of effectors. Other investigators have shown that IL2 activation can enhance ADCC with mouse monoclonal antibodies that are active with human targets and effectors (25, 50, 51), which we now extend to the example of chimeric antibodies with human constant regions. The target cells for our particular antibody, Tac-positive T-cells, are mostly IL2 responsive themselves, and use of IL2 with chimeric versions of anti-Tac for treatment of T-cell disorders may not be possible without exacerbating the original condition. The results, however, point to the potential of lymphokines with humanized antibodies in the more general case of antitumor therapies where IL2 (or another cytokine) does not itself stimulate the target cell.

Human-mouse chimeric and hyperchimeric antibodies to the Tac T-cell activation antigen have been prepared by genetic engineering which maintain the functions of high affinity binding and blockade of T-cell activation and proliferation. An important new feature of these products is their ADCC activity, which is absent in the parental mouse anti-Tac and may be critical to *in vivo* function. Moreover, the hyperchimeric version of anti-Tac (anti-Tac-H) is greater than 90% human, with the likelihood of minimal immunogenicity (10, 11). Anti-Tac-H thus provides us with a novel and potentially versatile therapeutic reagent, with possible applications in adult T-cell leukemia and other malignancies and in allograft, graft-versus-host, and autoimmune settings.

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