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Immunology

Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific

(species specificity/protein sequence)

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ABSTRACT **Complementary DNA clones encoding two distinct tumor necrosis factor receptors were isolated from a mouse macrophage cDNA library. The cDNA for murine tumor necrosis factor receptor type 1 (mTNF-R1) predicts a mature polypeptide of 425 amino acids that is 64% identical to its human counterpart, whereas the cDNA of murine tumor necrosis factor receptor type 2 (mTNF-R2) predicts a mature protein of 452 amino acids that is 62% identical to human tumor necrosis factor receptor type 2. The two murine tumor necrosis factor receptors have limited sequence homology (≈20% identity) in their extracellular regions but no apparent similarity in their cytoplasmic portions. Northern (RNA) analysis indicates a single 2.6-kilobase (kb) transcript for mTNF-R1; a 3.6-kb and a more predominant 4.5-kb transcript are observed for mTNF-R2. A human cell line transfected with either mTNF-R1 or mTNF-R2 expression vectors specifically bound ^{125}I-labeled recombinant murine tumor necrosis factor α (TNF-α). Although mTNF-R1 had a similar affinity for both recombinant murine TNF-α and human TNF-α, mTNF-R2 showed strong specificity for recombinant murine TNF-α. This result suggests that the various activities of human tumor necrosis factor α reported in mice or in murine cell lines are probably mediated by mTNF-R1.**

Tumor necrosis factor α (TNF-α), a protein initially characterized by its ability to cause hemorrhagic necrosis in certain transplanted tumors (1), is now understood to be a multifunctional cytokine (2–4). This factor is produced mainly by activated macrophages, T cells, mast cells, and some epithelial tumor cells (5, 6). TNF-α not only exhibits cytotoxic properties but also has a wide range of other biological effects, including growth proliferation of normal cells and immunoinflammatory, immunoregulatory, and antiviral responses, and appears to be associated with endotoxic shock and cachexia (2–4, 7). The related molecule tumor necrosis factor β (TNF-β) shows many of the properties of TNF-α and is produced by activated lymphocytes (4, 8).

The various activities of TNF-α and TNF-β appear mediated by the same specific cell-surface receptors (9). Tumor necrosis factor (TNF) receptors have been detected on a wide variety of normal tissues and cell lines sensitive or resistant to TNF-α (10–13). Taken together, these studies suggest that binding of TNF to its receptor(s) is necessary, but not sufficient, for a subsequent cytotoxic effect. Although these early studies reported a single human TNF receptor type, later investigations suggested the existence of two classes of TNF receptor (14–16). Recently, two immunologically distinct cell-surface-associated TNF-binding proteins of 55-kDa and 75-kDa were identified (17–19). Studies with anti-55-kDa receptor (human type 1) antibodies suggest that this receptor is involved in several TNF-mediated processes, such as cytotoxicity, resistance to chlamidiae, and synthesis of prostaglandin E_2 (17, 18, 20). Although the cDNAs for both human proteins have recently been cloned (21–23), the biological activity mediated by each has yet to be demonstrated through expression of the cloned genes.

Similarly, studies conducted on mouse cell lines have also suggested the presence of more than one receptor for TNF. While the reported range of K_d values for binding of recombinant murine TNF-α (mTNF-α) to a number of mouse cell lines varies only slightly, the variability in the affinity of recombinant human TNF-α (hTNF-α) binding seemed to imply that murine cells possess at least two distinct receptors (24–27). Here we describe the cloning and expression of the murine homologs of the 55-kDa and 75-kDa human TNF receptors.¶ Subsequent binding analysis confirms that the different affinities observed for the binding of hTNF-α to various mouse cell lines are due to the existence of these two distinct mouse TNF receptors, one of which displays specificity for murine TNF.

MATERIALS AND METHODS

Cells and Reagents. The murine cell lines used in this study included the WEHI 164 fibrosarcoma (ATCC CRL 1751), L-M connective tissue (ATCC CCL1.2), 231F_1 T-cell hybridoma (28), CT6 [interleukin 2-dependent cytotoxic T cells (27)] and the M1-T22 monocyte cell line (29). The TSA 201 cell line is a subclone of the human embryonic kidney cell line, 293 (ATCC CRL 1573). The hTNF-α (30) and mTNF-α (31) (>10^7 units/mg) were provided by Genentech.

Identification of TNF Receptor cDNA Clones. A λgt10 cDNA library (31), prepared by using mRNA from the murine PU5-1.8 cell line, was screened with ^{32}P-labeled human TNF receptor coding-region probes (21–23). Duplicate filters were hybridized as described (31). Positive clones were plaque-purified and analyzed by restriction mapping, Southern blotting, and DNA sequencing (32).

Mammalian Cell Expression of Recombinant Murine TNF Receptors. Full-length coding regions for both receptors were inserted into the mammalian expression vector pRK5 (21).

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Abbreviations: TNF, tumor necrosis factor; mTNF-α, recombinant murine tumor necrosis factor α; hTNF-α, recombinant human tumor necrosis factor α; mTNF-R1, murine tumor necrosis factor receptor type 1; mTNF-R2, murine tumor necrosis factor type 2; hTNF-R1, human tumor necrosis factor receptor type 1; hTNF-R2, human tumor necrosis factor receptor type 2; NGF, nerve growth factor.

¶The sequences of the cDNA clones reported in this paper have been deposited in the GenBank data base (accession nos. M60468 and M60469).

TSA 201 cells ($4–5 \times 10^6$) were transfected with either the pRK5 expression vector alone or one of the two pRK5-mTNF-R constructs using the lipofectin reagent (BRL), according to manufacturer's instructions (5 μg of DNA per 10-cm dish) and analyzed for receptor expression 36 hr after transfection.

Radioligand Binding Analysis. mTNF-α was iodinated with $Na^{125}I$ by the Iodo-Gen method to a specific activity of 2500 Ci/mmol (1 Ci = 37 GBq). Cell lines were harvested as described (21) and resuspended in phosphate buffered saline (PBS)/0.1% bovine serum albumin/0.02% sodium azide (PBSA buffer). Saturation isotherm studies were done by incubating $0.5–1 \times 10^6$ cells with increased concentrations of ^{125}I-labeled mTNF-α (10 pM–33 nM) with and without a 300-fold excess of unlabeled mTNF-α in a final volume of 500 μl. For competition assays, $0.5–1 \times 10^6$ cells were incubated with 15 pM of ^{125}I-labeled mTNF-α, with or without increased concentrations of unlabeled mTNF-α or hTNF-α (500-μl reaction volumes). Incubation times were 2 hr at 4°C for both experiments. Bound and free ligand were separated by centrifugation at $12000 \times g$ for 15 min at 4°C. Cell pellets were washed with 1 ml of PBSA buffer, and bound radioactivity was determined by counting in a γ counter. Computer analysis (33) of binding data was used to generate Scatchard plots.

Northern Analysis. Total cytoplasmic RNA was extracted from cells using RNAzol reagent (Cinna Biotecx Laboratories, Friendswood, TX), electrophoresed on a 1.2% formaldehyde/agarose gel, and transferred to a GeneScreen (New England Nuclear) nylon filter membrane. RNA isolated from tissues was electrophoresed on 1.2% formaldehyde/agarose gels and transferred to nitrocellulose filters (21). Filters were hybridized and washed as described (21). Probes used for filter hybridizations were a random-primed radiolabeled 1.8-kilobase (kb) fragment (entire coding region) of murine TNF receptor type 1 (mTNF-R1) cDNA, and a 780-base-pair (bp) fragment (5'-half) of murine TNF receptor type 2 (mTNF-R2) cDNA, respectively. Autoradiography was done at −70°C with Kodak intensifying screens.

RESULTS

Isolation of cDNA Clones for mTNF-R1. A murine macrophage cDNA library was screened at low stringency with a DNA fragment corresponding to the coding region of the human 55-kDa TNF receptor (21). Two partial clones, which together contained the predicted full reading frame, were combined at a unique *Bgl* II site yielding the composite cDNA clone used for subsequent expression experiments.

The nucleotide sequence contains two potential translation initiation codons. The first ATG encountered at the 5' end is followed 6 bp downstream by an in-frame termination codon. The second potential initiation site provides an open reading frame of 1362 nucleotides that codes for a protein of 454 amino acids (Fig. 1). The first 29 amino acids are largely hydrophobic and probably constitute a signal peptide. The predicted signal peptide cleavage site (34) lies at position −1/+1 with leucine representing the amino-terminal residue of a 425-amino acid mature protein (predicted molecular mass of 47,277 Da). The structure of the protein suggested by hydrophobicity data predicts a typical receptor molecule, consisting of an extracellular domain of 183 amino acids (70% identity to hTNF-R1) and a cytoplasmic domain of 219 amino acids (59% identity to hTNF-R1), bisected by a transmembrane region of 23 amino acids (74% identity to hTNF-R1). As previously observed for hTNF-R1, the presumed extracellular domain of mTNF-R1 contains 24 cysteine residues, which can be divided into four related subdomains (21). The extracellular domain also contains three potential sites for N-linked glycosylation (positions 25, 122, and 173), two of which are conserved in hTNF-R1. The intracellular region of mTNF-R1 contains potential tyrosine kinase (35), protein kinase C (36), and cyclic nucleotide-dependent kinase (37) phosphorylation sites.

Isolation of cDNA Clones for mTNF-R2. Screening of the murine cDNA library with a 400-bp fragment from the coding region of the human 75-kDa receptor (23) yielded several partial-length cDNA clones. Two overlapping cDNA clones were used to generate the full-length coding region of mTNF-R2. The nucleotide sequence contains an open reading frame of 1422 nucleotides that codes for a protein of 474 amino acids (Fig. 1). As with mTNF-R1, hydrophobicity data predicts a typical receptor molecule, beginning with a signal peptide of 22 amino acids (55% identity with hTNF-R2). Based on predictions for signal-peptide cleavage sites (34), the N-terminal residue of the 452-amino acid mature protein (predicted to be 47,912 Da) would be the valine lying at position +1. A potential 29-amino acid transmembrane domain (65% identity to hTNF-R2) separates the 235-amino acid extracellular domain (58% identity to hTNF-R2) from the 188-amino acid intracellular domain. The intracellular region shows 73% identity to hTNF-R2; however, mTNF-R2 has an additional 13 amino acids at its C terminus. The extracellular domain of mTNF-R2 contains 22 cysteines and also two potential sites for N-glycosylation (positions 47 and 173), one of which is conserved in hTNF-R2. The intracellular region exhibits no remarkable sequence similarities to other known proteins, except for the presence of motifs for potential cyclic nucleotide-dependent protein kinase (37) and protein kinase C (36) phosphorylation sites. The intracellular domains of mTNF-R1 and mTNF-R2 show no significant homology to one another.

Expression of mTNF Receptors in Mammalian Cells. The cloned cDNAs for both receptors were inserted into a mammalian expression vector and transfected into human TSA 201 cells. TSA 201 cells were used because they display low background binding to mTNF-α (<1000 sites per cell; K_d of $\approx$5–10 nM). Saturation binding assays with ^{125}I-labeled mTNF-α concentrations ranging from 10 pM to 33 nM were done on transiently transfected cells (Fig. 2). The amount of specifically bound ^{125}I-labeled mTNF-α to cells expressing mTNF-R1 appears near saturation levels at 25 nM. Scatchard analysis of this data reveals a curvilinear profile characteristic of two binding sites: a high affinity ($K_d \approx 0.2$ nM) component with 1800 sites per cell and a low affinity ($K_d \approx 10$ nM) component of $\approx$13,000 sites per cell (Fig. 2*A*). The binding of ligand to cells transfected with the mTNF-R2 construct reached saturation at $\approx$2 nM mTNF-α (Fig. 2*B*). Scatchard analysis indicated a single binding site of high affinity ($K_d \approx 50$ pM) with $\approx$13,000 receptors per cell.

Competition binding assays were performed on transfected cells by using a constant amount of ^{125}I-labeled mTNF-α with increased amounts of unlabeled mTNF-α or hTNF-α. The displacement of ^{125}I-labeled mTNF-α from mTNF-R1-expressing cells was very similar when using either mTNF-α or hTNF-α as competitor (Fig. 3*A*). However, even concentrations of hTNF-α as high as 10 nM exhibited little or no displacement of ^{125}I-labeled mTNF-α from cells expressing mTNF-R2 (Fig. 3*B*). Fluorescence-activated cell sorting (FACS) analysis of transfected TSA 201 cells confirmed the results of the whole-cell competition binding assay. The binding of biotinylated mTNF-α to mTNF-R2-expressing cells could not be displaced by excess hTNF-α (unpublished data).

To compare the relationship of the transiently expressed murine TNF receptors and endogenous TNF receptors for various mouse cell lines, binding experiments were done on the mouse CT6 and L-M cell lines. K_d values of 30 pM for CT6 cells and 5–10 pM for L-M cells were obtained. To examine the possibility that CT6 and L-M cells may possess different receptors, competition binding experiments were done. Bound ^{125}I-labeled mTNF-α was displaced by competition

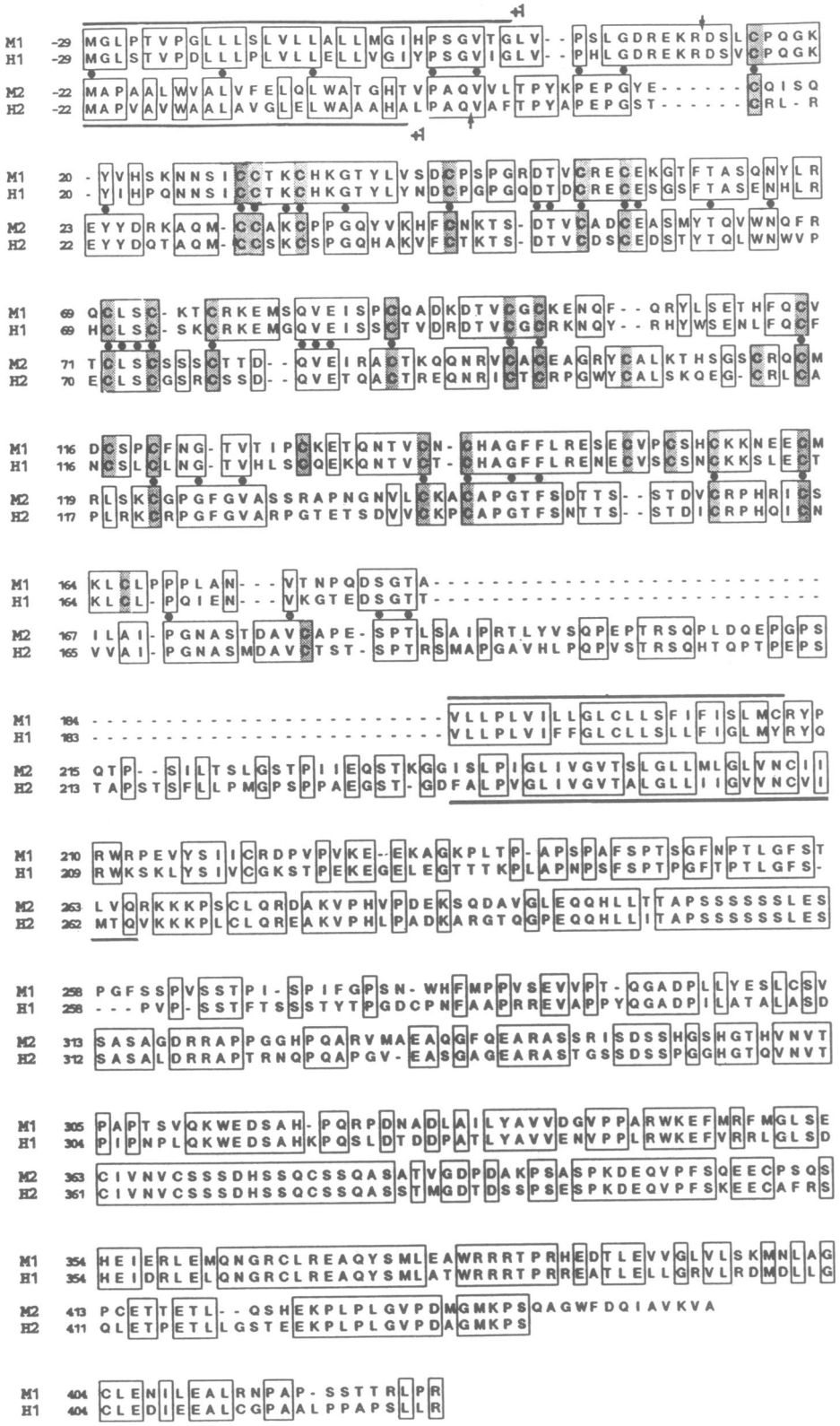

FIG. 1. Comparison of the deduced amino acid sequences of mTNF-R1 (M1) and mTNF-R2 (M2) to their human homologs (H1 and H2). Amino acids numbered −29 to −1 and −22 to −1 comprise the presumed signal peptide of TNF-R1 and -R2, respectively. Predicted N-terminal residues of mature receptors are indicated by +1. Identical residues between homologs are boxed, whereas residues shared by all four sequences are denoted by ● (alignment was not optimized for intracellular regions). Cysteine residues in the extracellular regions are shaded. Signal peptide and transmembrane domains are overlined for mTNF-R1 and human TNF receptor 1 (hTNF-R1) and underlined for mTNF-R2 and human TNF receptor 2 (hTNF-R2). Arrows, alternative N-terminal valine residues.

with mTNF-α from both cell lines, but when the competitor was hTNF-α, ≈40% displacement of bound radiolabeled ligand was observed for L-M cells, whereas no displacement was observed for CT6 cells (unpublished data).

Northern Blot Analysis. Several mouse cell lines were examined for the expression of mTNF-R1 and mTNF-R2 mRNA (Fig. 4*A*). A single mTNF-R1 mRNA species of 2.6 kb was detected in the WEHI fibrosarcoma, L-M fibroblast, 231F_1 T-cell hybridoma, and the M1 T22 mouse monocyte cell line. Only extremely low levels of this transcript were observed in the CT6 T-cell line. Analysis of the same cell lines for expression of mTNF-R2 revealed two mRNA transcripts of 3.6 kb and 4.5 kb, respectively. The amount of the 3.6-kb transcript, relative to the more predominant 4.5-kb transcript, remained the same for all cell lines examined. We also examined the level of mRNA for both receptors in normal mouse tissues (Fig. 4*B*). Both mTNF-R1 and mTNF-R2 mRNAs were seen in all tissues examined (brain, spleen, thymus, bone marrow, liver, and kidney) and were of the same size as those seen for cultured cell lines.

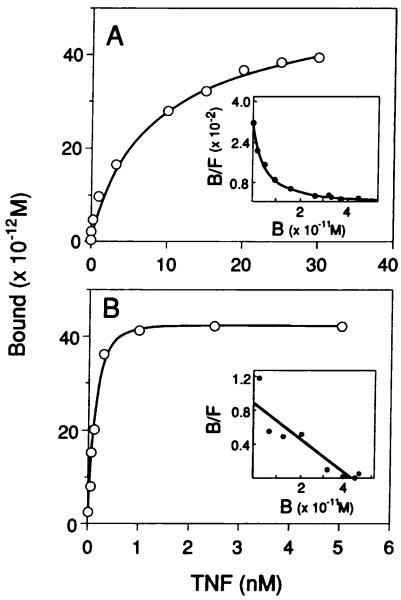

FIG. 2. Specific binding of ^{125}I-labeled mTNF-α to cells transfected with mTNF-R1 expression vector (*A*) or mTNF-R2 expression vector (*B*). Transiently transfected TSA 201 cells were incubated with increased concentrations of ^{125}I-labeled mTNF-α alone or with excess unlabeled mTNF-α. Specific binding of ^{125}I-labeled mTNF-α was determined in duplicate for each concentration. (*Inset*) Scatchard analysis of data. Nonspecific binding was ≈25% and <10% of total binding for mTNF-R1 and mTNF-R2, respectively. B/F, bound/free; B, bound.

DISCUSSION

Here we describe the cloning of the cDNAs for two distinct murine TNF receptors: mTNF-R1, the murine homolog of the 55-kDa human TNF receptor (21, 22), and mTNF-R2, the counterpart of the 75-kDa human TNF receptor (23). Post-translational removal of the signal peptides of the receptors probably produces an N-terminal leucine for mTNF-R1 and valine for mTNF-R2. The N-terminal sequence reported for the 55-kDa hTNF-R1 (22) agrees with this N-terminal designation for mature mTNF-R1. However, the N-terminal sequence of a soluble human TNF-binding protein (17, 18) begins at the aspartic acid at position 12 (Fig. 1). It is therefore possible that more than one form of the mTNF-R1 may exist. The predicted N-terminal residue for mTNF-R2 is the valine at position +1 that corresponds to the predicted

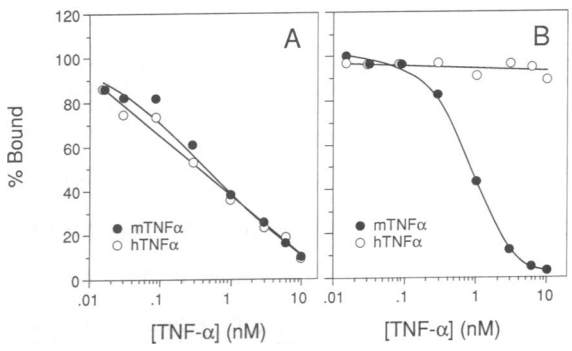

FIG. 3. Displacement curves showing inhibition of the specific binding of ^{125}I-labeled mTNF-α by unlabeled mTNF-α (●) or hTNF-α (○) to mTNF-R1 (*A*) and mTNF-R2 (*B*). TSA 201 cells transfected with the expression vectors were incubated with 15 pM ^{125}I-labeled mTNF-α with increased amounts of unlabeled mTNF-α or hTNF-α. Levels of nonspecific binding were the same as for Fig. 2. Results were from a single experiment repeated three times with duplicate determinations.

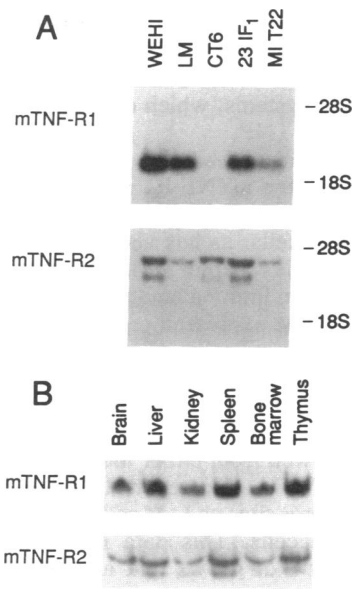

FIG. 4. Northern blot analysis of mTNF-R1 and mTNF-R2 RNA. (*A*) Total RNA (10 μg) from various mouse cell lines hybridized with mTNF-R1 and mTNF-R2 cDNA probes. Positions of the ribosomal RNA bands are denoted as 28S and 18S. (*B*) Poly(A)$^+$ RNA (3 μg) from various mouse tissues hybridized with mTNF-R1 and mTNF-R2 cDNA probes.

N-terminal residue for hTNF-R2 (23). However, the reported sequence for the N terminus of a corresponding soluble TNF-binding protein (17) suggests the removal of an additional four N-terminal amino acids may occur, producing an alternative N-terminal valine (indicated by arrows, Fig. 1).

Analysis of the extracellular domains of both receptors reveals a pattern of cysteine-rich repeats shared with the human and rat nerve growth factor (NGF) receptors (40, 41), the B-lymphocyte activation molecule Bp50 (39), the rat OX40 cell-surface molecule (38), cDNA clone 4-1BB, a murine cDNA from induced helper and cytolytic T-cell clones (42), and T2, a transcriptionally active open-reading frame from the Shope fibroma virus (43). Of the 19 cysteine residues shared between mTNF-R1 and mTNF-R2, 16 are conserved in the NGF receptor, Bp50, OX40, clone 4-IBB, and T2 proteins. Interestingly, the overall homology between the extracellular domains of the two murine TNF receptors is only ≈20%, which is approximately the same as that between each receptor and NGF receptor and OX40. In fact, Bp50 has a higher identity (≈30%) to each mTNF receptor than they have to each other. The evolutionary conservation of such repeats suggests that they form the general structural framework of the ligand-binding site for a family of related receptors. At physiologic pH both types of murine TNF receptors carry an overall positive charge, whereas TNF-α itself is a negatively charged molecule. The NGF receptor, on the other hand, has a negatively charged extracellular domain, and NGF itself is a positively charged molecule. Electrostatic interactions may, therefore, be important in ligand binding within this receptor family.

Examination of the identity between the extracellular and intracellular domains of the human and murine forms of the two TNF receptors shows that although the similarity of the extracellular domain is highest within the type 1 receptor, the intracellular domain (except for the 13-amino acid C-terminal extension) is more conserved within the type 2 receptor. The significantly lower homology between the extracellular domains of hTNF-R2 and mTNF-R2 compared with hTNF-R1 and mTNF-R1 may explain the greater species specificity of the receptor–ligand interaction seen for mTNF-R2 (discussed

below). We have not found significant similarities between the intracellular domains of the type 1 and type 2 receptors. This result may indicate that the two receptor types use different signaling systems, which contribute to the diverse biological effects of TNF.

Competitive radioligand-binding assays show that transfected cells expressing mTNF-R1 show similar affinity for mTNF-α and hTNF-α. Two different binding affinities were observed on these cells, as has also been reported for the human version of this receptor (21). We speculate that TSA 201 cells may contain limiting amounts of protein that interacts with some of the expressed receptors to give the high-affinity site. A similar hypothesis has been made for the NGF receptor (41). Binding studies of cells expressing mTNF-R2 show that this receptor is highly specific for mTNF-α. Additionally, it was found that ^{125}I-labeled mTNF-α could be displaced equally well from either murine L-M or CT6 cells by unlabeled mTNF-α. However, hTNF-α competes for $\approx$40% of the receptor sites on L-M cells but does not compete for sites on CT6 cells. This data is consistent with the Northern analysis that shows that although L-M contains mRNA for both receptor types, CT6 does not contain detectable mTNF-R1 mRNA levels.

Previous reports have described both species-specific and species-independent TNF effects on various mouse cell lines (24–27). The results of this study provide a possible explanation for these findings. Because mTNF-R2 was found to have very low affinity for hTNF-α, species-specific TNF responses are probably mediated by the type-2 receptor. This conclusion would include growth proliferation in the CT6 cell line (27) and in thymocytes (26). However, TNF-mediated effects that do not show species specificity, such as cytotoxicity in L-M cells, are more likely to be mediated by mTNF-R1. Thus, the different affinities of the two mouse receptors for human TNF may prove to be a valuable tool for distinguishing between mTNF-R1 and mTNF-R2-mediated effects. The low affinity of hTNF-α for mTNF-R2 may also have important clinical implications because potential therapeutic benefits or toxic activities signaled by the type-2 receptor might be missed in mouse experiments done with hTNF-α. Further studies directed toward understanding the individual roles of the two receptors should allow a more informed design of TNF-α therapies.

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