# Identification of a 60-kD Tumor Necrosis Factor (TNF) Receptor as the Major Signal Transducing Component in TNF Responses

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## Summary

We describe here a monoclonal antibody (H398) that immunoprecipitates a human 60-kD tumor necrosis factor (TNF) membrane receptor (p60) and competes with TNF binding to p60 but not to p85 TNF receptors. Despite partial inhibition of TNF binding capacity of cells coexpressing both TNF receptor molecules, H398 uniformly and completely inhibits very distinct TNF responses on a variety of cell lines. These data suggest a limited structural heterogeneity in those components actually contributing to TNF responsiveness and identify p60 as a common receptor molecule essential for TNF signal transduction. As H398 is a highly effective TNF antagonist in vitro, it might be useful as a therapeutic agent in the treatment of TNF-mediated acute toxicity.

TNF-$\alpha$ has been recognized as an essential element in the pathogenesis of several infectious and autoimmune diseases, necessitating the development of substances that antagonize harmful TNF actions (1–3). Specific membrane receptors initiate TNF's pleiotropic activities, and thus are the prime target of selective antagonists. Structural analysis of these receptors by ligand crosslinking and affinity purification revealed the existence of a major TNF binding protein of ~85 kD (4–9). However, recent studies using a variety of crosslinking reagents and anti-receptor antibodies have suggested a greater heterogeneity in TNF receptor molecules with apparent molecular masses of 55–60, 70, and 80 kD (10, 11). These receptor molecules might be expressed in a cell- or tissue-specific manner (10). Moreover, lower molecular mass TNF binding proteins have been found in human serum and urine, and most likely represent soluble forms of distinct TNF-membrane receptors (12–15). Accordingly, it is conceivable that in addition to diversification at the level of signal transduction, receptor heterogeneity accounts for the broad spectrum of TNF bioactivity. To gain information about the structure/function relationship of the distinct TNF binding proteins, and in search of antagonists of TNF action, we have developed mAbs against affinity-purified TNF receptor preparations. We describe here an antibody (H398) that specifically recognizes a human 60-kD TNF receptor (p60) and uniformly acts as a strong antagonist of TNF action even on cells that are heterogeneous in TNF receptors. These data suggest that the p60 TNF receptor is an essential component for signal transduction of TNF responses, whereas TNF binding to p85 appears on its own not sufficient to elicit a biological response.

## Materials and Methods

*Materials.* All reagents, if not otherwise stated, were from Sigma Chemical Co. (Munich, FRG). Purified recombinant human TNF-$\alpha$ and IFN-$\gamma$ were kindly provided by G. Adolf, Boehringer Ingelheim (Vienna, Austria).

*Cell Lines.* All cell lines used were obtained from American Type Culture Collection (Rockville, MD) and were maintained in Click's/RPMI tissue culture medium (Biochrom, Berlin, FRG) supplemented with 5% FCS, 10 mM Hepes, penicillin, and streptomycin (7). YT cells were a generous gift of R. Robb (I.E. duPont de Nemours, Glenolden, PA).

*Production of mAbs.* About 220 $\mu$g affinity-purified receptor material from HL-60 cells containing ~9 $\mu$g actively binding receptor were used to immunize a mouse of the BALB/c strain (first injection [3 $\mu$g] with CFA, second injection with IFA, and a booster injection with soluble protein alone). Spleen cells were fused with NSO cells (16) followed by the usual HAT selection protocol. One of the clones obtained produced an IgG2a able to significantly downmodulate TNF binding capacity on HL-60 cells. This clone, H398, was subcloned twice and used to prepare ascites fluid. IgG2a was purified using a protein A column (Promab; Proton, Maidenhead, England) and used for further studies.

*Binding Studies.* TNF and H398 antibody were iodinated by the lactoperoxidase method, as described, resulting in material without significant loss in bioactivity and a specific radioactivity of 40–100 $\mu$Ci/$\mu$g protein (17). Determination of specific binding capacities with iodinated TNF or with purified iodinated H398 antibody were performed in duplicates at various concentrations

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of the respective ligand (0.8–40 ng/ml $^{125}$I-TNF and 3–150 ng/ml $^{125}$I-H398 antibody). Cells were incubated for 3 h at 0°C in PBS supplemented with 2% FCS, 10 μg/ml mouse IgG, and 0.02% sodium azide. Nonspecific binding was determined in each case in the presence of a 200-fold excess of the respective unlabeled molecules. Saturation binding studies were analyzed using the program Enzfitter (Elsevier, Biosoft, London).

*TNF Bioassays.* For determination of antagonistic activity of mAb H398, the following bioassays have been used. Modulation of cell surface antigens was determined by indirect immunofluorescence analysis on an EPICS C cytofluorograph (Coulter Electronics Inc., Krefeld, FRG) as described (18), using the antibodies W6/32, L243, and anti-Tac (19) for determination of HLA-A,B,C, HLA-DR, and IL-2R expression, respectively (18, 20). TNF-mediated growth inhibition and cytotoxic activity of TNF were determined by [$^3$H]thymidine incorporation and crystal violet staining, respectively, according to established protocols (7, 21), and are detailed in the legends.

*Immunoprecipitation and SDS Gel Electrophoresis.* HL-60 cells ($3 \times 10^8$) were surface labeled with $^{125}$I and lysed on ice with 1% Triton X-100 as described (17). After removal of a 12,000-*g* pellet, the supernatant was precleared with protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) for 2 h and then incubated with either 2 μg/ml mAb H398 or control antibody for 16 h at 0°C in the presence of protein A–Sepharose and 1 M NaCl adjusted to pH 8.0. The beads were washed three times with 20 mM Tris-HCl, pH 8.0, 1 M NaCl, 5 mM $MgCl_2$, 0.2% Triton X-100, and 1 mM PMSF, and once with 50 mM Tris-HCl, pH 7.8, were heated in sample buffer (5 min, 95°C), and subjected to SDS-PAGE (7.5% gel) according to Laemmli (22). Electroelution was performed in an Elucon (Biometra, Göttingen, FRG) with a recovery of 60% of the radioactivity. Electroblotting onto nitrocellulose was carried out in a discontinuous buffer system essentially as described (17). The membranes were incubated with $^{125}$I-TNF (20 ng/ml) for 2 h at room temperature, washed, and exposed to Kodak XAR films.

*Crosslinking Studies.* Crosslinking experiments were performed essentially as described (17). Cells were incubated with 20 ng/ml $^{125}$I-TNF for 1 h at 0°C and subsequently treated with 1 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) for 30 min. After immunoprecipitation with an anti-TNF antibody (23), samples were analyzed by SDS-PAGE and autoradiography.

## Results and Discussion

An anti–human TNF receptor mAb (H398) was obtained by immunization of mice with HL-60-derived affinity-purified TNF receptor material. H398 specifically precipitates a 60-kD membrane protein from lysates of surface-iodinated HL-60 cells (Fig. 1, lane *2*), capable of specific TNF binding, as demonstrated by subsequent ligand blotting (Fig. 1, lane 7). Moreover, H398 exhibited high affinity binding to HL-60 cells ($K_d = 2.5 \times 10^{-10}$ M; Fig. 2 *A*) and competed with $^{125}$I-TNF binding with half-maximum inhibition at 0.01 μg/ml (Fig. 2 *B*). Taken together, these data show that H398 identifies a TNF membrane receptor. Western blot analysis of H398 immunoprecipitates from whole cell lysates of HL-60 cells revealed, in addition to the 60-kD receptor, specific bands at 45 and ~120 kD, suggesting that these products are antigenically related to p60 and probably represent intracellular precursors and/or degradation products (data not shown).

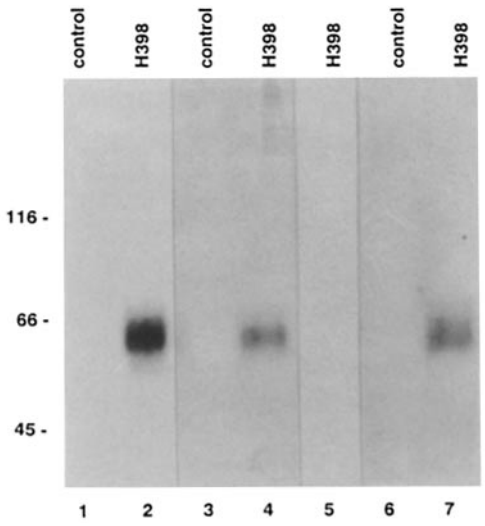


**Figure 1.** H398 antibody defines a 60-kD TNF membrane receptor. HL-60 cells were surface iodinated, and H398 (lanes *2*, *4*, *5*, and 7) or control IgG (lanes *1*, *3*, and *6*) immunoprecipitates were prepared from cell lysates. Separation by SDS-PAGE in the presence (lanes *1* and *2*) or absence (not shown) of 2-ME reveals a specific band of ~60 kD. To demonstrate specific TNF binding capacity, which is abolished by reducing agents (data not shown; 11), aliquots from the same immunoprecipitates were subjected to nonreducing SDS-PAGE, and gel pieces in the range of 50–70 kD were excised. After electroelution, radioactivity was quantitated by scintillation counting (H398 vs. control antibody; 1,210 specific cpm). The two samples were rerun on SDS-PAGE and blotted electrophoretically onto nitrocellulose membranes before autoradiography (lane *3*: control antibody, 90-h exposure time; lanes *4* and *5*: H398, 90 and 14 h, respectively). Subsequently, the nitrocellulose sheets were blotted with $^{125}$I-TNF resulting in additional specifically bound radioactivity, as determined by autoradiography (lane *6*, control antibody; lane 7, H398, both 14-h exposure time) and scintillation counting (7,150 specific cpm compared with 1,210 specific cpm before blotting, see above).

Partial competition of $^{125}$I-TNF binding, with a plateau at ~60% of total TNF binding capacity of HL-60 cells (Fig. 2 *B*, Table 1), is in accordance with the view of the existence of distinct TNF receptors. Comparative quantitative binding studies with $^{125}$I-labeled TNF and H398 indeed showed expression of two distinct TNF receptors. Whereas in most cell lines investigated, the number of H398-defined epitopes was in the range of TNF binding sites (calculated on the basis of trimeric TNF as ligand), in YT cells and dibutyric cAMP (DBcAMP)[1]-pretreated HL-60 cells, a clear segregation was observed (Table 1). Crosslinking studies further support these data. In accordance with earlier findings (9), in HL-60 cells, as in most cell lines, an 85-kD protein is found as a major TNF receptor protein (Fig. 3) that is not recognized by H398 (Fig. 1). In addition, in HeLa cells, a distinct crosslinking product of ~78 kD can be revealed (Fig. 3, lane *3*), which

[1] *Abbreviation used in this paper:* DBcAMP, dibutyric cAMP.

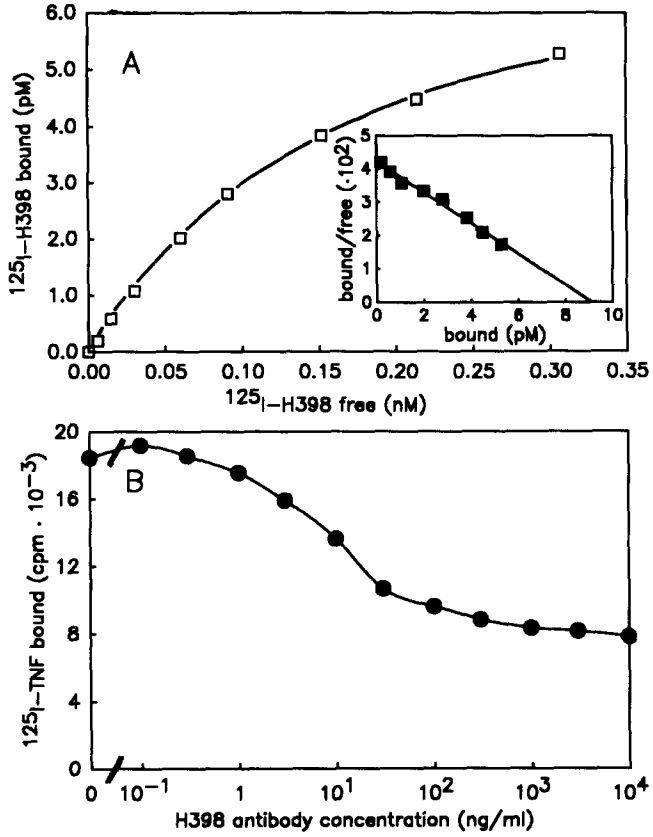


**Figure 2.** Saturation binding of $^{125}$I-H398 on HL-60 cells and partial competition of H398 with $^{125}$I-TNF binding. (*A*) Specific $^{125}$I-H398 binding of HL-60 cells was determined as described in Material and Methods. The apparent dissociation constant of H398 binding ($K_d$ = 2.5 × $10^{-10}$ M) and the number of H398 epitopes (1,556/cell) were calculated from Scatchard analyses (see *inset*). (*B*) $10^6$ HL-60 cells were incubated in duplicates with 20 ng/ml $^{125}$I-TNF and the indicated concentrations of H398 antibody for 3 h at 0°C. Nonspecific binding in the presence of a 200-fold excess of unlabeled TNF was 2,150 cpm and is subtracted in the graph.

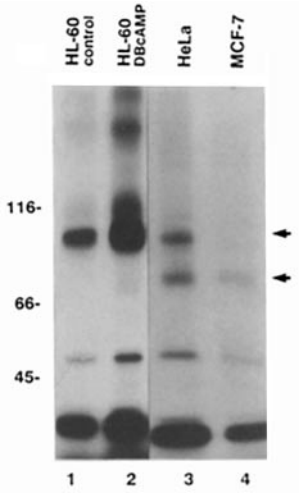


**Figure 3.** Chemical crosslinking studies with $^{125}$I-TNF on different cell lines reveal two distinct receptor proteins. Membrane-bound $^{125}$I-TNF was crosslinked with disuccinimidyl suberate and analyzed by SDS-PAGE as described in Materials and Methods. Arrows indicate the two major crosslinking products of 100 and 78 kD, respectively.

in HL-60 cells is detectable only as a faint band after long exposures (data not shown; 10). The latter is the dominant and probably exclusive crosslinking product in MCF-7 cells (Fig. 3, lane 4), most likely composed of monomeric TNF crosslinked to the H398-defined receptor p60. Together, these data show the existence of two distinct TNF receptor proteins of ~60 and ~85 kD that are coexpressed in several cell lines of distinct tissue origin.

Evidence for an independent regulation of these two TNF receptor proteins was obtained in HL-60 cells, in which TNF binding capacity is under positive control of protein kinase A (24). We show here that the DBcAMP-mediated 13-fold upregulation of TNF binding capacity is associated with a strong increase in p85 with no change in p60 levels, as revealed from formation of the 100-kD crosslinking product of p85 (Fig. 3, lanes *1* and *2*) and Scatchard analyses of H398-defined vs. total TNF receptors, respectively (Table 1).

The observation of competition of H398 with TNF binding prompted studies on the interference of the antibody with cellular TNF responses. On MCF-7 and HeLa cells, where TNF binding can be blocked to a large extent, if not totally, pretreatment with H398 indeed abrogated TNF-induced cytotoxicity (Table 1). Unexpectedly, however, H398 proved to be a potent TNF antagonist for distinct cellular TNF responses, even in a cell line in which p60 comprises only a small portion of total TNF receptors. H398 inhibited the TNF-induced enhancement of IL-2Rs (Tac antigen) in YT cells, though p60-mediated TNF binding only accounts for ~15%

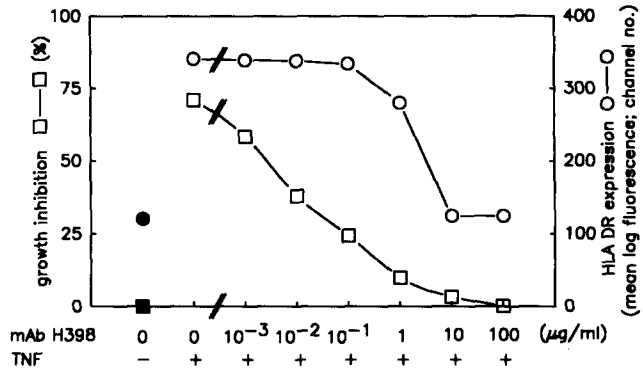


**Figure 4.** H398 antibody is a TNF antagonist. Shown is the dose-dependent inhibition of TNF-mediated cytostasis on U937 cells (□) (*left axis*) and of TNF-mediated enhancement of HLA-DR expression in Colo 205 cells (○) (*right axis*). In both assay systems, the presence of IFN-γ is required to induce the respective TNF responses (18, 27, 28). Modulation of TNF cytostatic activity on U937 cells is given in percent growth inhibition of IFN-γ-treated U937 cells (■) (8,191 cpm). HLA-DR antigen expression in Colo 205 cells is given in arbitrary fluorescence units on a log scale. Untreated Colo 205 cells are HLA-DR$^-$ (mean log fluorescence intensity of 54 vs. 52 with control antibody). IFN-γ induces gene expression (●) (mean log fluorescence intensity of 121), which is enhanced by TNF (27). U937 and Colo 205 cells were cultured for 40 h in the presence of 10 and 0.02 ng/ml of IFN-γ, respectively, with or without 1 ng/ml TNF and log $_{10}$ dilutions of H398 antibody. U937 cells were then pulsed with [$^3$H]thymidine for 6 h (28). Each value represents the mean of six replicates (SD <18%). Colo 205 cells were analyzed for HLA-DR expression by immunofluorescence flow cytometry.

**Table 1.** *Interference of H398 with TNF Action and Binding in Comparison with the Expression of TNF Binding Sites vs. H398 Epitopes*

| Cell line | Percent inhibition of TNF response | Assay system | Percent inhibition of $^{125}$I-TNF binding | Binding sites per cell | |
|---|---|---|---|---|---|
| | | | | $^{125}$I-TNF | $^{125}$I-H398 |
| | | | ± *SD* | ± *SD* | |
| YT | 100 | IL-2R expression | 15 ± 7 | 7,116 ± 1,477 | 911 ± 99 |
| HL-60 (untreated cells) | 100 | Growth inhibition | 43 ± 14 | 1,864 ± 412 | 2,463 ± 457 |
| HL-60 (cAMP-treated cells) | ND | ND | 6 ± 5 | 24,026 ± 1,114 | 1,428 ± 168 |
| U937 | 100 | Growth inhibition | 40 ± 8 | 1,439 ± 261 | 1,727 ± 206 |
| Colo 205 | 100 | HLA-DR expression | 20 ± 7 | 1,770 ± 210 | 1,250 ± 380 |
| HeLa | 100 | Cytotoxicity | 88 ± 8 | 4,152 ± 1,075 | 4,412 ± 1,250 |
| MCF-7 | >82 | Cytotoxicity | 94 ± 5 | ND | ND |
| K562 | 100 | HLA-A,B,C expression | 16 ± 12 | ND | ND |
| L929 | <5 | Cytotoxicity | 0 | ND | ND |

Data represent means of three independent experiments ± SD. TNF and H398 binding sites were calculated from Scatchard analyses of specific binding data. To quantitate maximum competition of H398 with $^{125}$I-TNF, the respective cells were incubated for 3 h at 0°C in triplicates with 40 ng/ml $^{125}$I-TNF in the presence and absence of increasing concentrations (0.01–10 μg/ml) of H398, respectively. Unspecific binding was determined in the presence of a 200-fold excess of unlabeled TNF. Assessment of antagonistic activity of H398 antibody in TNF bioassays was performed at TNF concentrations from 1 to 10 ng/ml, dependent on the assay system, in the presence or absence of 10 μg/ml of H398 antibody. U937 and Colo 205 cells were treated as described in Fig. 4. HL-60 cells were cultured in six replicates (200 cells per microwell) for 10 d in the presence of 1 ng/ml TNF, and proliferation was determined by [$^3$H]thymidine incorporation. K562 cells were cultured for 48 h in the presence of 10 ng/ml of IFN-γ to induce HLA-A,B,C expression and 3 ng/ml of TNF. HLA-A,B,C antigen expression was determined by immunofluorescence flow cytometry. Confluent HeLa and MCF-7 cells were cultured in six replicates in the presence of 1 ng/ml TNF and 5 μg/ml cycloheximide (7 h) or 2.5 μg/ml cycloheximide (20 h). Viability of cells was determined by staining with cristal violet as described (21). YT cells were cultured for 24 h in the presence of 10 ng/ml of TNF, and thereafter, expression of the Tac antigen was quantitated by immunofluorescence flow cytometry using anti-Tac antibody (19). Effects of H398 antibody on TNF-mediated cytotoxicity in murine L929 cells was studied in the standardized assay system in the presence of 1 μg/ml of actinomycin D (26).

of total TNF binding sites of this cell line (Table 1). Likewise, in K562 (Table 1) and Colo 205 cells (Fig. 4), TNF-mediated enhancement of class I and class II MHC antigen expression, respectively, was completely inhibited in the presence of H398. Moreover, TNF-induced cytostasis of U937 (Fig. 4) and HL-60 cells (Table 1) was also fully antagonized by H398 treatment.

The finding that H398 uniformly blocks various gene regulatory as well as cytostatic and cytotoxic activities of TNF is very intriguing, and suggests that H398 is an antagonist of TNF action in general. According to this view, p60 must represent the biologically relevant TNF receptor or a necessary component thereof, whereas binding of TNF to p85 is not sufficient to initiate TNF responses. However, it is conceivable that in analogy to the IL-2R system (25), in cells coexpressing both types of TNF receptors, p85 may cooperate with p60, e.g., by modulation of ligand binding affinity. Irrespective of a possible cooperation with other TNF binding proteins, p60 appears essential for TNF receptor function, suggesting that it mediates a common primary signal across the membrane. Therefore, p60-specific antibody H398 appears a valuable tool to elucidate these initial signal transduction events giving rise to distinct TNF responses. Moreover, as H398 is a highly effective TNF antagonist, it is potentially useful as a therapeutic agent in the treatment of TNF-mediated acute toxic effects.

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