Monoclonal Antibodies Specific for Murine p55 and p75 Tumor Necrosis Factor Receptors: Identification of a Novel In Vivo Role for p75

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Summary

Monoclonal antibodies (mAbs) specific for the murine p55 and p75 tumor necrosis factor (TNF) receptors were produced after immunization of Armenian hamsters with the purified soluble extracellular domains of each receptor protein. Four p55- (55R) and five p75 (TR75)-reactive mAbs immunoprecipitated the appropriate receptor from the surface of L929 cells. None of the mAbs cross-reacted with the other TNF receptor form. The mAbs were functionally characterized by their ability to inhibit ligand binding and influence TNF-dependent L cell cytolytic activity or proliferation of the murine cytolytic T cell clone CT6. One p55-specific mAb, 55R-593, displayed agonist activity, while two other p55-specific mAbs (55R-170 and -176) were found to be TNF antagonists. The fourth mAb (55R-286) had no functional effects on cells. Several antibodies specific for the p75 TNF receptor partially inhibited recombinant murine TNF-α–dependent cytolytic activity (60%). Blocking mAbs specific for p75 but not anti-p55 inhibited TNF-mediated proliferation of CT6 T cells. When used in vivo, p55- but not p75-specific mAbs protected mice from lethal endotoxin shock and blocked development of a protective response against Listeria monocytogenes infection. In contrast, both p55 and p75 mAbs individually blocked development of skin necrosis in mice treated with murine TNF-α. These data thus demonstrate the utility of the two families of murine TNF receptor–specific mAbs and identify a novel function of the p75 TNF receptor in vivo.

T NF, originally identified by its ability to effect hemorrhagic necrosis of specific tumors (1), is now known to play a major role in promoting immunologic, inflammatory, and pathobiologic reactions (2). TNF's pleiotropic activities are induced after binding of the homotrimeric ligand to either of two distinct cell surface receptors of 55 and 75 kD that are independently expressed on a variety of different cell types (3, 4). The cDNAs and genes for the murine and human p55 and p75 TNF receptors have been cloned and expressed and the proteins they encode characterized (5–9). Although the extracellular domains of the two TNF receptors are 28% identical, no homology has been found between the intracellular domains of the two proteins (10). This observation suggests that the two receptors may be responsible for inducing distinct cellular responses. The murine p55 TNF receptor binds both murine and human TNF-α. In contrast, the murine p75 receptor binds only its homologous murine ligand and not the human homologue (8, 9).

Work is ongoing to define the functional roles of the p55 and p75 TNF receptors on various cell types. The p55 receptor is now known to be important for inducing in cells a wide variety of functions such as cytolytic activity (11, 12), antiviral activity (13), expression of manganous superoxide dismutase (14) and intercellular adhesion molecule (ICAM)[1] 1 (15, 16), IL-6 mRNA accumulation (17, 18), and NF-κB induction (12, 15, 19, 20). Recent experiments using p55-deficient mice have demonstrated that the p55 TNF receptor plays a physiologically important role in promoting lethal shock induced by LPS and galactosamine and in effecting antimicrobial responses to Listeria monocytogenes (21, 22). In contrast, little information is available concerning the biologic responses mediated by the p75 TNF receptor. In vitro experiments have suggested that p75 enhances p55-induced biologic responses by facilitating binding of TNF to p55 (23, 24). In addition, p75 induces proliferative responses in certain cells of hematopoietic origin and enhances expression of certain adhesion molecules such as ICAM-1 (11, 14, 25–27).

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[1] Abbreviations used in this paper: HuTNF, MuTNF, and MuIFN, recombinant human and murine TNF-α and IFN-γ, respectively; ICAM, intracellular adhesion molecule.

Although receptor-specific mAbs have been generated against the human TNF receptors, no mAbs are currently available that are reactive with the murine TNF receptor proteins. Herein, we report the production and characterization of hamster mAbs specific for the murine p55 and p75 TNF receptors. The functional activities of these antibodies are demonstrated in well-defined in vitro systems and subsequently by use of in vivo murine models of lethal shock and anti-*Listeria* responses. Finally, using an in vivo model of TNF-induced skin necrosis in mice, we identify a novel physiologic role of p75.

Materials and Methods

Animals. C57Bl/6J, CBA/J, BALB/cByJ, and C3H/HeJ female mice 6–12 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. Male Armenian hamsters were obtained from Cytogen Research and Development, Inc. (Cambridge, MA). Goats were purchased through and maintained at the Washington University School of Medicine Tyson Research Center.

Reagents and Materials. Media, supplements, and buffers used in these experiments were purchased or prepared as previously described (28, 29) and contained <0.005 endotoxin units/ml as determined by the *Limulus* amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD). Purified recombinant murine TNF-α (MuTNF) (1.2 $\times$ 10^7 U/mg), recombinant human TNF-α (HuTNF) (5.6 $\times$ 10^7 U/mg) and recombinant murine IFN-γ (MuIFN) (4.7 $\times$ 10^6 U/mg) were produced by Genentech, Inc. (South San Francisco, CA). MuTNF was radiolabeled to a specific activity of 10.8 μCi/μg by use of Na^{125}I (ICN Biomedicals, Inc., Irvine, CA) and Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturers' directions. Iodinated HuTNF (800 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Purified soluble extracellular domains of the p55 and p75 murine TNF receptors were prepared as described (8, 30) and radioiodinated by use of the Bolton-Hunter reagent (ICN Biomedicals, Inc.) according to the manufacturer's directions to a specific activity of 3.2 and 9.2 μCi/μg, respectively. LPS (*Escherichia coli* strain 0111:B4) and D-galactosamine were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies. The following previously described and characterized hamster mAbs were used in this study: TN3-19.12 (anti-murine TNF-α) (29), JAMA-147 (anti-murine type I IL-1 receptor) (31), and H22 (anti-murine IFN-γ) (28); L2-3D9 (29) and 6C8 (32) are control hamster mAbs not reactive with naturally occurring murine proteins. Polyvalent rabbit p55- and p75-specific antisera were produced as previously described (11). Anti–hamster IgG was produced by immunization of goats with purified Armenian hamster IgG. Hamster IgG–specific antibodies were purified from immune sera purified by affinity chromatography by use of agarose-bound Armenian hamster IgG. Purified antibodies were biotinylated by use of the Enzotin reagent (Enzo Biochemical, Inc., New York) according to the manufacturer's instructions.

Production of Anti-TNF Receptor Antibodies. Hamster mAbs were generated from Armenian hamsters as previously described (28, 29). Animals were immunized intraperitoneally with the soluble purified extracellular domains of either the p55 or p75 murine TNF receptor (10 μg) emulsified in CFA and boosted three times with the same quantity of antigen in IFA at 2-wk intervals. 3 wk after the last boost, each animal was given an i.v. injection of 10 μg of the appropriate antigen diluted in sterile saline. Cultures were screened

by immunoprecipitation of radiolabeled soluble receptor protein (2 ng/well) as previously described (33).

For the anti-p55 fusion, 565/612 growth-positive wells were obtained, which yielded 20 separate cultures producing p55-specific antibody (3.54%). The p75 fusion produced 236/408 growth-positive wells, and 12 produced p75-specific antibodies (5.08%). Antibody-producing hybridomas were cloned by limiting dilution. mAbs were purified from clone culture supernatants by use of Affi-Prep Protein A Silica (Bio-Rad Laboratories, Richmond, CA) chromatography as previously described (29) and HPLC gel filtration. All antibody stocks contained <200 pg endotoxin per milligram of protein when analyzed by the *Limulus* amebocyte lysate assay.

Ligand Binding Inhibition. The ability of anti-p55 and anti-p75 mAbs to block ligand binding to L929 was determined by use of a protocol similar to that described previously (34). 4 million L929 cells were preincubated for 1 h at 4°C with serial dilutions of mAb and then incubated with radiolabeled recombinant TNF in a final total volume of 200 μl. To monitor binding of TNF to p55, 4 ng ^{125}I-labeled human TNF was used in the experiment since human TNF binds only to murine p55 TNF receptors (8). To monitor binding of TNF to p75 TNF receptors, cells were first incubated with 5 μg unlabeled human TNF for 1 h at 4°C to block the p55 TNF receptor, exposed to mAb, and then incubated with 20 ng ^{125}I-labeled murine TNF. The level of nonspecific binding in each experiment was determined by adding a 100-fold excess of unlabeled human or murine TNF to control wells. Cell-associated and free radioactivity were separated by centrifugation over a phthalate oil mixture (34) and the percent inhibition of specific binding determined.

Immunoprecipitation, SDS-PAGE, and Immunoblotting. L929 cells (2 $\times$ 10^7) were lysed in 1 ml PBS containing 1% Triton X-100 and protease inhibitors as previously described (35). Samples were precleared by 1-h incubation with 50 μl protein A–Sepharose slurry (Pharmacia Fine Chemicals, Piscataway, NJ) and then incubated overnight at 4°C with 20 μg mAb. 50 μl of protein A–Sepharose was added to each sample and incubated an additional 4 h. Beads were pelleted by centrifugation and washed three times with 1 ml immunomix (35) and once with 1 ml PBS. Beads were resuspended in 70 μl of Laemmli sample buffer (36), boiled 10 min, and centrifuged.

Half of each sample was applied to a 12% polyacrylamide gel and subjected to SDS-PAGE according to the method of Laemmli by use of a mini-Protean II gel apparatus (Bio-Rad Laboratories) (36). Western blot analysis was performed as previously described (35). Filters were washed in PBS containing 0.05% Tween-20 and incubated 2 h with biotin-conjugated anti-p55 (55R-593) or anti-p75 (TR75-54) at 2 μg/ml. After washing, filters were incubated 15 min with peroxidase-conjugated streptavidin (1:4,000), washed, and developed by use of the enhanced chemiluminescence system (ECL; Amersham).

L929 Cytolytic Assay. TNF-dependent L929 killing activity was quantitated as previously described (29). Briefly, L929 cell cultures (80% confluent) were harvested in EDTA, washed, resuspended to 7.5 $\times$ 10^5 cells/ml in medium, and treated with actinomycin D (2 μg/ml final concentration). After 2 h at 37°C, 100 μl of the cell suspension was mixed with 100 μl of serial dilutions of either mAb or TNF in 96-well flat-bottom culture plates. In assays in which cell-bound mAb was cross-linked, 20 μg of affinity-purified goat anti–hamster Ig was added per well. After 18 h of culture at 37°C, viability of the cell cultures was determined by vital dye staining with crystal violet as previously described (29).

Neutralization of TNF Activity. Neutralization of TNF-dependent cytolytic activity by mAb was quantitated by use of a modi-

fication of the TNF bioassay described above. Different concentrations of mAb were preincubated with actinomycin D–treated L929 cells (7.5 × 10⁴/well) for 2 h at 37°C. A constant amount of TNF was then added to each well and the cultures incubated an additional 18 h at 37°C. Cell viability was assessed by crystal violet staining.

Nitric Oxide Assay. Nitric oxide induction in murine L929 fibroblasts by MuIFN and TNF was assayed essentially as described previously (37, 38).

CT6 Proliferation. TNF-dependent proliferation of the murine CT6 cytotoxic T cell line was assayed as previously described (11). For inhibition studies, cells were preincubated with mAb for 2 h at 37°C before addition of 2 ng/well MuTNF. Cultures were pulsed with [³H]thymidine (0.5 μCi/well) during the final 4–6 h of a 24-h culture period and cpm incorporated into DNA determined.

LPS-Galactosamine-induced Shock. Groups of 10 C57Bl/6J mice were injected intraperitoneally with either pyrogen-free saline or mAb (250 μg) 24 h before treatment with a combination of 600 ng LPS and 10 mg D-galactosamine (intraperitoneally in 0.5 ml). Survival was monitored at 4- to 6-h intervals over a period of 72 h (39).

Listeria Infection. Mice were pretreated with either saline or mAb injected intraperitoneally 6 h before i.p. administration of 3,000 CFU of *Listeria monocytogenes* as described (40). Survival was monitored twice daily over a 2-wk period.

TNF-mediated Skin Necrosis. Mice were pretreated with either pyrogen-free saline or mAb (100 μg i.p.), anesthetized with sodium pentobarbital (80 mg/kg), and the hair removed from the back. 24 h later, the animals were injected subcutaneously with either saline, MuTNF (3 μg), HuTNF (3 or 30 μg), or a mixture of 3 μg TNF and mAb (50 μg) in 0.2 ml. Injections were continued on a daily basis. Mice were observed twice daily for the appearance of local skin hemorrhage and necrosis. At various times after treatment, the mice were bled, killed, and tissue prepared for histology according to standard procedures.

Results

Immunoprecipitation of Receptor from Cell Surfaces. Purified mAbs were examined for their ability to specifically precipitate intact receptor from cell surfaces. Western blot analysis showed that three of the anti-p55 mAbs (55R-593, -176, and -170) precipitated a protein of the appropriate molecular mass from L929 cell lysates (Fig. 1, *top*). In a separate experiment, similar results were obtained with a fourth p55-specific mAb, 55R-286 (not shown). No p55 was precipitated by any of the anti-p75 mAbs (TR75-32, TR75-45, or TR75-54) or control hamster mAbs specific for the murine type I IL-1 receptor (JAMA-147) or TNF-α (TN3-19.12). Conversely, the p75 receptor was precipitated only by mAb generated against the murine p75 TNF receptor extracellular domain (Fig. 1, *bottom*). In this case, Western blot analysis revealed that the anti-p75 mAb precipitated two components, a major component of 70 kD and a minor component of 60 kD. Similar patterns were seen with two additional p75-specific mAbs, TR75-4 and TR-75-89 (data not shown). No TR75-reactive bands were identified after precipitation with either the anti-p55 mAb or control IgG. The antigenic specificity of each mAb was verified by an ELISA that used immobilized purified p55 or p75 extracellular domain (data not shown). These
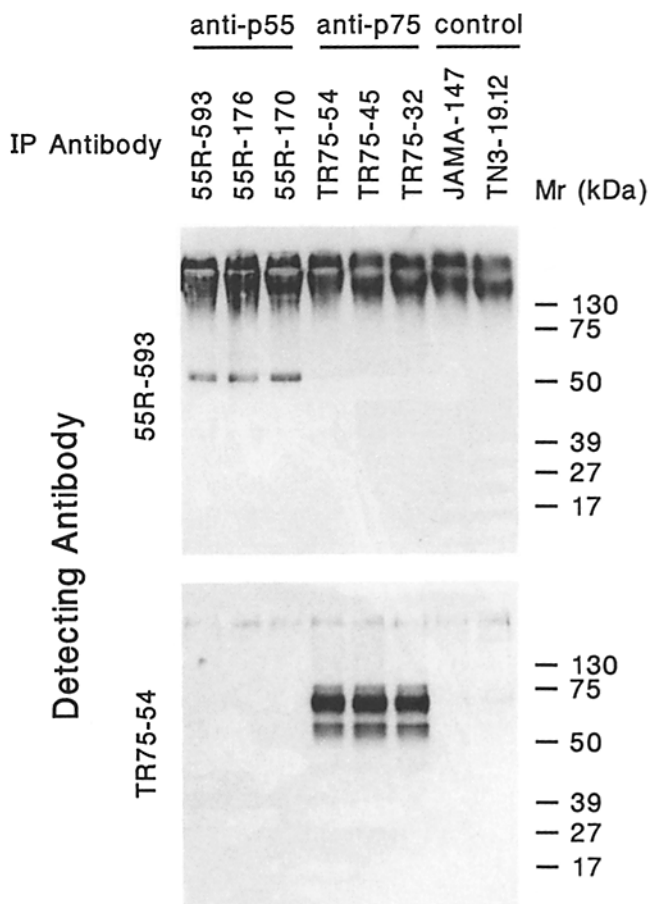

Figure 1. Immunoprecipitation of TNF receptors from L929 cells with anti-p55 and anti-p75 TNF receptor mAbs. Murine L929 lysates were incubated with 20 μg of p55- or p75-specific mAbs and protein A–Sepharose. Precipitates from 10⁷ cells were separated by SDS-PAGE on 12% polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose, and detected with 2 μg/ml either biotin-conjugated 55R-593 (*top*) or TR75-54 (*bottom*). Western blots were developed with streptavidin-HRP (1:4,000) and the ECL system.

results thus established that each family of mAb reacted only with the class of TNF receptor used for immunization and that the antibodies recognized the natural receptor molecules derived from whole cells.

Inhibition of Ligand Binding. Radioligand-binding studies were performed on murine L929 cells, which express both p55 and p75 TNF receptors to further characterize the specificity of each mAb. To monitor effects on the p55 TNF receptor, the antibodies were tested for their ability to inhibit binding of radiolabeled HuTNF to murine L929 cells since the human ligand binds exclusively to the murine p55 TNF receptor. Incubation of L929 with 55R-593, 55R-176, or 55R-170 produced a dose-dependent inhibition of binding of 4ng ¹²⁵I-HuTNF (Fig. 2 *A*). 50% inhibition of HuTNF binding was achieved at mAb doses of 4, 5, and 1,500 ng, respectively. No inhibition was detected with 55R-286, even at mAb doses >75 μg/well. Binding of HuTNF was not inhibited with the 6C8 control hamster IgG (Fig. 2 *A*) or p75-specific mAb (data not shown).
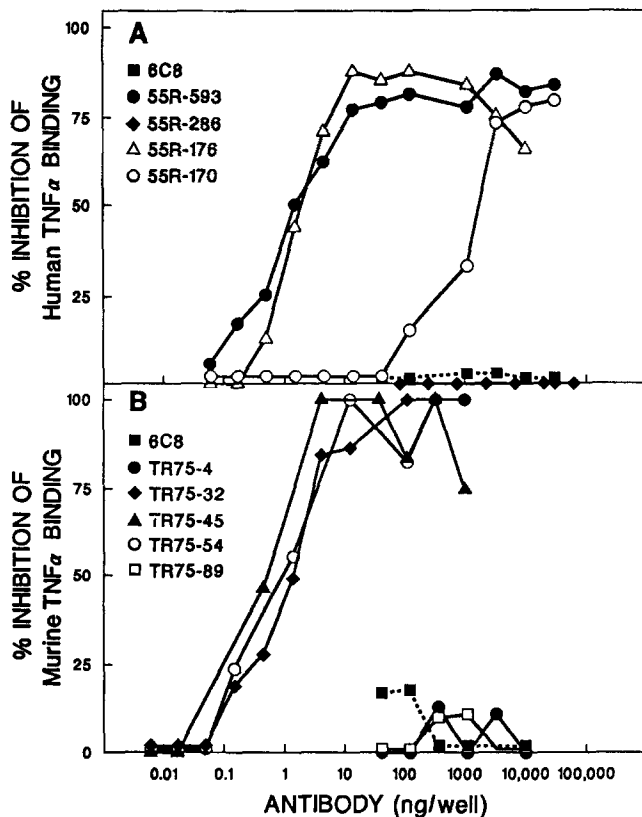
Figure 2. Inhibition of ligand binding by p55- or p75-specific TNF receptor mAbs. (*A*) L929 cells (4 × 10⁶/well) were incubated with increasing concentrations of 55R-593, -286, -176, -170, or control hamster Ig 6C8 for 1 h at 4°C before addition of 4 ng radioiodinated HuTNF. Cell-associated and free radioactivity were separated by centrifugation through oil. Maximum binding of ¹²⁵I-HuTNF was 15,000 cpm, while nonspecific binding in the presence of 5 μg unlabeled HuTNF was <2%. (*B*) To assess p75-specific ligand binding, L929 cells were first incubated with saturating concentrations of HuTNF (5 μg) for 1 h at 4°C before addition of various doses of TR75-4, -32, -45, -54, and -89 or control hamster Ig 6C8. After 1 h at 4°C, 20 ng radioiodinated MuTNF was added for an additional hour. Samples were harvested as above and the percent binding inhibition determined. Binding of 20 ng labeled MuTNF yielded 5,000 cpm, and nonspecific binding in the presence of an additional 5 μg unlabeled MuTNF was 1.89%.

To assess the ability of the anti-p75 receptor mAb to inhibit ligand binding, L929 cells were pretreated with saturating concentrations (5 μg) of unlabeled HuTNF (to block all of the p55 receptors), followed by addition of ¹²⁵I-murine TNF, which thus bound exclusively to the p75 TNF receptor. Three p75-specific mAbs inhibited binding of ¹²⁵I-MuTNF in a dose-dependent manner (Fig. 2 *B*). 50% inhibition of binding of 20 ng radiolabeled MuTNF was achieved with 0.5, 0.9, and 1.5 ng of TR75-45, -54, and -32, respectively. No inhibition was observed with the TR75-4 and TR75-89 mAbs or control hamster IgG (6C8). Thus, each family of murine TNF receptor-specific reagents contained both receptor blocking and nonblocking mAbs.

Effect of p55 and p75 mAbs on In Vitro Cellular Responses to TNF. Fig. 3, *A* and *B*, depict the results of experiments to determine the agonist activity of the TNF receptor-specific

mAb in the in vitro L929 killing assay in the absence of exogenous TNF. Only 55R-593 induced L929 cell lysis in a dose-dependent fashion (Fig. 3 *A*). However, addition of goat anti-hamster Ig to cultures pretreated with 55R-170 and -176 induced L929 lysis, while cross-linking of 55R-286-treated cultures or cultures treated with the 6C8 control mAb failed to induce cell killing (data not shown). The efficacy of 55R-593-induced L929 killing was not affected by antibody cross-linking. None of the anti-p75 mAbs induced cytolytic activity when used alone (Fi. 3 *B*), in combination, or when cross-linked with anti-hamster Ig (data not shown). These results thus demonstrate that 55R-593 displays TNF agonist activity and confirms that L929 cell-killing activity can be effected solely through p55 receptor engagement, but not p75 engagement.

The antibodies were also tested for their capacity to inhibit TNF-dependent L929 killing. The p55-specific mAbs 55R-170 and -176 blocked the lytic activity of 30 pg MuTNF in a dose-dependent fashion. 50% inhibition of TNF activity was obtained with 1,000 and 20 ng of the mAbs, respectively (Fig. 3 *C*), and corresponds to their relative abilities to inhibit ligand-receptor interaction. The p55 agonist mAb 55R-593, the nonblocking p55 mAb 55R-286, and control mAb failed to inhibit MuTNF-dependent cytolytic activity. When the experiments were conducted with HuTNF (which binds only to the murine p55 TNF receptor), similar results were obtained, except that 55R-170 and -176 were more efficient inhibitors (ID₅₀ = 35 and 2 ng, respectively) (Fig. 3 *E*). Thus, mAbs 55R-170 and -176 act as TNF antagonists in the L929 killing assay. When similar experiments were performed with anti-p75 mAbs, partial inhibition of MuTNF lytic activity (up to ∼60%) was noted (Fig. 3 *D*). However, the anti-p75 mAbs, even at doses of 30 μg/well, failed to inhibit L cell killing mediated by HuTNF (Fig. 3 *F*). An identical pattern of results was obtained when these two mAb families were tested for their ability to induce or inhibit TNF-mediated nitric oxide production (data not shown).

To examine whether the anti-p75 mAbs affected biologic responses mediated solely by the p75 TNF receptor, we used an assay that monitors murine TNF-induced proliferation of the murine CT6 cytotoxic T cell line. This cell line proliferates in response to polyvalent p75-specific antisera, but not p55-specific antisera (11). While none of the p75-specific mAbs elicited a proliferative response alone, all of the p75-specific mAbs induced significant CT6 expansion when cross-linked with goat anti-hamster Ig (data not shown). None of the p55-specific mAbs, either alone or after cross-linking, induced proliferation of CT6. These data thus confirm that clustering of the p75 TNF receptor is sufficient to induce a proliferative response in these cells. In contrast, TR75-45, -54, and -32 inhibited CT6 proliferative responses induced by 2 ng MuTNF in a dose-dependent manner (Fig. 4 *B*). The ID₅₀ values for each mAb (2, 5, and 22 ng, respectively) correlated with the ability of each mAb to block ligand-receptor interaction. TR75-4 and -89 did not display functional inhibitory activity. Antibodies reactive with p55 TNF receptor had no significant inhibitory effect on TNF-dependent CT6 proliferation (Fig. 4 *A*).
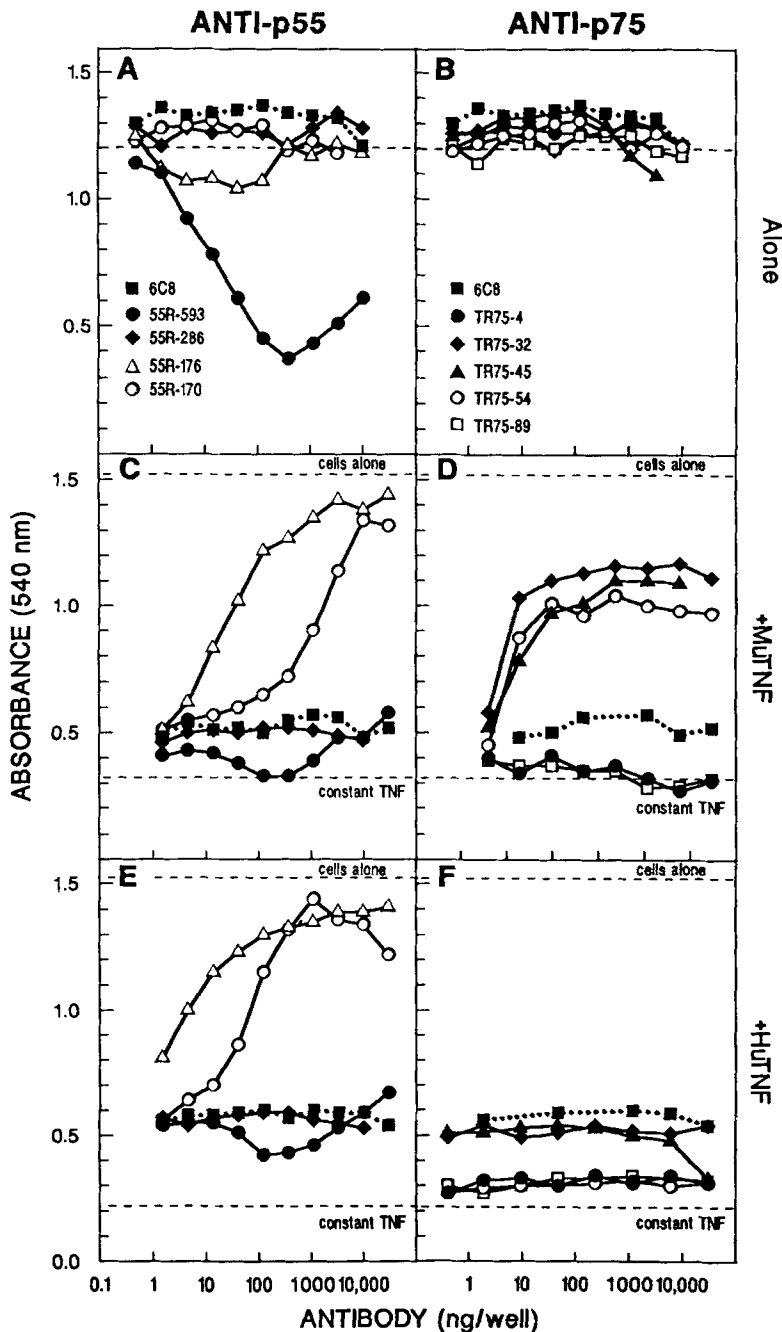
Figure 3. Agonist/antagonist activity of TNF receptor mAbs for L929 killing. Various dilutions of anti-p55 (*A*, *C*, and *E*), anti-p75 (*B*, *D*, and *F*) or control Ig 6C8 were preincubated with actinomycin D–treated L929 cells for 2 h at 37°C before addition of either medium (*A* and *B*) or 30 pg/well MuTNF (*C* and *E*) or 700 pg/well HuTNF (*D* and *F*). Monolayer viability was assessed after 18 h by vital dye uptake. Untreated control monolayers exhibited an absorbance at 540 nm of 1.2 (*A* and *B*) or 1.5 (*C–F*). Incubation with murine or human TNF resulted in readings of 0.32 and 0.22, respectively.

Effects of p55 and p75 mAb In Vivo. Pharmacokinetic studies performed on each antibody in naive BALB/c mice established that all mAbs displayed serum half-lives of ~2 d (data not shown). The half-life of each antibody remained unchanged in mice that received four mAb injections over a 16-wk period. Thus the hamster anti-TNF receptor mAbs were not immunogenic in mice.

To examine the efficacy of the receptor-specific mAbs in vivo, we initially used two experimental systems (LPS/galactosamine–mediated lethal shock and resistance to infection by *L. monocytogenes*), which, based on experiments with p55-deficient mice, are known to be obligatorily dependent on p55 TNF receptor engagement (21, 22). Animals pretreated

with either saline or control hamster IgG succumbed to the lethal combination of LPS and galactosamine within 24 h (Fig. 5 *A*). Pretreatment of mice with 250 μg TN3-19.12 (a neutralizing hamster mAb specific for murine TNF-α) completely protected mice from lethal shock. Mice pretreated with the antagonistic 55R-170 mAb were also protected from lethal shock. Similar results were obtained by use of the other p55 antagonist mAb 55R-176 (data not shown). In separate dose–response experiments, 40 μg of 55R-170 was sufficient to provide complete protection (data not shown). In contrast, the antagonistic anti-p75 TNF receptor mAb TR75-54 was not protective at any dose.

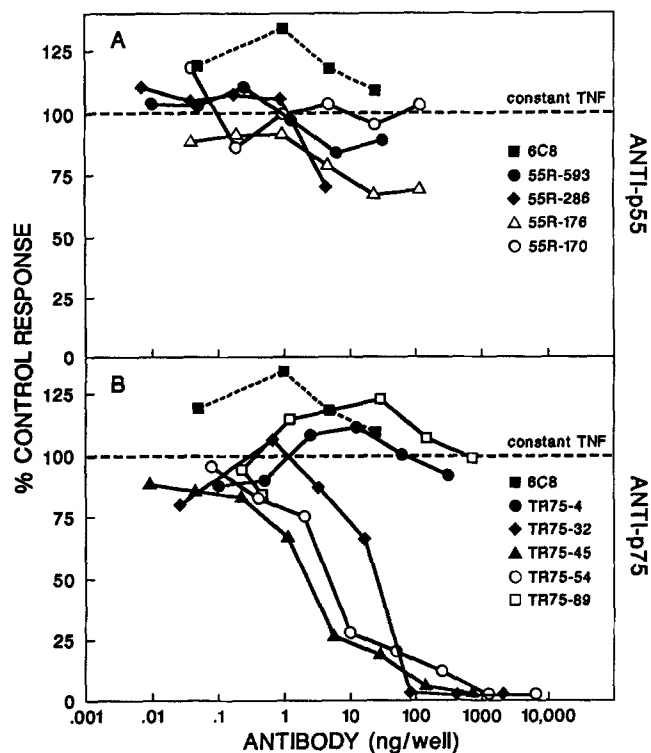In the *Listeria* infection model, mice treated either with

Figure 4. Anti-p75 inhibition of TNF-mediated CT6 proliferation. CT6 cells (5 × 10⁴) were incubated with anti-p55 (*A*), anti-p75 (*B*), or control hamster mAb 6C8 for 2 h at 37°C before addition of 2 ng MuTNF. Cultures were pulsed after 20 h with [³H]thymidine for an additional 4–6 h and the percent of control response determined. Cultures stimulated with 2 ng MuTNF alone yielded 12,300 cpm with background counts <3,500.

Figure 5. In vivo activity of TNF receptor–specific mAbs. (*A*) LPS/galactosamine–mediated lethal shock. Groups of 10 C57Bl/6 mice were treated with saline or 250 µg mAb 24 h before challenge with a combination of LPS (600 ng) and D-galactosamine (10 mg). Survival was monitored over a 48-h period. (*B*) *Listeria* infection. Groups of 5 C3H/HeJ mice were injected intraperitoneally with 250 µg antibody 6 h before infection with 3,000 live *Listeria* organisms. Survival was monitored twice daily over a 10-d period. Depicted is one of six representative experiments.

saline or irrelevant hamster mAb mounted a protective anti-*Listeria* response and survived infection with 3,000 live *Listeria* organisms (Fig. 5 *B*). Mice treated with anti-TNF became susceptible to lethal infection and died. Animals treated with the antagonist p55-specific mAb (55R-170) failed to generate an anti-*Listeria* response and succumbed to the infection. As little as 25 µg of 55R-170 was sufficient to render all the animals susceptible to infection (data not shown). In contrast, TR75-54 did not inhibit the development of an anti-*Listeria* response in mice. Subsequent testing of TR75-32, -45, and -54 at doses of 250 and 500 µg per mouse showed that none of the p75-specific mAbs affected development of anti-*Listeria* responses (not shown). Thus, in both in vivo models, anti-p55–treated mice responded similarly to mice lacking the p55 TNF receptor. Moreover, antibody blockade of p75 was without functional consequences.

p75 TNF Receptor Engagement Is Required for MuTNF-mediated Skin Necrosis. Whereas the aforementioned studies confirmed the physiologic requirement of p55 TNF receptors in promoting protective and immunopathologic responses to bacteria and their products in vivo, no information was obtained that helped define the physiologic role of p75 TNF receptors. Insights into p75's biologic role in vivo were gained from experiments being conducted to explore TNF's im-
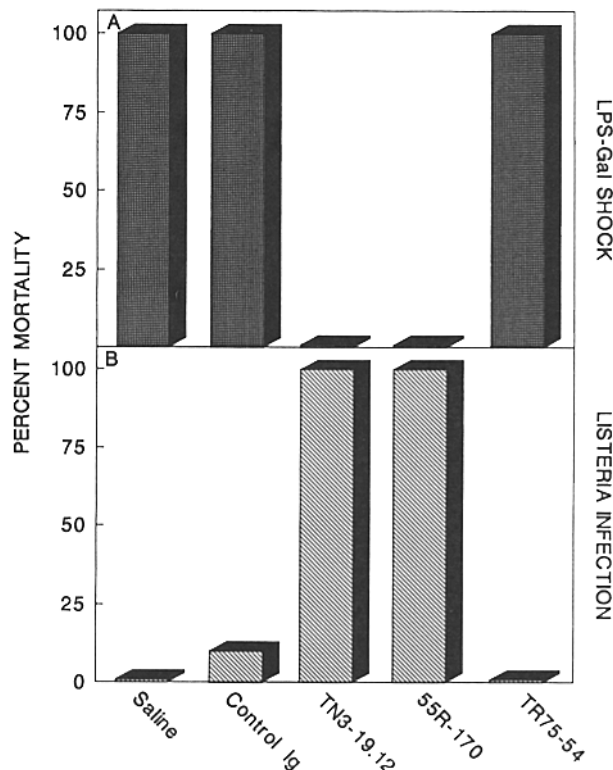
munosuppressive activities. In these studies, daily subcutaneous injections of either murine or human TNF into mice led to a state of immunosuppression that blocked xenograft rejection (41) and inhibited T cell priming to the soluble protein antigen, hen egg lysozyme (Sheehan, K., and R. Schreiber, unpublished observations). During the course of these experiments, we observed that subcutaneous injection of 3 µg MuTNF produced severe hemorrhage in the skin and subsequent necrosis at the injection site (Fig. 6 *B*), while s.c. injection of 3 or 30 µg HuTNF did not (Fig. 6 *C*). In CBA/J mice, the lesion induced with MuTNF was observed after three to five daily injections. The lesion initially presented as a reddening of the skin, followed by intradermal hemorrhage and skin necrosis. Histologically, the full-thickness skin and subcutaneous sections of the saline- and HuTNF-treated mice disclosed an absence of microscopic abnormalities (Fig. 7, *A* and *C*) to correspond with the unremarkable gross features depicted in Fig. 6, *A* and *C*. In contrast, early epidermal necrosis, transdermal neutrophilia and hemorrhage, subcutaneous hemorrhage, and recent thrombi were present in the MuTNF-treated mice (Fig. 7 *B*, *inset*). This result thus suggested that the p75 TNF receptor was involved in this process.
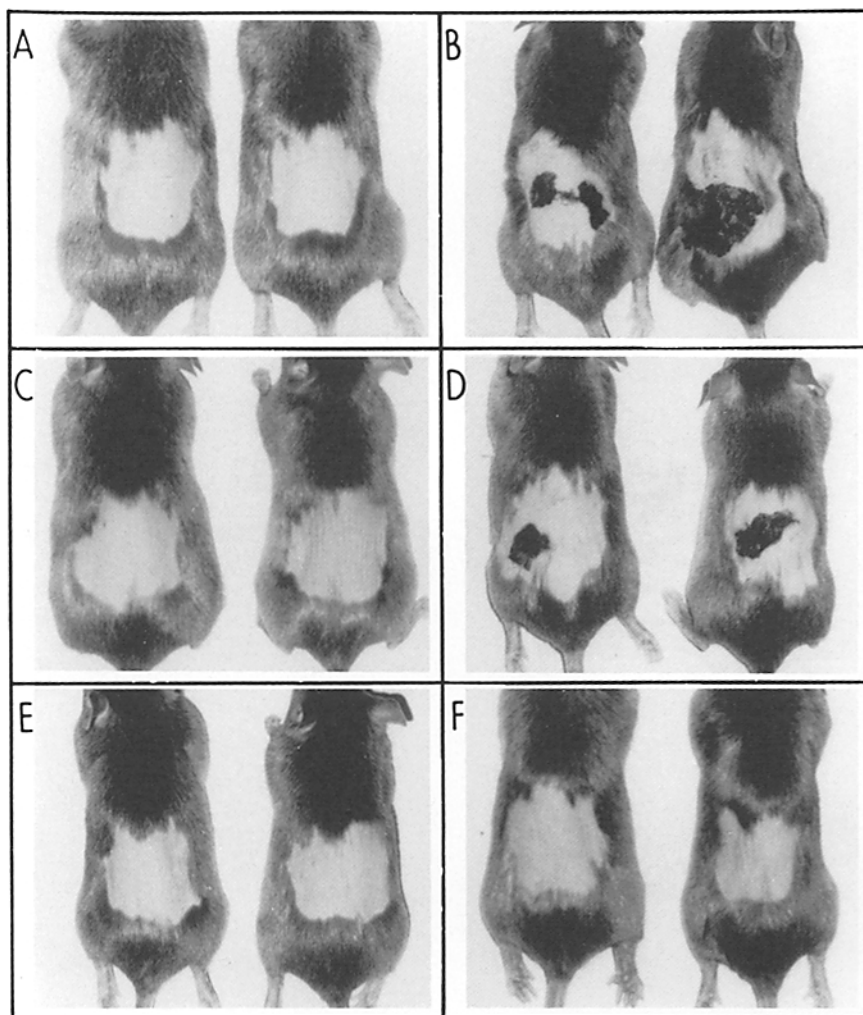
Figure 6. Prevention of MuTNF-induced skin necrosis by anti-p55 and anti-p75 TNF receptor-specific mAbs. Mice were pretreated on day −1 with either saline or 100 μg of antibody intraperitoneally and injected daily with 3 μg TNF alone or combined with 50 μg mAb subcutaneously over a period of 7 d. (A) Saline alone; (B) MuTNF only; (C) HuTNF only; (D) MuTNF + 6C8 control IgG; (E) MuTNF + 55R-170; and (F) MuTNF + TR75-54.

To further confirm the roles of the p55 and p75 TNF receptors in this process, mice were treated with MuTNF either alone or in combination with control or antagonistic p55- or p75-specific mAbs. None of the mAbs individually or collectively induced the skin necrosis reaction (data not shown). Pretreatment of mice with antagonistic p75 mAbs (TR75-32 or -54) completely blocked the development of the skin reaction (Fig. 6 F) as well as any histologic changes (Fig. 7 F). Similar results were obtained by use of the p55-specific antagonistic mAb 55R-170 (Figs. 6 E and 7 E). Control hamster Ig was without effect, resulting in a composite of microscopic changes virtually identical to those in mice treated with MuTNF alone (Fig. 7 B) with epidermal necrosis, hemorrhage, neutrophilia, and thrombosis (Figs. 6 D and 7 D). These results thus demonstrate, for the first time, an in vivo role for the p75 TNF receptor and suggest that p75 engagement may be directly involved in certain types of acute inflammatory and hemorrhagic reactions.

Discussion

The studies presented here document the production of hamster mAbs specific for the extracellular domains of the p55 and p75 murine TNF receptors. These reagents represent the first murine TNF receptor-specific mAbs to be generated. Each member of the anti-p55 or anti-p75 mAb family displayed strict specificity for the receptor form used for immunization. Within the anti-p55 family, 55R-286 did not interfere with p55-TNF interaction, while 55R-170, -176, and -593 blocked the ability of p55 to bind ligand in a dose-dependent manner. Three p75-specific mAbs (TR75-32, -45, and -54) were identified that blocked ligand–receptor interaction, while two others (TR75-4 and -89) did not.

Functionally, the antibodies could be distinguished by their effects in well-defined in vitro TNF bioassays. By use of the L cell–killing assay, the anti-p55 mAb family was found to contain one mAb (55R-593) that acted as a TNF agonist, two (55R-170 and -176) that acted as antagonists, and one (55R-286) that did not affect TNF's actions on p55. The production of an agonistic anti-murine p55 TNF receptor mAb is notable because its functional activity makes it unique among anti-p55 mAbs. Although it has been shown that signaling through the p55 TNF receptor is initiated by oligomerization of surface receptors using either polyvalent anti-p55 sera (11, 42) or homotrimeric ligand (43), the activity of the 55R-593 mAb presented in this report suggests that dimerization of the p55 TNF receptor may be sufficient to
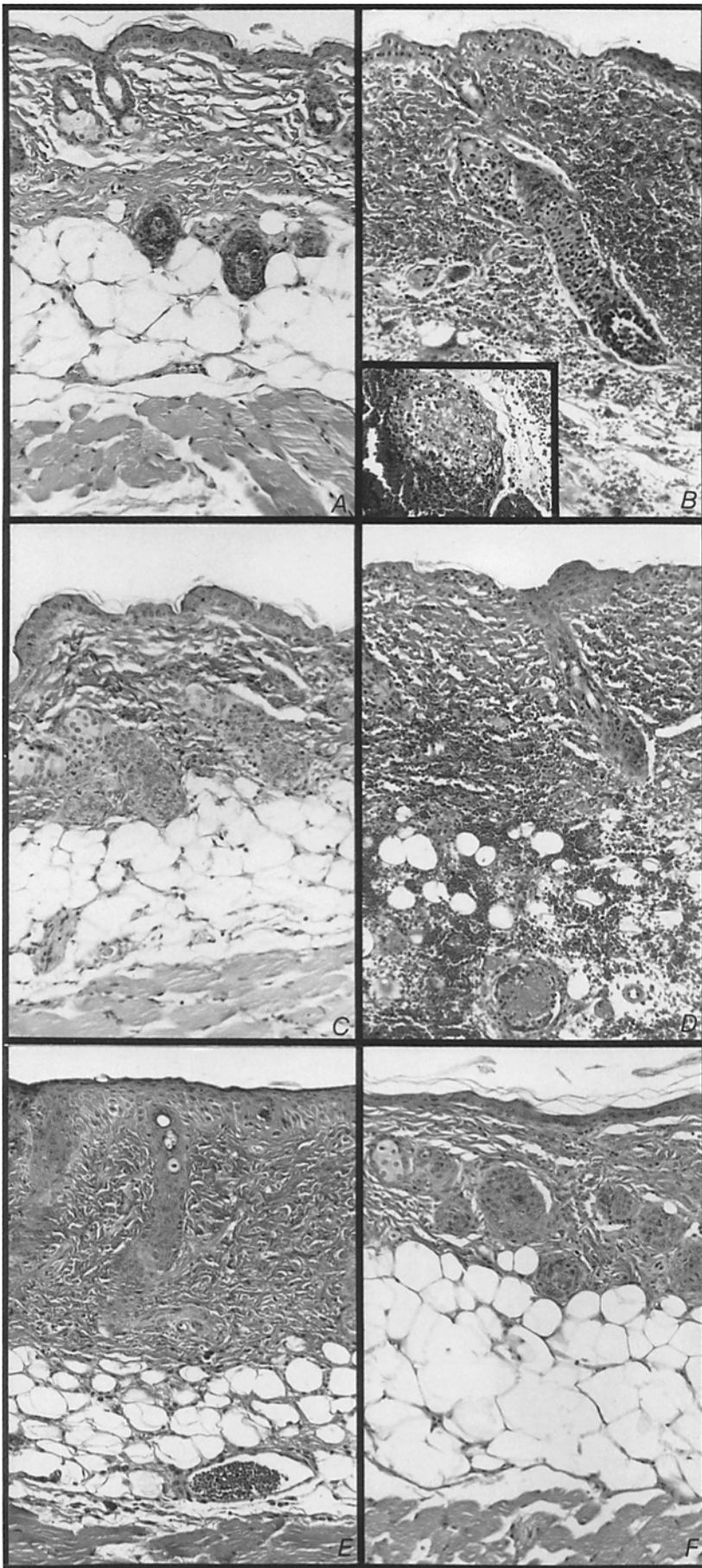
Figure 7. Histologic examination of TNF-mediated skin necrosis. Mice were treated as above with 100 μg antibody intraperitoneally on day -1 and 3 μg TNF alone or in combination with 50 μg antibody subcutaneously daily. After 4 d, mice were killed and tissue processed and stained with hematoxalin and eosin. In vivo treatments are as follows: (*A*) saline only; (*B*) MuTNF alone; (*C*) HuTNF alone; (*D*) MuTNF + 6C8 control IgG; (*E*) MuTNF + 55R-170; and (*F*) MuTNF + TR75-32. ×200.

induce a biologic response in these cells. Importantly, the antagonistic p55-specific mAb could be converted into agonists after cross-linking. In fact, in early experiments, small amounts of high molecular weight mAb aggregates present in the purified antibody preparations led to an agonist action of 55R-170 and -176. Addition of a gel filtration step to the mAb purification procedure separated the high molecular mass aggregates that displayed potent agonist activity from the bulk of the monomeric mAbs that displayed antagonistic activity. When 55R-593 was subjected to the same gel filtration procedure, the agonist activity coeluted with monomeric antibody, con- firming that 55R-593 acted as an agonist in its native form. Thus, these results not only document the functional activity of the p55-specific mAbs, but also confirm the concept that engagement of p55 is both necessary and sufficient to induce killing of L929 cells.

The same assays were used to show that the anti-p75 mAb family contained three members that acted as p75 antagonists (TR75-32, -45, and -54) and two that were unable by themselves to affect TNF-dependent biologic responses in cells (TR75-4 and -89). By use of the CT6 proliferation assay, TR75-32, -45, and -54 were found to inhibit MuTNF-driven proliferative responses in a dose-dependent manner, while TR75-4 and -89 did not. These mAbs represent the only reagents currently available that display antagonist activity for the murine p75 TNF receptor and thus document that engagement of p75 is both necessary and sufficient to induce proliferative responses in CT6 cells.

The p75 antagonist mAbs also partially inhibited MuTNF-dependent L cell killing. Inhibition was never complete (40–60%) and could be overcome if higher concentrations of murine TNF were used. Moreover, none of the anti-p75 reagents effected L cell killing when cross-linked in the absence of TNF. These results thus best support the model where p75 plays an accessory role in the killing process. The precise role of p75 in mediating L cell killing remains controversial. Heller et al. (23, 24) proposed that p75 can directly signal for cytotoxicity, while Tartaglia et al. (12, 30) proposed that p75 facilitates ligand uptake onto p55 and that p55 is the sole TNF receptor responsible for initiating the L cell–killing response. Recently, the latter "ligand-passing model" has been supported by the results of structural analyses of the two TNF receptors' intracellular domains. The p55 intracellular domain contains a "death domain" similar to that found in the intracellular domain of the Fas antigen that is important in effecting cell killing (44), while the p75 TNF receptor does not. Moreover, additional support for a ligand-passing function has been derived from the characterization of mice with selective genetic deficiencies of either the p55 (21, 22) or p75 (44a) TNF receptors.

To initially examine the potential usefulness of the anti-p55 and -p75 mAbs in vivo, we used two well-characterized murine models (39, 45) that are known to be unequivocally dependent on endogenous TNF production. As little as 25 or 40 μg of antagonistic anti-p55 inhibited development of a protective immune response to *L. monocytogenes* in naive mice and protected normal mice from the lethal effects of LPS/galactosamine, respectively. In contrast, up to 500 μg

quantities of antagonistic p75 TNF receptor–specific mAbs were without biologic effects. Importantly, these results recapitulate the observations made by others using mice lacking p55 (21, 22) or p75 (44a) and thereby validate the in vivo effects of our anti-p55 and anti-p75 mAbs.

The characterization of the specificities and functional activities of the murine TNF receptor–specific mAbs was based on the known structure and function of the two receptors. Whereas definitive experiments have now been performed that define at least some of the physiologic roles of the p55 TNF receptor as they occur in vivo, little information is available concerning the physiologic function of the p75 TNF receptor. The availability of antagonistic mAbs specific for the murine p75 TNF receptor has permitted us to identify a heretofore unrecognized role of this protein in promoting inflammatory responses. Previous studies by others (46) have indicated that TNF plays an important role in the development of naturally occurring cutaneous diseases. Moreover, subcutaneous injection of highly purified recombinant TNF into the skin of normal mice and rabbits has been shown by some groups to induce intradermal hemorrhage and skin necrosis (47, 48). In studies in which human TNF was administered to experimental animals, the development of the cutaneous inflammatory response was found to require the additional presence of bacterial products (49), whereas repetitive subcutaneous administration of LPS-free murine TNF to mice led to generation of clear necrotic skin lesions (46, 48).

Based on the recognized differential species specificity displayed by the p55 and p75 murine TNF receptors for human versus murine TNF, the previous discrepant results suggested that both p55 and p75 TNF receptors might be involved in the process. In this report, we show that whereas repeated injections of MuTNF induced frank intradermal hemorrhage and severe skin necrosis, repeated injections of up to 10 times larger quantities of human TNF were without effect. This result suggested that p75 was involved in the inflammatory skin reaction. This hypothesis was supported by our observation that TNF-induced skin necrosis could be inhibited by administration of antagonistic p75-specific mAbs. Moreover, since skin necrosis was also inhibited by blocking p55-specific mAbs, the ultimate development of in vivo inflammatory responses in the skin must be due to engagement of both TNF receptor forms.

At this time, the identity of the cells whose function is regulated by the p75 and p55 TNF receptors remains unknown. It is also unclear whether the dual requirement for p55 and p75 in this process involves a single cell population that is stimulated by engagement of both types of TNF receptors or multiple cell populations that are specifically induced to perform their function through engagement of one or the other TNF receptors. The gross and histologic findings in this study, in particular the presence of neutrophilia, intense hemorrhage, and thrombosis, would suggest that endothelial cells and neutrophils may be important cellular participants in this model. It is noteworthy that, based on in vitro experiments, others (50) have suggested that p75 may be important in effecting biologic response induction in endothelial cells. Studies are currently underway in the labora-

tory to define the precise physiologic roles of p55 and p75 in promoting inflammatory reactions.

Thus, we have documented the production and antigen-binding characteristics of two novel families of hamster mAbs specific for the murine p55 and p75 TNF receptors. The ability of these mAbs to function in vitro and in vivo coupled with their lack of immunogenicity in vivo provides the opportu-nity to modulate TNF-mediated responses in a receptor-specific fashion. The availability of these monoclonal murine TNF receptor–specific reagents will thus facilitate investigations aimed at defining the molecular basis for TNF's pleiotropic actions and the physiologic roles of the two TNF receptors in vivo.

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