Immunomodulatory Properties of Recombinant Murine and Human Tumor Necrosis Factor

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ABSTRACT

Tumor necrosis factor has traditionally been thought to have direct cytostatic and cytotoxic properties with little or no direct immunomodulatory activity. We report here that tumor necrosis factor is able to activate macrophages both in vitro and in vivo and can increase a mixed lymphocyte response and act as an adjuvant for both T- and B-cells in vivo. Adjuvant activity in T-cells occurred in conjunction with the administration of a suboptimal syngeneic tumor cell vaccine. In addition, tumor necrosis factor demonstrated a potent dose-dependent effect on bone marrow stem cell number, dramatically depressing cellularity and thus total stem cell number. An appreciable interval is required for recovery from such stem cell depletion. Therefore, the study of the therapeutic activity of tumor necrosis factor must include a consideration of its immunomodulatory properties.

INTRODUCTION

The cytokine that became known as TNF was first reported by Carswell et al. (1). This protein is found in the serum of primed, endotoxin-treated animals (2) and induces hemorrhagic necrosis in some murine sarcomas. Because of this activity, the protein was named tumor necrosis factor. Priming with a variety of agents, including Bacillus Calmette-Guérin and Propionibacterium acnes, causes proliferation of macrophages that can be stimulated by LPS to secrete TNF (3-5). TNFs are secreted by both human and murine myelomonocytic cells and have been purified to homogeneity, sequenced, and produced by recombinant DNA technology (6-8). A similar factor, which induces cachexia in chronically injected animals, is also found in serum by both human and murine myelomonocytic cells and has been purified to homogeneity, sequenced, and produced by recombinant DNA technology (6-8). A similar factor, which induces cachexia in chronically injected animals, is also found in serum and is secreted by macrophages (9-12). This factor was originally designated cachectin. The NH2-terminal sequence of murine cachectin is similar to that of human TNF, suggesting that cachectin and TNF are homologous proteins (11). Partially purified and recombinant preparations of TNF have been tested for direct cytotoxicity against murine and human cells both in vitro and in vivo (2, 13-15). Tumor cell lines, but not normal cell lines from either species, are susceptible to the cytotoxic activity of murine and human TNF. Furthermore, murine TNF is active against human and murine tumor transplants in nude and normal mice, respectively (1, 4, 13, 15-21). TNF also demonstrates other biological effects; for example, it suppresses lipoprotein lipase activity and inhibits biosynthesis of differentiation-specific mRNAs in adipocytes (10), decreases prostaglandin E2, and collagenase production by human synovial cells and dermal fibroblasts (22), and induces a procoagulant activity in vascular endothelial cells (23, 24). TNF may also mediate the cytostatic properties of NK cells (25) and macrophages (26-28). The aim of the present study was to expand our understanding of the immunomodulatory properties of TNF, which we studied by examining a wide variety of effector cells, including T-cells, macrophages, NK cells, and B-cells.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C57BL/6N mice (H-2b) and C3H/HeN (mammary tumor virus-negative) H-2b mice, 3 or 4 weeks old, were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility.

Tumors. In these studies, we used the radiation-induced fibrosarcoma (29) UV-2237, syngeneic to the H-2b mouse, and UV-2237 Cl-46, a regressor clone (30) obtained from UV-2237. Lymphomas included the methylcholanthrene-induced mastocytoma P815 (31), syngeneic to the DBA/2 mouse (H-2b), and the Moloney virus-induced lymphoma YAC-1 (32) of A/SN-H-2b origin. We also used the metastatic melanoma variant B16-BL6 (33), which was selected in vitro from the B16 melanoma, a spontaneous tumor from a C57BL/6N mouse. The fibrosarcoma and melanoma were maintained as monolayers in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, glutamine, sodium pyruvate, 2-fold vitamins, and nonessential amino acids (34). The P815 and YAC-1 tumor cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and the same medium supplements added to Eagle's minimum essential medium (complete minimum essential medium). All cell lines were free of Mycoplasma and pathogenic murine viruses (34). The media, supplements, and serum contained less than 0.1 ng/ml of endotoxin (34), as assessed by the Limulus lysate assay.

Agents. rH TNF was obtained from Genentech (South San Francisco, CA (~6.72 x 107 units/mg), as well as Biogen (Cambridge, MA (~2 x 107 units/mg)). rM TNF was also supplied by Genentech (~2.9 x 107 units/mg) and Biogen (~5 x 106 units/mg). We obtained rH LT TNF-β from Genetech (~1.2 x 104 units/mg). The specific activity of these various materials varies from lot to lot and was standardized in each experiment on the basis of a previously aliquoted source. The "in laboratory" standardized units were used for all analyses.

Technical Approach. The experiments were repeated at least 3 times, and any study in which either the negative or positive control did not function was performed again. In vitro assays were analyzed with the paired Student t test.

MLR. Responder spleen cells were admixed with irradiated stimulator spleen cells in flat-bottomed, 96-well plates (34) in culture medium containing various doses of the test BRM or thymosin fraction 5, which served as a positive control. We used the culture medium described by Clinkscales et al. (35), supplemented with 0.5% murine serum. The cultures were pulse labeled with [3H]thymidine (1 μCi/well) for 24 h before harvest, which we performed with an automatic cell harvester 4 or 5 days after culture initiation.

Cytotoxic Effector Cells Stimulated in an Allogeneic MLTR-CMC Assay. In the MLTR-CMC assay, effector cells were stimulated by
culturing C3H/HeN-H-2b splenic lymphocytes with irradiated P815 (H-2b) tumor cells (34). Experimental cultures contained various concentrations of the test BRM, whereas control cultures included spleen cells cultured in normal medium or in medium supplemented with the test BRM. After a 5-day incubation, the effector lymphocytes were washed several times, lymphocyte viability was assessed, and we determined their cytotoxic properties in a 4-h 51Cr release assay with P815 targets. The cytotoxic effector cell induced was specific for P815 and sensitive to anti-Thy antiserum but not to anti-asialo-monoganglioside antiserum (36). Cytotoxicity was calculated as

\[
\text{% of cytotoxicity} = \frac{\text{cpm released by lymphocytes} - \text{cpm released spontaneously}}{\text{Total Triton-released cpm}} \times 100
\]

Induction of Tumor-specific Cytotoxic T-Lymphocytes. Syngeneic mice were immunized with \(5 \times 10^5\) irradiated, collagenase-dissociated UV-2237 tumor cells, with or without an adjuvant, by injecting 0.05 ml of HBSS containing \(2 \times 10^5\) cells into 5 i.d. sites as the primary vaccine. Some mice also received i.p. injections of the adjuvant on days 1 and 2. Ten days after primary immunizations, the animals received a secondary immunization of \(5 \times 10^5\) cells total in 5 i.d. injections with the adjuvant as an admixture; this was followed by an i.p. injection on days 11 and 12. We used HBSS or vaccines consisting of the test BRM alone as controls. Target cells for the cytotoxic T-lymphocyte assays were added to a flat-bottomed, 96-well plate in 0.1 ml of complete minimum essential medium containing 5% fetal bovine serum and 5000 viable tumor cells radiolabeled with \(^{35}\)Se-methionine (34). Five days after the secondary immunization or 15 days after the primary immunization, specific effector cells at various effectortarget cell ratios were added in triplicate. The cocultures were incubated at 37°C for 18 h and centrifuged for 3 min. We removed 0.1-ml aliquots and determined the amount of radioactivity released. The percentage of cytotoxicity was calculated as

\[
\text{% of cytotoxicity} = \frac{\text{cpm released in cultures with effector cells}}{\text{cpm released spontaneously}} \times 100
\]

Assay for Macrophage-mediated Cytotoxicity. Thioglycollate-elicited peritoneal exudate macrophages from normal mice were collected and the macrophage cytotoxicity assay was performed as previously described (34). Briefly, macrophage suspensions were plated into a flat-bottomed, 96-well plate and incubated for 2 h, and nonadherent cells were removed. The monolayers were incubated with control medium or an activating agent for 24 h, after which we removed the medium and replaced it with medium containing \([^{32}\text{P}]\)iododeoxyuridine-radiolabeled B16-BL6 target cells. In experiments using in vivo-activated macrophages, mice were given i.p. injections of saline, poly(I,C)-LC (which served as a positive control), or the test BRMs. Twenty-four h later, these peritoneal cells were harvested, adhered purified, and cocultured with radiolabeled target cells. The cytotoxicity assays from both the in vitro and in vivo macrophage augmentation studies were added to a flat-bottomed, 96-well plate in 0.1 ml of complete medium supplemented with sodium pyruvate, equine serum (10%), fetal bovine serum (20%), L-serine, and tryptic soy broth (0.6%). For colony growth, medium was supplemented with 0.3% Bacto-Agar and 100 units/ml of rM colony-stimulating factor-granulocyte macrophage (Difco Laboratories, Detroit, MI). Ninety-six-well (0.2 ml) flat-bottomed microtiter plates were used for culture, and 24 wells were set up for each dilution of the cell suspension tested. The plates were incubated for 8 or 9 days in 100% humidity, in a 5% CO2-air atmosphere. The wells were scored as positive or negative with a dissecting microscope at \(\times 100\) magnification. The CFUCl precursor frequency was calculated from the limiting dilution data by probability distributions. Lines were fitted to the data points using the BMDP-JR program on an IBM-AT computer.

RESULTS

Assessment of NK Cell Activity. The ability of rH TNF to activate NK cells in vitro or in vivo (Table 1). In these studies, using endotoxin-free rH TNF, no augmentation of NK cell activity was observed either in vitro or in vivo. The positive control, poly(I,C)-LC, significantly stimulated splenic NK cell activity, whereas rH TNF at 0.1 to 1,000 units/ml in vitro or 5,000 to 5,000,000 units/animal in vivo did not stimulate NK cell activity. No NK cell augmentation was observed in vivo from 1 to 3 days after the i.v. administration of rH TNF. Therefore, the inability of rH TNF to augment NK cell activity did not appear to be associated with the delayed NK cell augmentation that is characteristic of immune modulators such as...
as methyl divinyl ether (37). However, multiple injections of toxic doses of rH TNF resulted in the activation of pulmonary NK cells, an augmentation that presumably was associated with the inflammatory-repair process rather than with direct NK cell augmentation (results not shown).

MLR. The allogeneic MLR (34) was performed at a suboptimal responder:stimulator cell ratio (10:1) in order to demonstrate maximal immunomodulation (36). When spleen cells were cocultured with the positive control (100 μg/ml of thymosin fraction 5), a significant increase was observed in the incorporation of [3H]thymidine in the allogeneic reaction (Table 2). Similarly, when 1 to 10,000 units of rH TNF or rM TNF was cocultured with the allogeneic MLR, it significantly increased the incorporation of [3H]thymidine. The optimal incorporation of [3H]thymidine occurred at 10 units/ml of rH TNF and 100 units of rM TNF. Neither rTNF appeared to be blastogenic to responder cells cultured alone. In addition, there seemed to be a trend for doses of TNF greater than 1000 units/ml to decrease the incorporation of [3H]thymidine as compared to the optimal dose. This occurred in responder cells cultured with both allogeneic stimulator cells and rH TNF or rM TNF. Similar results were obtained with rH LT (results not shown).

Allogeneic MLTR-CMC Assay. The ability of rH TNF to stimulate the generation of cytotoxic T-effector cells was also examined during an allogeneic MLTR-CMC assay (Table 3). This assay was performed at a suboptimal stimulator:effector cell ratio (1:300) to optimize the detection of immunostimulatation (36). However, none of the doses of rH TNF significantly increased effector cell activity. At doses of 1 to 1,000 units/ml, rH TNF was nontoxic to the lymphocytes after a 5-day cult ure; there was, however, a nonsignificant trend for a decrease in cell viability and [3H]thymidine incorporation at 10,000 units/ml. Similar results have also been obtained with rM TNF and rH LT (results not shown). The positive control in this experiment, thymosin fraction 5, significantly stimulated cytotoxic effector cells, demonstrating that immunostimulation did occur in this experimental system.

Specific Adjuvant Activity of rTNF. The immunoadjuvant activity of rTNF was assessed by examining its effect on the in vivo generation of splenic cytotoxic T-lymphocyte activity (34). Spleen cells from mice immunized with a suboptimal tumor vaccine did not have significantly increased splenic cytotoxic activity compared to control spleen cells (Table 4). However, the addition of 25 mg/kg of FK-565 (the positive control), rM TNF, rH TNF, or rH LT to the suboptimal tumor cell vaccine significantly increased the level of cytotoxic effector cell activity compared to a vaccine of tumor cells alone (Table 4). This adjuvant activity was dose dependent, such that lower doses had no adjuvant-like activity for the tumor vaccine. Administering any of these cytokines alone at adjuvant doses, in the absence of the tumor vaccine, did not increase levels of splenic cytotoxic T-lymphocytes when compared to spleen cells from mice receiving HBSS alone. The immune effector cells induced by the vaccines had a specific cytotoxicity such that no increase in cytotoxicity was observed against specificity control, B16-BL6 tumor targets in parallel cytotoxicity (results not shown).

It is possible that the foreign antigenicity, rather than the biological activities of the rH TNF and rH LT, was the source of the adjuvant activity. Thus, it was important to demonstrate that the rM TNF also had adjuvant activity for the tumor vaccines. It is possible that the lack of glycosylation on the recombinant molecules provides some foreign antigenicity to the host and is the source of the adjuvancy. However, similar results were obtained in other studies using natural murine TNF (obtained from Dr. Old of Memorial Sloan Kettering Institute) as a partially purified preparation. Thus, it appears that both murine and human TNF do have adjuvant activities.

Adjuvant Activity of TNF for Antibody Production. The ability of TNF to augment anti-BSA antibody production was investigated using an enzyme-linked immunosorbent assay. BSA was injected i.p. as an antigen on day 0 at a suboptimal vaccine dose. Various doses of rH TNF were administered 48 h following the second injection of BSA. The mice were bled 3 days later and serum antibody levels were determined using routine enzyme-linked immunosorbent assay techniques. The i.v. injection of either methyl divinyl ether (positive control) or rH TNF augmented the production of antibody directed against BSA in a dose-dependent manner (Fig. 1). However, the injection of TNF alone did not induce detectable serum antibody levels.

Macrophage Activation. The ability of rTNF to activate macrophages in vitro was studied by incubating C57BL/6NJ thiglycollate-elicited macrophages with either rH TNF, rM TNF (Table 5), or poly(I:C)-LC (positive control) for 24 h. Cytotoxicity was assessed using syngeneic [125I]iododeoxyuridine-labeled B16-BL6 target cells in a 72-h assay (34). At levels of TNF greater than 0.01 units/ml, significant activation of macrol

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**Table 3** Effect of rH TNF on in vitro generation of alloreactive cytotoxic T-lymphocytes (MLTR-CMC)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/ml)</th>
<th>Stimulator</th>
<th>% viable (day 5)</th>
<th>% of cytotoxicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>–</td>
<td>–</td>
<td>76 0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Thymosin fraction 5</td>
<td>100 μg</td>
<td>–</td>
<td>88 4 3 3</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>1 unit</td>
<td>–</td>
<td>79 2 2 3</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rM TNF</td>
<td>10 units</td>
<td>–</td>
<td>79 1 4 4</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>100 units</td>
<td>–</td>
<td>70 4 3 5</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rM TNF</td>
<td>1,000 units</td>
<td>–</td>
<td>70 4 5 4</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>10,000 units</td>
<td>–</td>
<td>78 5 5 5</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Medium</td>
<td>+</td>
<td>–</td>
<td>78 54 7 3</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Thymosin fraction 5</td>
<td>100 μg</td>
<td>+</td>
<td>80 57 30</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>1 unit</td>
<td>+</td>
<td>82 46 24 14</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>10 units</td>
<td>+</td>
<td>74 59 27 20</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rM TNF</td>
<td>100 units</td>
<td>+</td>
<td>57 46 26 20</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>1,000 units</td>
<td>+</td>
<td>60 58 26 16</td>
<td>0 0 0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>10,000 units</td>
<td>+</td>
<td>56 53 22 13</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

* Cytotoxicity was determined at effector:target cell ratios of 50:1, 25:1, and 12:1.

* Significant difference in cytotoxicity compared to medium control, as determined with Student's t test (P < 0.01).

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**Table 2** Effect of rH and rM TNF on induction of MLR and in vitro generation of alloreactive cytotoxic T-lymphocytes (MLTR-CMC)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (units/ml)</th>
<th>Stimulator</th>
<th>% viable (day 5)</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>–</td>
<td>–</td>
<td>2,389 7,829 8,264 8,134</td>
<td>10,834</td>
</tr>
<tr>
<td>Thymosin fraction 5</td>
<td>50 μg</td>
<td>–</td>
<td>2,374 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rTNF</td>
<td>1 unit</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rTFN</td>
<td>10 units</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rM TNF</td>
<td>10 units</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rM TNF</td>
<td>100 units</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rM TNF</td>
<td>1,000 units</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
<tr>
<td>rM TNF</td>
<td>10,000 units</td>
<td>–</td>
<td>2,788 24,402 8 2,709 14,706</td>
<td>10,834</td>
</tr>
</tbody>
</table>

* Significant change in the incorporation of [3H]thymidine compared to medium control, as determined by the paired Student t test (P < 0.01).
Table 4 Adjuvant activity of TNFs on the development of syngeneic cytotoxic T-lymphocytes

<table>
<thead>
<tr>
<th>Dose (mg/kg)*</th>
<th>Agent</th>
<th>rM TNF</th>
<th>rH LT</th>
<th>rH TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UV-2237</td>
<td>200:1</td>
<td>100:1</td>
</tr>
</tbody>
</table>
| C3H mice were immunized with collagenase-DNase-dissociated syngeneic UV-2237 tumor cells (5 x 10^6 cells total in 5 i.d. injections) in HBSS, recombinant lymphokine, or FK-565 (positive control). The adjuvant was administered at time 0 as an admixture and on days 1 and 2 as a i.p. injection. Ten days after primary immunization, a secondary immunization was administered; 5 days later, effector cell activity was examined in a 24-h radiorelease assay with ^35S-methionine-labeled Cl-46 cells. These were obtained from clonal subpopulations of UV-2237 and are more sensitive to cytotoxic T-cell activity than the parent tumor. Each group contained 3 mice.

Table 5 In vivo activation of thioglycollate-elicited peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were incubated for 24 h with medium, poly(I:C)-LC (positive control), or rTNF at various doses. [125I]iododeoxyuridine-labeled B16-BL6 tumor cells were plated onto the peritoneal macrophage monolayers in triplicate, incubated for 72 h, and assayed for cytotoxicity.

Table 6 In vivo activation of peritoneal macrophage-mediated cytotoxicity by rTNF

C57BL/6 mice (N = 5/group) received i.p. injections of poly(I:C)-LC or rTNF. Twenty-four h later, peritoneal exudate cells were harvested. The macrophages were purified by adherence and used as effector cells with [125I]iododeoxyuridine-labeled B16-BL6 tumor cells in a 72-h assay.

Fig. 1. Adjuvant activity of rH TNF for the production of IgG directed against the BSA vaccine. Mice received a priming vaccine of BSA and, 10 days later, a second vaccine of BSA with or without various doses of rH TNF. On day 15, the mice were bled and the serum antibody was determined in an enzyme-linked immunosorbent assay.

rTNF also activated peritoneal macrophages to become cytotoxic in vivo (Table 6). Mice were given i.p. injections of poly(I:C)-LC, rH TNF, or rH LT and peritoneal exudate macrophages were obtained by lavage 24 h later. In these studies, there was a dramatic bell-shaped dose-response curve, such that high doses of rH TNF (>0.5 mg/kg) had minimal but significant macrophage-activating properties, while significantly greater augmentation was observed at lower doses. Results with rH LT were similar. This experiment revealed macrophage activation at doses of 0.005 to 0.0005 mg/kg for rH TNF or rH LT as well as rM TNF (results not shown). In other studies, we observed that multiple daily injections of rTNFs (see rM TNF data in Table 7) significantly augmented macrophage activation compared to a single injection. Studies of the kinetics of peritoneal macrophage activation by rH TNF (Table 8) revealed that peak activation occurs approximately 48 h following i.p.

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IMMUNOMODULATORY PROPERTIES OF RECOMBINANT TNF

Table 7 Effect of multiple injections of rM TNF on in vivo activation of peritoneal macrophage-mediated cytotoxicity

C57BL/6 mice (N = 5/group) received either 1 or 4 i.p. injections of rM TNF. Twenty-four h later, peritoneal exudate cells were harvested. The macrophages were purified by adherence and used as effector cells with [125I]iododeoxyuridine-labeled B16-BL6 tumor cells in a 72-h assay.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 injection</td>
<td>4 injections</td>
</tr>
<tr>
<td></td>
<td>-LPS</td>
<td>+LPS</td>
</tr>
<tr>
<td></td>
<td>-LPS</td>
<td>+LPS</td>
</tr>
<tr>
<td>HBSS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rM TNF</td>
<td>0.5</td>
<td>17*</td>
</tr>
<tr>
<td>rM TNF</td>
<td>0.05</td>
<td>28*</td>
</tr>
<tr>
<td>rM TNF</td>
<td>0.005</td>
<td>32*</td>
</tr>
<tr>
<td>rM TNF</td>
<td>0.0005</td>
<td>7*</td>
</tr>
</tbody>
</table>

* Significantly decreased cpm compared to HBSS control (P < 0.005).

Table 8 Kinetics of the in vivo activation of peritoneal macrophage-mediated cytotoxicity by rH TNF

C57BL/6 mice (N = 5/group) received i.p. injections of rH TNF (0.5 mg/kg) at various times. Peritoneal exudate cells were harvested by peritoneal lavage at time 0 and tumoricidal activity was determined with [125I]iododeoxyuridine-labeled B16-BL6 tumor cells in a 72-h assay.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Time</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-LPS</td>
<td>+LPS</td>
</tr>
<tr>
<td></td>
<td>-LPS</td>
<td>+LPS</td>
</tr>
<tr>
<td>HBSS</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-9</td>
<td>10</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-7</td>
<td>12*</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-4</td>
<td>17*</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-3</td>
<td>27*</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-2</td>
<td>36*</td>
</tr>
<tr>
<td>rH TNF</td>
<td>-1</td>
<td>24*</td>
</tr>
</tbody>
</table>

* Significantly decreased cpm compared to HBSS control (P < 0.005).

administration, with activity returning to near background levels by days 7 to 9. Thus, rM TNF, rH TNF-α, and rH LT seem to have similar abilities to activate murine macrophages both in vitro and in vivo. It appears that maximal activation occurs following multiple injections of TNF and that relatively high levels of activation are maintained for approximately 96 h.

Effect of TNF on Bone Marrow Stem Cells. The i.v. injection of rH TNF has a dramatic effect in vivo on bone marrow cellularity and on the number of CFUCs per femur. Fig. 2A demonstrates the ability of a single injection of rH TNF to significantly depress the number of cells per femur at 1, 0.5, and 0.05 mg/kg of rH TNF 3 days later. In addition, there was a depression in the number of CFUCs per femur when 0.05 to 1 mg/kg of rH TNF was injected i.v. (Fig. 2B). However, the CFUC frequency per 10^6 bone marrow cells was only slightly depressed, suggesting that much of the effect of TNF on stem cell activity is due to the depression in cellularity with only a minor effect on stem cell frequency (results not shown). The maximum depression in stem cell activity occurs 3 days following i.v. administration of TNF (Fig. 3), and animals that received 50 μg/kg had recovered 18 days following administration. However, the administration of 1 mg/kg by i.v. injection still significantly depressed the number of CFUCs per femur at this time. In other studies, we found that the myeloid depression associated with TNF could be partially reduced with cyclooxygenase inhibitors such as aspirin. This treatment affected bone marrow cellularity as well as the number of CFUCs per femur, as shown in Fig. 4. In this study, animals received aspirin 30 min prior to and 4 h after the injection of various doses of rH TNF. In every case, the injections provided significant protection to bone marrow stem cell populations.

DISCUSSION

TNF has been reported to have direct cytotoxic-cytostatic activity in approximately 30% of all human and murine tumor
TNF cannot activate NK cells, demonstrating that this agent 
tumors (1,4, 13, 15-21) and, in recent studies from our labo-
been found to have significant therapeutic properties for i.d.
Fig. 4. Mice were given injections of various doses of rH TNF with or without 
with aspirin, which was administered 30 min before and 4 h after rH TNF treatment.
Twenty-four h later, the number of CFUC per femur was determined for the 2 
groups of animals.

cell lines (4-8, 13-15). In addition, recent studies by Cerami 
(9-11) have revealed that the activity of TNF is identical to 
that of cachectin, causing increased metabolism of lipids by 
adipocytes and a wasting phenomenon in vivo (10). TNF has 
been found to have significant therapeutic properties for i.d. 
tumors (1, 4, 13, 15-21) and, in recent studies from our labo-
atory, somewhat limited therapeutic properties for metastatic 
disease (15). Recent studies have also revealed that TNF can 
activate polymorphonuclear cells (37, 38) and increase their 
adherence to endothelial cells (39).

We report here that TNF has significant immunomodulatory 
properties for macrophages and adjuvant activity for both T-
cell and B-cell responses. We also observed the augmentation of 
an allogeneic MLR in vitro. TNF can significantly activate 
macrophages in vitro and in vivo in a dose-dependent manner, 
and LPS acts as a secondary signal to produce increased mac-
rophage activation. However, significant tumoricidal activity is 
also observed in the absence of exogenous endotoxin as a second 
signal. In vivo, multiple injections of TNF result in higher levels 
of macrophage activation than a single administration. Maxi-
mal macrophage activation occurs approximately 48 h follow-
ing i.p. administration of TNF; levels of activity remain elevated 
for at least 96 h and return to near background by 7 days. In 
addition, TNF has potent adjuvant activity for tumor cell 
vaccines and stimulates serum antibody production in response 
to a foreign antigen. Whether or not this is associated with its 
ability to induce interleukin 1 activity [demonstrated following 
in vitro coculture of macrophages (results not shown) or in 
spleen cell cultures (40)] or is due to direct activity of TNF 
remains to be determined. TNF also increased an allogeneic 
MLR in vitro but did not increase an allogeneic MLTR, as 
assessed by measuring cell-mediated cytotoxicity. However, 
TNF cannot activate NK cells, demonstrating that this agent 
distinguishes between macrophage and NK cell augmentation. 
Thus, TNF has a number of immunomodulatory properties 
which may have a role in its therapeutic and direct cytotoxic-
cytostatic activities. The importance of its adjuvant activity has 
been shown in studies from the laboratory of Dr. Mulé,* which 
revealed that antigenic tumors respond to TNF better than less 
antigenic tumors. We have also found that TNF at low doses 
has significant therapeutic activity for UV-induced autochto-
ous skin tumors, which in the absence of suppressor cells are 
also highly antigenic.

One potential drawback to the clinical utilization of TNF is 
its dramatic effect on bone marrow stem cells. In part, this 
effect is associated with the ischemic necrosis that we have 
observed during histological examination (results not shown). 
This toxic response can be partially inhibited with cyclooxy-
genase inhibitors and corticosteroids. Thus, as in previous in vitro 
studies demonstrating that TNF directly affects bone marrow 
cells (41-44), we have found that TNF has activity in bone 
marrow cells. This appears to be due in part to direct cytotoxic-
ity, resulting in a decreased number of stem cells as well as 
microthrombi-associated ischemic necrosis, which can be par-
tially ameliorated by cyclooxygenase inhibitors. Recovery from 
myelosuppression is observed approximately 2.5 to 3 weeks 
following the injection of even moderate doses of TNF. Thus, 
combining chemoimmunotherapy or radioimmunotherapy 
with TNF must be cautiously undertaken. Indeed, preliminary 
studies in our laboratory have demonstrated a significant in-
crease in toxicity when radiation and TNF are combined.

In summary, rH TNF, rH LT, and rM TNF have similar 
immunomodulatory properties. Indeed, in several assays in 
which we directly compared these agents, equisidine responses 
were observed, suggesting that the various TNFs have similar 
properties regardless of their amino acid sequences. The rTNFs 
can augment an allogeneic MLR, activate macrophages both in 
and in vivo, serve as potent adjuvants for suboptimal tumor 
vaccines, and increase the serum antibody response to foreign 
antigens. They also adversely affect bone marrow cellularly 
and the number and frequency of CFUCs (stem cells). This last 
toxic effect requires a prolonged recovery period and may 
impede combination chemoimmunotherapy or radioimmuno-
thrapy.

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* Mulé, personal communication.

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IMMUNOMODULATORY PROPERTIES OF RECOMBINANT TNF


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