

THE ANTITUMOR FUNCTION OF TUMOR NECROSIS FACTOR (TNF)

I. Therapeutic Action of TNF against an Established Murine Sarcoma Is Indirect, Immunologically Dependent, and Limited by Severe Toxicity

BY EDWARD A. HAVELL, WALTER FIER, AND ROBERT J. NORTH

*From The Trudeau Institute, Saranac Lake, New York 12983; and the Laboratory of
Molecular Biology, the State University of Ghent, Ghent, Belgium*

Tumor necrosis factor (TNF) is a protein synthesized and secreted by mononuclear phagocytes in response to stimulation with bacterial endotoxin and other agents. Originally TNF was defined functionally as the factor in postendotoxin serum from *Propionibacterium acnes*-treated mice that mediated hemorrhagic necrosis of established fibrosarcomas in recipient mice (1). More recently, it has been established that TNF, and cachectin, the molecule responsible for the toxic symptoms of endotoxin, are one and the same molecule (2, 3). The genes for human (4–7) and mouse (7, 8) TNF have been cloned and expressed in *Escherichia coli*. Consequently, adequate quantities of pure human and mouse rTNF are now available for study.

For the most part, the antitumor function of rTNF has been studied in terms of its cytotoxic activity for neoplastic cells in vitro. Such studies are an extension, therefore, of those originally performed (9) with TNF-containing postendotoxin serum (TNS)¹ from *P. acnes*-treated mice. TNS was shown to be capable of killing certain neoplastic cells in vitro, as well as causing hemorrhagic necrosis of established tumors in vivo. Indeed, it has been often assumed, since these original findings, that the in vivo therapeutic action of TNF is based on its ability to directly kill cells of the tumor. However, the evidence that TNF is therapeutic in vivo is not particularly convincing in that it is not based on the ability of TNF to cause tumor regression, but on its ability to cause hemorrhagic necrosis of the centers of established tumors. In other words, treatment with TNF rarely results in complete regression of the ring of living tumor tissue that survives central hemorrhagic necrosis.

The same shortcoming applies to therapy with bacterial endotoxin, which has been known for many years to be capable of causing central hemorrhagic necrosis of most tumors, but complete regression of only some (10). The possible reason for the limited therapeutic effect of endotoxin was supplied by Parr et al. (11), who demonstrated that only immunogenic tumors are susceptible to endotoxin-

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¹ Abbreviation used in this paper: TNS, tumor-necrotizing serum.

induced regression, and that immunogenic tumors fail to undergo complete regression if they are growing in immunosuppressed mice. These findings were essentially confirmed by others (12, 13), who postulated that endotoxin-induced tumor regression, as opposed to hemorrhagic necrosis, depends on the possession by the host of an underlying mechanism of antitumor immunity. It was demonstrated more recently (14) that endotoxin-induced regression of an immunogenic sarcoma depends on the preceding generation by the host of a subtherapeutic number of tumor-sensitized L3T4⁺ T cells. Given the belief that TNF is the mediator of endotoxin-induced tumor regression, it might be expected that TNF-induced tumor regression is also immunologically dependent.

The purpose of this paper is to present results in keeping with the view that the therapeutic action of TNF against an established immunogenic sarcoma does not depend on the ability of TNF to directly destroy cells of this tumor in vivo, but on its ability to directly or indirectly destroy the tumor's vasculature. In this way destruction of most of the center of the tumor results from ischemia. It will be shown, in addition, that TNF-induced hemorrhagic necrosis is reduced, and complete regression of the outer ring of living tumor tissue fails to occur, if the host is made incapable of generating an antitumor immune response. Moreover, in all cases, almost lethal quantities of TNF were required to cause tumor regression in immunocompetent mice.

Materials and Methods

Mice. A/Tru and AB6F1 (A × C57BL/6) female mice were used at 12 wk of age. They were supplied by the Trudeau Institute Animal Breeding Facility, and were free of common viral pathogens, as evidenced by the results of routine screening provided by the Diagnostic Testing Service of Microbiological Associates, Bethesda, MD.

Tumors. The SA1 spindle cell sarcoma syngeneic in A/J mice was obtained some years ago from The Jackson Laboratory, Bar Harbor, ME. It was grown as an ascites in A/Tru mice, harvested in PBS, suspended in Fishers' medium (Gibco Laboratories, Grand Island, NY) containing 10% DMSO and 20% FCS, and stored in small vials over liquid nitrogen to serve as stock tumor. Experiments were performed in semisyngeneic AB6F₁ mice because they were more plentiful and less expensive than the parental strains. There was no evidence of hybrid resistance against the SA1 sarcoma. Before each experiment the tumor was grown as an ascites intraperitoneally in AB6F₁ mice, harvested and washed in PBS, and resuspended in PBS for implantation. Tumors were initiated by intradermal injection of 10⁶ tumor cells in the midventral region. Tumor growth was monitored against time by measuring changes in the mean of two perpendicular diameters.

In Vitro Cytotoxic Assay for rTNF and TNS. A modified assay based on the procedure described by Wang et al. (6) was used. Briefly, 1.5 × 10⁴ murine L929B cells in 100 μl of Eagle's MEM (Microbiological Associates, Walkersville, MD) containing 5% FCS and 100 U/ml of penicillin and 100 μg/ml streptomycin, were placed in individual wells of 96-well microtiter plates, and incubated overnight at 37°C in a 5% CO₂ humidified incubator. Sequential twofold dilutions of the rTNF solution or TNS were made in the above medium containing 2 μg/ml of actinomycin D (Calbiochem-Behring Corp., La Jolla, CA), and 100 μl of each dilution was added to replicate wells of microtest plates seeded the day before with L929B cells. After 24 h of incubation, cytotoxicity was scored microscopically. The cytotoxicity titer (U/ml) is defined as the highest dilution of the test material causing 50% or more destruction of the monolayer of L929B cells. To assess the relative sensitivities of the L929B assay cells at different times an internal rTNF laboratory standard was included in all assays. All given TNF titers (U/ml) have been corrected to this standard preparation.

rTNF and TNS. Murine rTNF was produced in *E. coli* and purified (sp act 1.37 × 10⁸

U/mg protein; <10 ng endotoxin/mg protein) as described previously (7). SDS-PAGE showed that the purified preparation consisted of a single band corresponding to a mol wt of 17×10^3 . This rTNF was generously supplied by Dr. Jan Tavernier (Ghent, Belgium). TNS was prepared from the blood of mice that were injected intravenously 2 h earlier with 50 μ g of *Salmonella enteritidis* endotoxin (batch 3105-25, Difco Laboratories, Detroit, MI). The mice had been injected intravenously 2 wk earlier with 1 mg of formalin-killed *P. acnes* (Trudeau Institute), according to the standard procedure for priming mice for endotoxin-induced TNF production (9).

Anti-rTNF Antibody. Specific neutralizing antibodies to pure murine rTNF were raised in rabbits according to previously reported procedures (15). Initially, rabbits received a total of 2 ml of an rTNF (10^7 U)/adjuvant mixture at multiple subcutaneous sites. The first two biweekly immunizations were done with CFA, and all subsequent booster immunizations were carried out with IFA. Immune sera were collected, heat inactivated at 56°C for 30 min, and the IgG was purified and concentrated by three cycles of $(\text{NH}_4)_2\text{SO}_4$ precipitation (33% saturation), exhaustively dialyzed, and then sterilized by filtration. 1 ml of the resulting IgG preparation was capable of neutralizing 3×10^6 TNF units.

Quantitation of the TNF-induced Tumor Hemorrhagic Reaction. The TNF-induced intratumor hemorrhaging was quantified by measuring the intratumor extravasation of ^{51}Cr -labeled syngeneic red cells against time. To prepare ^{51}Cr -labeled red cells, syngeneic mice were bled by cardiac puncture into a syringe containing a solution of citric acid-dextrose solution. The red cells were pelleted by centrifugation and washed three times in RPMI 1640 medium (Gibco Laboratories). To each 0.5 ml of packed red cells was added 0.5 ml of RPMI 1640 medium containing 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ having a sp act of 250–500 mCi/mg (Amersham Corp., Arlington Heights, IL). The RBCs were suspended and incubated for 1 h at 37°C, after which they were washed three times in RPMI 1640, and resuspended in 2 ml of PBS for intravenous infusion. Each mouse was infused with 0.2 ml of the red cell suspension. This resulted in the labeling of ~ 1 in 20 circulating red cells. The mice were used in TNF experiments not earlier than 30 min after red cell infusion. To measure TNF-induced intratumor hemorrhaging, mice were injected intravenously with rTNF or TNS, and at 5 min, and 1, 2, 4, and 6 h later, three to four mice were bled by cardiac puncture, killed by cervical dislocation, and their tumors were excised. Each tumor was placed in a glass vial and its content of ^{51}Cr was determined with a Rack Gamma II Counter (LKB Instruments, Inc., Gaithersburg, MD). The results are given as total counts per minute per tumor. The reaction was followed for only 4–6 h, because the results of a study in progress (Havell, E. A., and R. J. North, submitted for publication) show that extravasation of red cells does not continue beyond this time.

T Cell-deficient (TXB) Mice. Mice were made T cell deficient by thymectomy at 6 wk of age followed 1 wk later by exposure to 1,000 rad of γ -radiation from a ^{137}Cs source at a midphantom dose rate of 30 rad/min. Immediately after irradiation, the mice were infused with 2×10^6 syngeneic bone marrow cells, and used in experiments 6 wk or more later.

Results

In Vitro Toxicity of rTNF and TNS for SA1 Tumor Cells and L929B Cells. Although TNF was originally defined in terms of its ability to cause necrosis of established tumors in vivo (1), it is currently being studied in most laboratories in terms of its in vitro toxicity for neoplastic cells. Consequently, different preparations of TNF are compared in terms of their in vitro cytotoxic activity. Therefore, before investigating the therapeutic action of the rTNF and TNS preparations in this laboratory against an established SA1 sarcoma, it was necessary to determine their cytotoxicity for L929B cells in vitro. It was also necessary to determine whether they were capable of killing cells of the SA1 sarcoma in vitro.

TABLE I
In Vitro Cytotoxic Activities of Murine Tumor Necrotizing Serum (TNS) and Recombinant Murine Tumor Necrosis Factor (rMuTNF)

Cells	Cytotoxicity titer*	
	TNS	rMuTNF
L929B cells + actinomycin D [†]	2.1×10^5	4.1×10^7
L929B cells	3.2×10^2	6.4×10^4
SA-1 sarcoma	1.0×10^3	8.2×10^5
Murine embryo fibroblasts	<8	<8

* 1 U of cytotoxic activity is the concentration of test preparation that causes 50% destruction of a given cell type, and the titer of cytotoxic activity (U/ml) is the reciprocal of the highest twofold dilution of the preparation that causes $\geq 50\%$ cell destruction.

[†] All cells were preseeded 1 d before the addition of the given preparation. Actinomycin D was added to a final concentration of 1 $\mu\text{g/ml}$ along with the test preparation to preseeded cultures.

TABLE II
Specific Activities of TNS and rTNF

Sample	Cytotoxic activity on actinomycin D-treated L929B cells	Protein	Specific activity (U/mg protein)
	U/ml	mg/ml	
rMuTNF	4.1×10^7	0.3	1.37×10^8
TNS	2.1×10^5	62.5	3.40×10^5

Table I shows the toxicity of the rTNF and TNS preparations under study in this laboratory for SA1 cells, L929B cells, and diploid AB6F₁ embryonic fibroblasts. Both preparations were highly toxic for SA1 cells, as well as for L929B cells, but showed no cytotoxicity at all for embryonic fibroblasts. In keeping with the general finding of others, treatment of L929B cells with actinomycin D increased their sensitivity to rTNF and TNS by 500–1,000-fold. The rTNF proved 200–800 times more active than TNS (U/ml) on the cells that were susceptible. In Table II are given the specific activities of the pure rTNF and TNS, based on their cytotoxic activities on actinomycin D-treated L929B cells. The specific activity of the rTNF was 4×10^4 times greater than that of the TNS.

rTNF and TNS Cause Regression of the SA1 Sarcoma But Only When Given at Near Lethal Doses. Figs. 1 and 2 show the results of attempts to cause complete regression of a 9-d SA1 sarcoma with intravenous injection of decreasing twofold dilutions of rTNF and TNS, respectively, starting at 10^6 U of rTNF and 4×10^5 U of TNS. It can be seen in the case of rTNF that 10^6 U killed all mice; 5×10^5 U killed three of five, with the tumor in one of the survivors undergoing complete regression; 2.5×10^5 was not lethal for any mice, but tumor regression occurred in only three of five mice. The therapeutic effect of lower doses of rTNF was even less impressive. It should be mentioned in regard to these

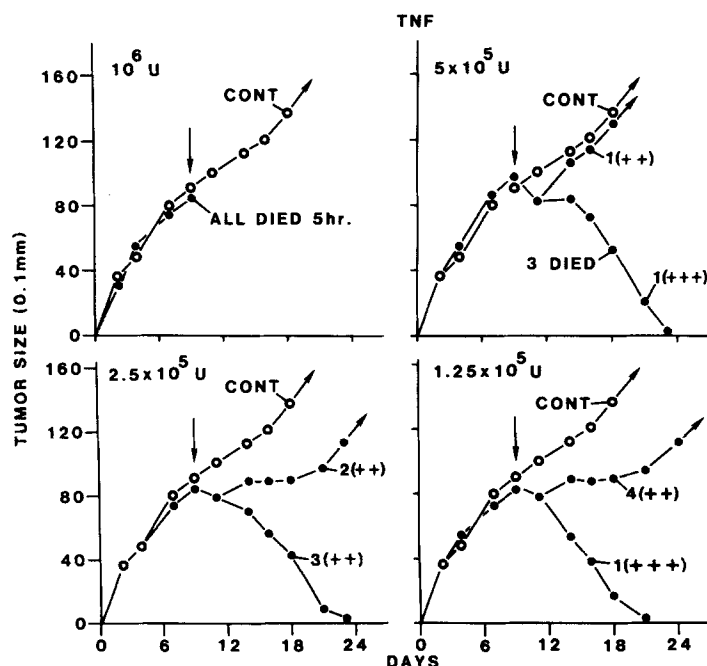


FIGURE 1. Effect of intravenous injection of 10^6 , 5×10^5 , 2.5×10^5 , or 1.25×10^5 U of rTNF on growth of the SA1 sarcoma growing from an intradermal implant. The rTNF was injected on day 9 of tumor growth. In this experiment the most therapeutic dose of rTNF was 2.5×10^5 U, in that it caused complete regression of the tumor in three of five mice. Higher doses were too toxic, whereas lower doses failed to cause tumor regression. All doses tested were capable of causing hemorrhagic necrosis of >75% (+++) or 50% (++) of the center of the tumor.

findings that the half-life of intravenously infused rTNF was determined to be 7–8 min, which is in agreement with the findings of Buetler and Cerami (3).

In the case of TNS (Fig. 2), the most therapeutic dose was 10^5 U, which was highly toxic but not lethal, and caused regression of tumors in five of five mice. In other experiments, however, this dose of TNS killed a significant proportion of mice, and higher doses were more lethal.

In all of the above experiments, tumors were examined 24 h after giving rTNF or TNS to subjectively assess the degree of central hemorrhagic necrosis. In general, complete tumor regression was preceded by extensive hemorrhagic necrosis involving >75% of the tumor (scored as +++). Smaller degrees of hemorrhagic necrosis (scored as ++) resulting from lower doses of rTNF and TNS left wider rims of living tumor tissue which, more often than not, did not undergo complete regression.

Mice that died from the toxic effects of rTNF and TNS did so before 24 h. However, those that survived therapeutic doses showed symptoms of extreme toxicity, including hypothermia, lethargy, piloerection, and severe diarrhea.

The Effect of rTNF Was no Greater against Smaller Tumors or when Injected Intratumorally. The foregoing experiments tested the ability of rTNF and TNS given intravenously to cause a regression of a relatively large (8–9-mm diameter) 9-d tumor. A 9-d SA1 sarcoma was chosen because previous studies had shown

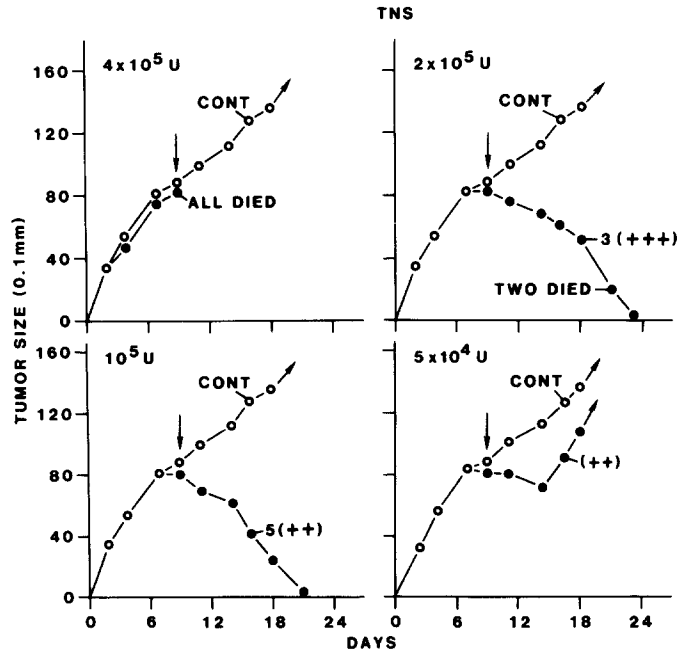


FIGURE 2. The same experiment as shown in Fig. 1, except that TNS was used. TNS proved more reliable than rTNF at causing regression in that it was capable, in a dose of 10^5 cytotoxic units, of routinely causing regression of the SA1 sarcoma in five of five mice. Higher doses were lethal, and lower doses were less therapeutic. All doses of TNS tested caused hemorrhagic necrosis that involved $>75\%$ (+++), or $>50\%$ (++) of the tumor's center.

(12, 13) that the tumor was most susceptible to endotoxin-induced regression at this stage of its growth. The possibility existed, however, that tumor regression could be achieved with less toxic amounts of rTNF or TNS if the tumor was smaller at the time of treatment. It also seemed possible that more impressive therapeutic results would be obtained if the agents were injected directly into the tumor, rather than intravenously. It can be seen in Fig. 3 that neither possibility turned out to be true, in that a dose that was partially therapeutic against a 9-d tumor when given intravenously, was even less therapeutic against a 3-d tumor. Again, giving this same dose of rTNF intralesionally did not improve its therapeutic action against a 9-d or 3-d tumor. If anything, rTNF tended to be less therapeutic when given intralesionally.

TNF-induced Tumor Regression Requires that the Host be Immunocompetent. TNF is supposed to be the molecular mediator of tumor hemorrhagic necrosis and regression caused by parenteral injection of bacterial endotoxin. However, endotoxin-induced regression of the SA1 sarcoma is dependent on an underlying antitumor immune response (12–14). It was anticipated, therefore, that rTNF-induced and TNS-induced regression of the SA1 sarcoma also would require that the host be capable of generating an antitumor immune response. This was tested by determining whether rTNF or TNS could cause regression of the SA1 growing in TXB mice. The results in Figs. 4 and 5 show that, whereas a dose of 2.5×10^5 U of rTNF or 10^5 U of TNS caused either partial or complete

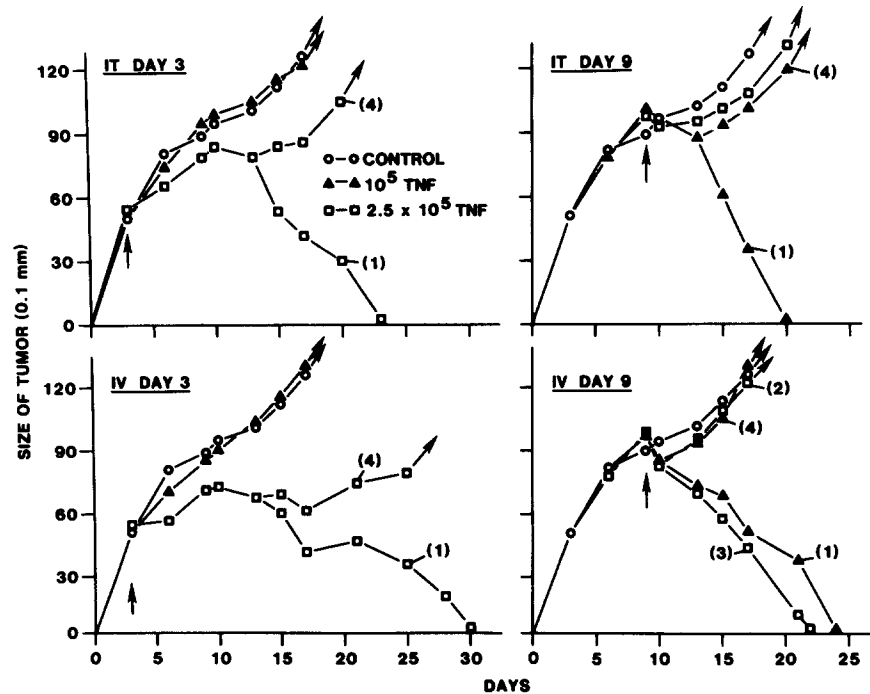


FIGURE 3. Evidence that 10^5 or 2.5×10^5 U of rTNF was not significantly more therapeutic when injected intratumorally than when injected intravenously. Nor was it more therapeutic against a 3-d than a 9-d tumor, as judged by complete tumor regression. In this experiment, higher doses of rTNF were lethal for all mice.

regression of a 9-d SA1 in immunocompetent mice, respectively, neither preparation caused regression of the tumor growing in TXB mice. It will be noted that although rTNF caused complete regression of the tumor in only two of five immunocompetent mice, it nevertheless caused appreciable regression of the tumor in the remaining three mice. The temporary brief cessation of growth of tumors in all TXB mice treated with TNS was the result of a central hemorrhagic reaction.

Quantitation of the rTNF-induced Hemorrhagic Reaction by Measuring Intratumor Extravasation of ^{51}Cr -labeled Red Cells. The therapeutic action of rTNF and TNS against established tumors in vivo is subjectively assessed for the most part in terms of the extensiveness of central hemorrhagic necrosis. On the other hand, complete regression of the tumor is not commonly measured because it rarely occurs. The same can be said for assessing the therapeutic action of endotoxin, which also commonly causes hemorrhagic necrosis, but rarely complete tumor regression. According to an elegant study performed by Algire et al. 40 years ago (16), tumor hemorrhagic necrosis results from the destruction of most of the tumor's vasculature, rather than from a direct toxic action of endotoxin on tumor cells.

A functional collapse of the tumor's vasculature is almost certainly the way that rTNF and TNS causes central hemorrhagic necrosis of the SA1 sarcoma. This is indicated by the appearance of a 9-d SA1 sarcoma undergoing a hemor-

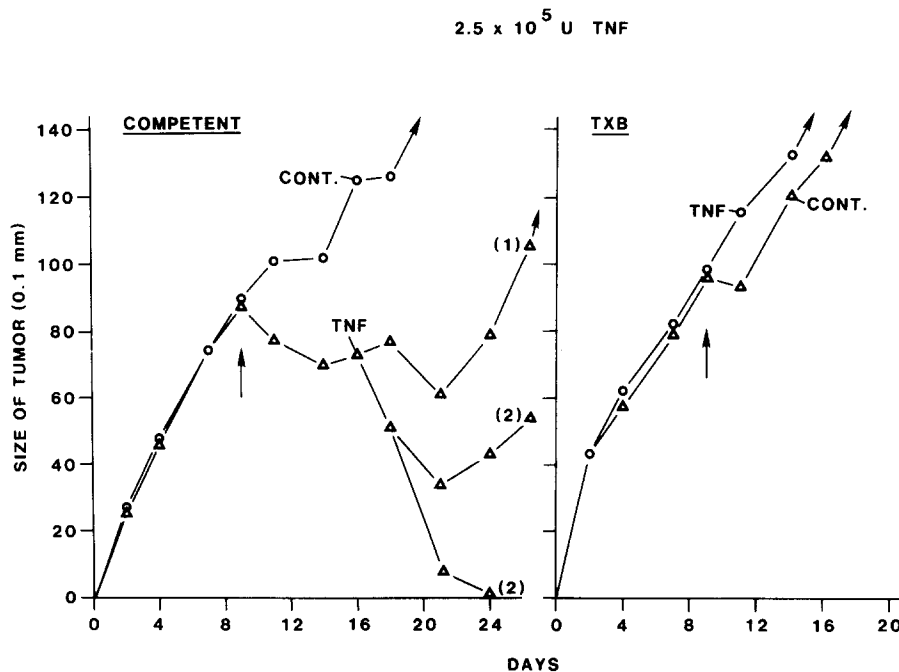


FIGURE 4. Evidence that the ability of rTNF to cause tumor regression depends on host immunocompetence. A dose of 2.5×10^5 U of rTNF caused either complete or partial regression of the SA1 growing in immunocompetent mice (left), but not if it was growing in T cell-deficient (TXB) mice (right).

rhagic reaction in response to injection of 2.5×10^5 U of rTNF, as shown in Fig. 6. It can be seen that the overall darkening of most of the tumor, as seen from the external side, was caused by multiple petechial hemorrhages in the tumor's vascular bed, as seen from the internal side. It is obvious that it is the tumor's vascular bed and tumor tissue internal to it that is consequently destroyed by the ischemia resulting from loss of blood supply.

It was found that the intratumor hemorrhagic reaction shown in Fig. 6 could be quantified by measuring the intratumor extravasation and accumulation of ^{51}Cr -labeled syngeneic red cells infused 30 min earlier, as described under Materials and Methods. The results of one of several experiments are shown in Fig. 7 where it can be seen that while tumors of control mice infused with ^{51}Cr -labeled red cells contained an approximately constant level of ^{51}Cr over the 6-h period of the assay, the tumors of mice given rTNF intravenously accumulated a large quantity of ^{51}Cr during the same period. It will be noted that extravasation of ^{51}Cr -labeled red cells did not begin until ~ 1 h after giving rTNF, and that by 6 h the tumors of rTNF-treated mice contained ~ 10 times more ^{51}Cr -labeled red cells than control tumors. Extravasation of red cells did not increase significantly after 6 h.

TNF-induced Intratumor Hemorrhaging Partly Depends on Host Immunocompetence. It can be seen in Fig. 8 that the ability of 2.5×10^5 U of rTNF to cause intratumor extravasation of ^{51}Cr -labeled syngeneic red cells was substantially

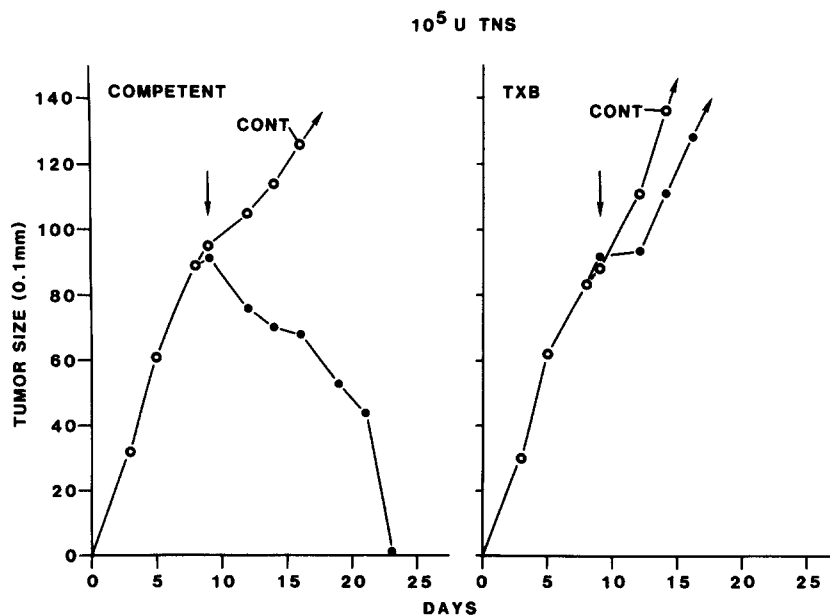


FIGURE 5. The same experiment as shown in Fig. 4, but performed with TNS instead of rTNF. Means of five mice per group.

reduced if the tumor was growing in TXB mice. Whereas ^{51}Cr accumulated progressively for 6 h in the tumors of rTNF-treated immunocompetent mice, it accumulated for only 2 h in the tumors of TXB mice. A similar result was obtained when TNS was used, except that in this case the biggest difference was seen 4 h after giving TNS. There can be no doubt, in view of these results, that host immunocompetence is needed for full development of the TNF-induced intratumor hemorrhagic reaction.

Neutralization of In Vitro Cytotoxicity and In Vivo Antitumor Function of rTNF and TNS by an Anti-rTNF Antibody. For future analysis of the in vitro and in vivo antitumor function of TNF, it was considered necessary to raise an anti-rTNF neutralizing antibody. A neutralizing antibody was also considered a necessary tool to determine whether endogenous TNF is the mediator molecule responsible for hemorrhagic necrosis and regression of established tumors caused by endotoxin and other agents, as is generally assumed (1, 9). Therefore, a monospecific, polyvalent anti-rTNF antiserum was raised in rabbits, according to the description given under Materials and Methods.

Table III shows that the antiserum was highly efficient at neutralizing the in vitro cytotoxicity of both rTNF and TNS for L929B cells. However, it was six times less efficient at neutralizing TNS than rTNF, and 100 times less efficient at neutralizing human rTNF. It was completely incapable, on the other hand, of neutralizing the cytotoxicity of recombinant human lymphotoxin.

A preparation of purified anti-rTNF IgG was also highly efficient at blocking the ability of rTNF and TNS to cause tumor hemorrhagic necrosis in vivo. It can be seen in Fig. 9 that giving anti-rTNF IgG intravenously to tumor-bearing mice 1 h before giving them 2.5×10^5 U of rTNF or 10^5 U of TNS completely

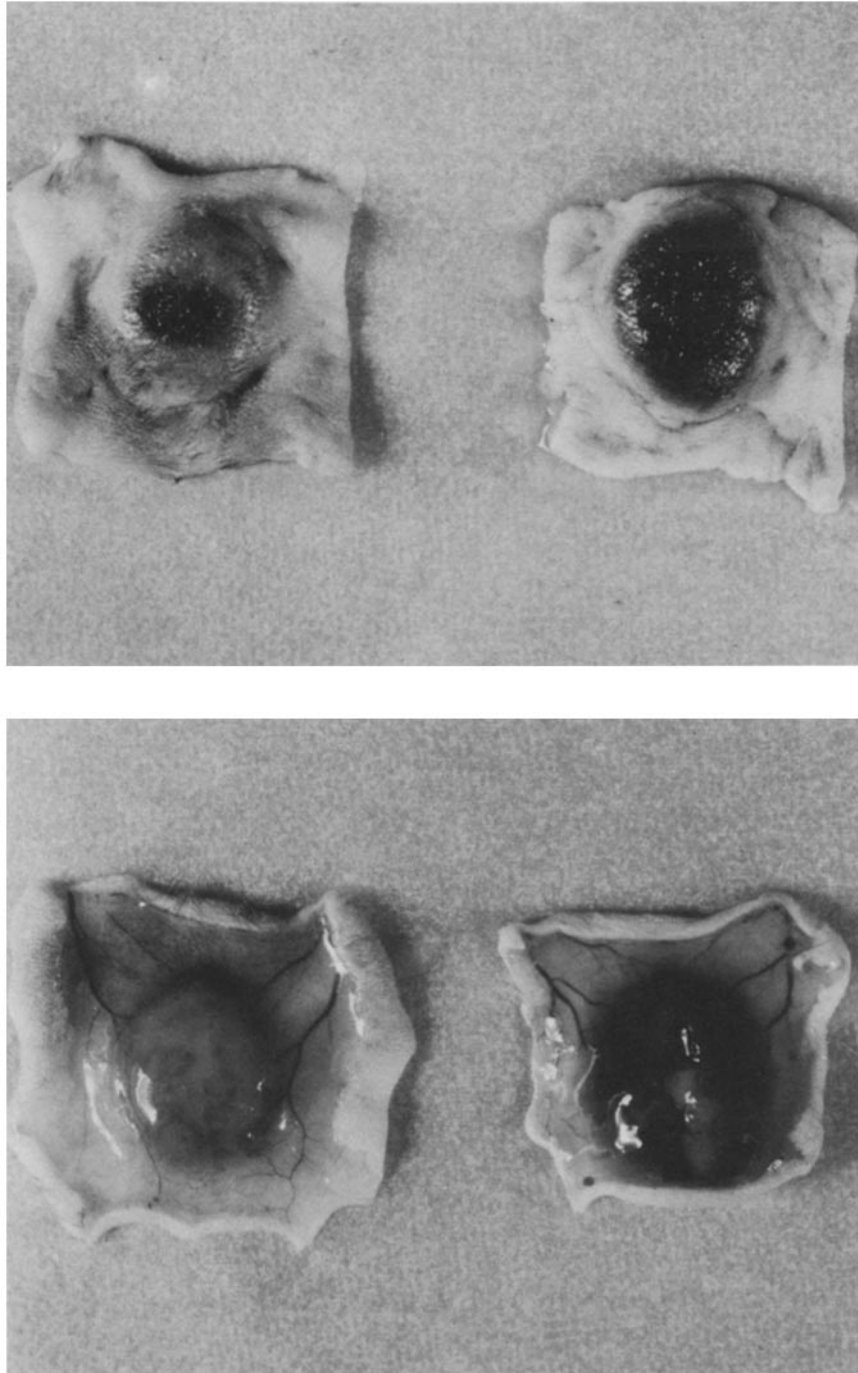


FIGURE 6. Tumor from a control mouse (*left*) and from a mouse treated 6 h earlier with 2.5×10^5 U of rTNF (*right*) photographed from external side (*top*) and internal side (*bottom*). Central darkening of the treated tumor seen from the external side was caused by numerous hemorrhages in the tumor's vascular bed. A small central necrotic core was already present at the time of treatment.

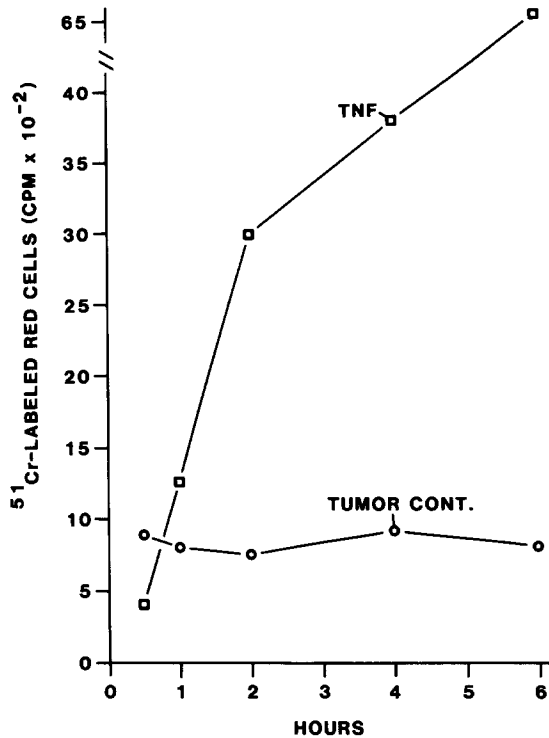


FIGURE 7. Quantitation of the rTNF-induced hemorrhagic reaction shown in Fig. 6, as determined by measuring intratumor extravasation of ^{51}Cr -labeled syngeneic red cells infused intravenously 30 min before giving 2.5×10^5 U of rTNF. Means of four tumors per group per time point.

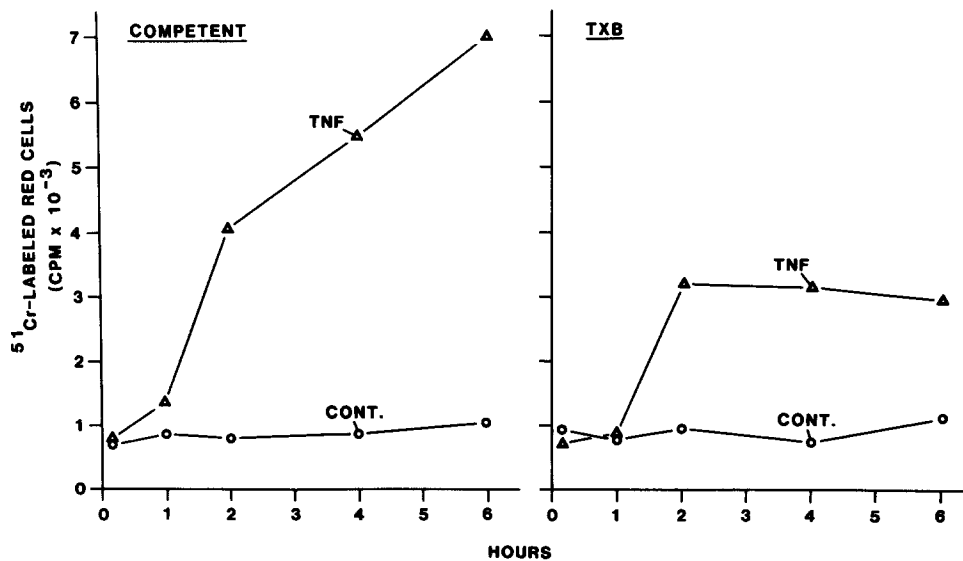


FIGURE 8. Evidence that the rTNF-induced intratumor hemorrhagic reaction, as measured by intratumor extravasation of ^{51}Cr -labeled red cells is partly dependent on host immunocompetence. Red cell extravasation continued for at least a 6-h period in rTNF-treated immunocompetent mice (*left*), but for only 2 h in TXB mice (*right*). Means of four mice per group per time point.

TABLE III
Neutralization of the Cytotoxic Activities of rTNF, TNS, and Other Cytotoxic Factors by Rabbit Anti-rMuTNF Serum

Cytotoxic factor	Source	Neutralizing titer*
		U/ml
rMuTNF	Biogent/Fiers [‡]	40,141
TNS	Trudeau/North [§]	6,554
rHuTNF	Cetus/Lin	410
Human lymphotoxin (natural)	RPMI/Sulkowski	<26
Human lymphotoxin (recombinant)	Genentech/Shepard	<26

* The rabbit anti-rMuTNF neutralizing titer is defined as the reciprocal of the highest dilution of the antiserum, which when reacted with an equal volume of test sample containing 20 cytotoxic units/ml, neutralizes 50% of the activity on actinomycin D-treated L929B murine fibroblasts.

[‡] This rMuTNF served as immunogen. Rabbit serum obtained before immunization possessed no anti-rMuTNF neutralizing activity (<16).

[§] Serum collected from *P. acnes*-primed mice 2 h after the intravenous infusion of 50 μ g of *S. enteritidis* endotoxin.

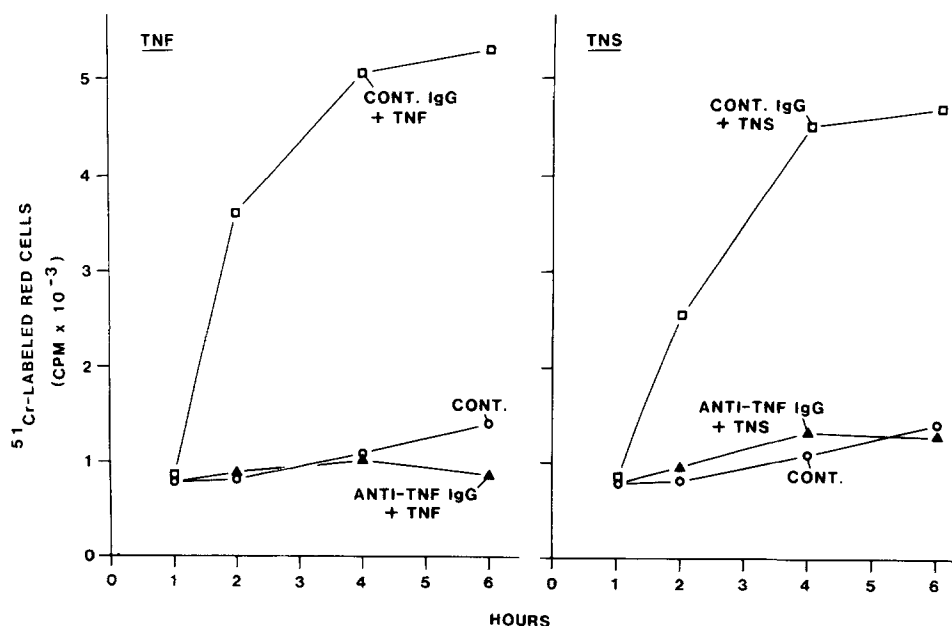


FIGURE 9. Evidence that anti-rTNF antibody given intravenously in a dose of 3.2×10^4 neutralizing units was capable of completely preventing the ability of 2.5×10^5 U of rTNF (left), or 10^5 U of TNS (right) to cause intratumor hemorrhaging, as measured by intratumor extravasation of ⁵¹Cr-labeled red cells. Mean counts per tumor of four tumors per group.

blocked the development of the tumor hemorrhagic reaction, as measured by intratumor extravasation and accumulation of ⁵¹Cr-labeled red cells. This result is shown visually in Fig. 10, where it can be seen that anti-rTNF IgG completely inhibited the ability of rTNF to cause hemorrhaging in the tumor's vascular bed.

Not surprisingly, therefore, the same 1-h pretreatment with anti-rTNF com-

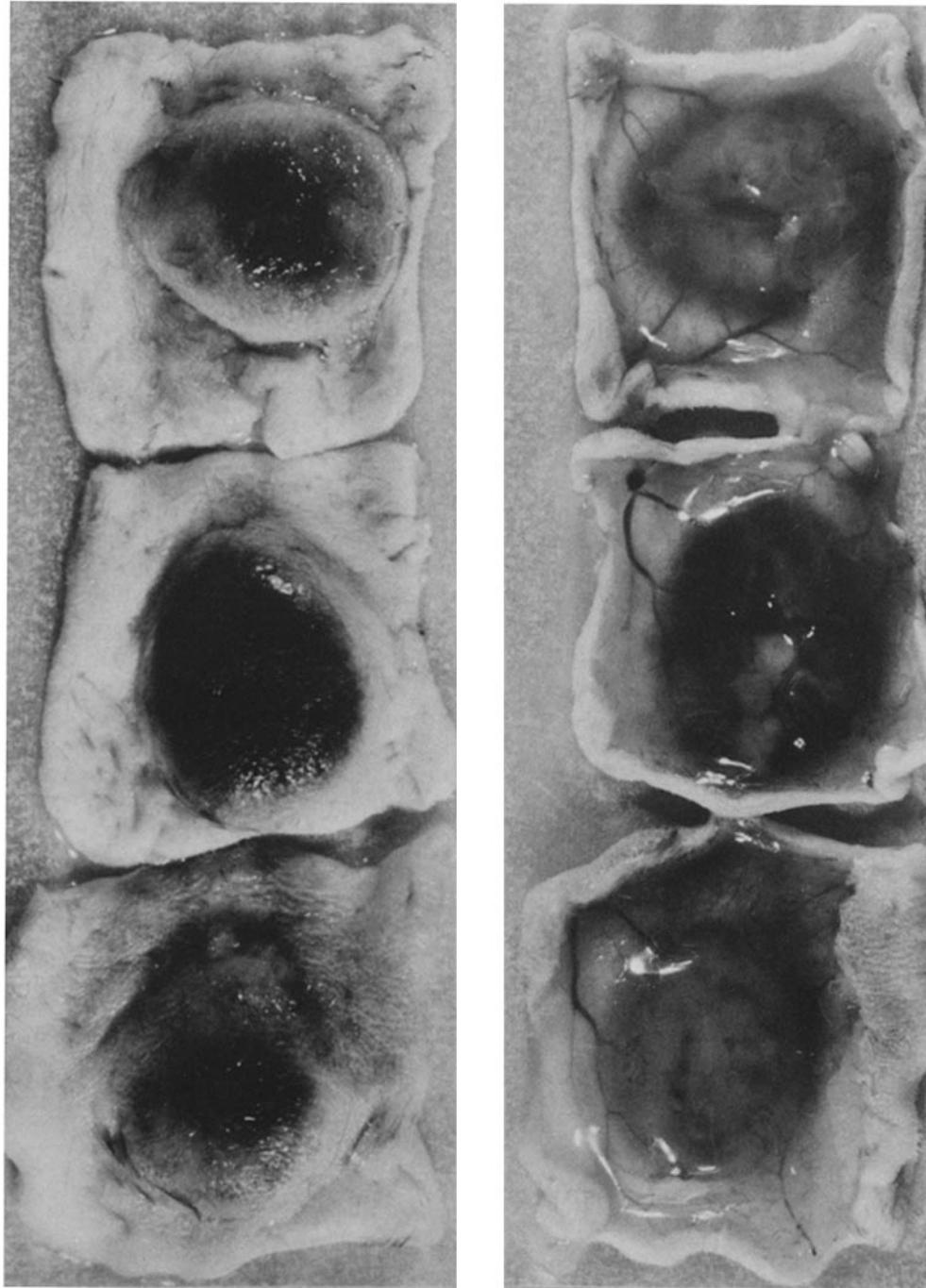


FIGURE 10. Appearance of a 9-d control tumor (*top*), a tumor undergoing a hemorrhagic reaction in response to rTNF given 6 h earlier (*middle*), and a tumor in which a rTNF-induced hemorrhagic reaction was blocked by treating the host with anti-rTNF antibody 30 min before giving rTNF (*bottom*). The left panel shows tumors photographed from the external side, and the right panel shows the same tumors photographed from the internal side.

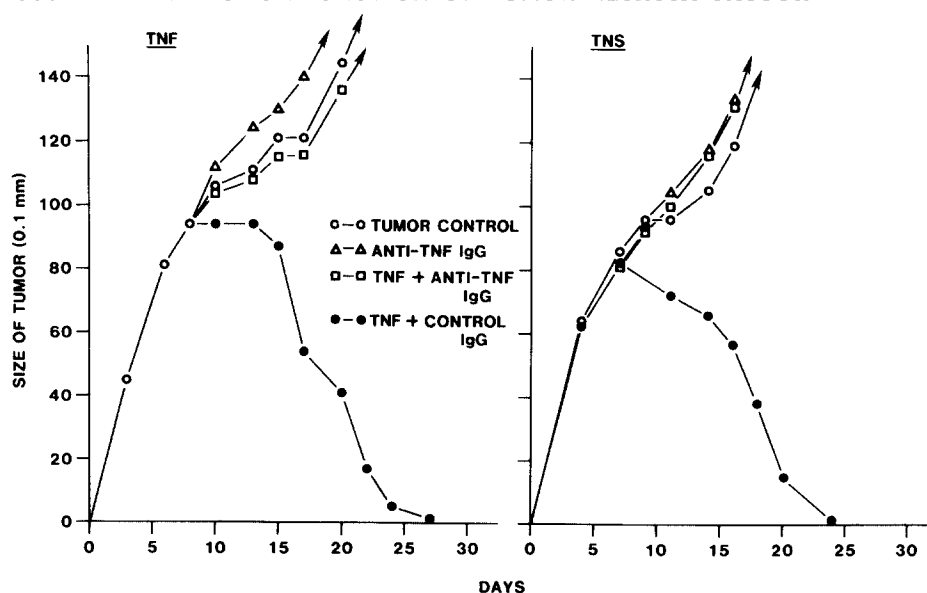


FIGURE 11. Evidence that pretreatment of mice with 3.2×10^4 neutralizing units of anti-rTNF antibody completely inhibited the ability of 2.5×10^5 U of rTNF (*left*), and 10^5 U of TNS (*right*) to cause regression of the SA1 sarcoma. The anti-rTNF was given 1 h before rTNF on day 9 of tumor growth. Infusion of the same quantity of control IgG was without effect. Means of five mice per group.

pletely inhibited the ability of therapeutic doses of rTNF and TNS to cause complete regression of a 9-d SA1 sarcoma (Fig. 11). It should be mentioned, moreover, that treatment with anti-rTNF IgG protected the tumor-bearing mice from the toxic effects of rTNF and TNS, in that antibody-treated mice showed no signs of lethargy, hypothermia, or diarrhea.

Discussion

The results of this study with an immunogenic tumor that is highly susceptible to endotoxin therapy (12, 13) show that murine rTNF is not an impressive antitumor agent when used as a single agent in mice, in that it cannot be relied on to cause complete regression of this tumor in all mice, even when given in highly toxic, near lethal doses. Natural TNF contained in TNS seems more therapeutic, but the doses needed to cause tumor regression are also extremely toxic. However, in the case of TNS, the participation of other monokines cannot be ruled out. Multiple subtoxic doses of rTNF or TNS were no more therapeutic than single toxic doses (North, R. J., and E. A. Havell, manuscript in preparation). In our experience, therefore, rTNF is less therapeutic against the SA1 sarcoma than endotoxin itself (14, 17), even though TNF is supposed to mediate the therapeutic effects of endotoxin (1, 9). It is also apparent (12–14, 17) that parenterally injected endotoxin routinely causes complete regression of the SA1 sarcoma with much less severe symptoms of toxicity than reported here for TNF.

The need for highly toxic doses of TNF to cause regression of an endotoxin-susceptible tumor is in agreement with the recent findings of Bloksma and

Hofhuis (18) and of Palladino et al. (19), who investigated the therapeutic efficacy of human rTNF against the Meth A fibrosarcoma. These authors were not impressed with the ability of rTNF to cause regression of this tumor, in that they found that the incidence of complete regressions in response to highly toxic doses of rTNF was quite low. Indeed, it is apparent that in spite of the current extensive interest in the cytotoxic function of rTNF, very few published descriptions exist of rTNF-induced regression of established tumors. Instead, the *in vivo* antitumor function of TNF is measured in terms of its ability to cause tumor hemorrhagic necrosis. In other words, as a single agent TNF would have to be considered to be of limited therapeutic value, if judged by its ability to cause complete tumor regression.

It was shown in the present study, moreover, that the therapeutic efficacy of murine rTNF could not be improved on by testing it against smaller tumors. On the contrary, a 3-d (4–5-mm diameter) SA1 sarcoma was less susceptible to a 2.5×10^5 U dose of rTNF than a 9-d (8–9-mm diameter) SA1, regardless of whether rTNF was injected intravenously or directly into the tumor. With regard to tumor size, it has been known for many years (10, 11) that parenterally injected endotoxin is therapeutic only against tumors above a certain critical size. This must surely be related to the reason why the Meth A fibrosarcoma invariably is allowed to grow for 6–7 d before attempts are made to cause it to undergo regression with TNS (1, 9) or rTNF (18, 19).

One possible reason why rTNF is active only against well-established tumors might be that a therapeutic effect requires that the host possess an underlying mechanism of antitumor immunity, the generation of which is not induced until the tumor grows large enough to supply an adequate quantity of antigen. Evidence that this is the case with endotoxin-induced tumor regression is supplied in publications from this laboratory (14, 17), which show that until the host has generated an adequate level of concomitant antitumor immunity, endotoxin is ineffective in causing regression of the SA1 sarcoma. In addition, these publications show that endotoxin fails to cause regression of the SA1 sarcoma growing in a TXB host, but does so if the host is supplied with L3T4⁺-sensitized T cells from a concomitantly immune donor. Because it was shown here that neither rTNF nor TNS is capable of causing regression of the SA1 sarcoma growing in TXB mice, it seems reasonable to conclude that regression of this tumor by these agents is also dependent on an adequate level of underlying antitumor immunity. This argues against the notion that TNF causes tumor regression by directly killing cells of the tumor. Other evidence against direct cytotoxic destruction of tumor cells by TNF *in vivo* is seen in the publications of others (18, 19) showing that tumor cells that are resistant to the cytotoxic action of TNF *in vitro* nevertheless form tumors that undergo hemorrhagic necrosis and occasional regression in response to TNF *in vivo*.

Again, it was established here that the ability of TNF to cause tumor hemorrhagic necrosis, as opposed to tumor regression, is also partly dependent on host immunocompetence. The results show that, whereas rTNF-induced intratumor extravasation of ⁵¹Cr-labeled red cells progressed for 6 h in tumors growing in immunocompetent mice, it progressed for only 2 h in tumors growing in TXB mice. This indicates that the TNF-induced tumor hemorrhagic reaction consists

of an initial immunologically independent phase, and a subsequent immunologically dependent phase. The meaning of this is unknown.

However, the therapeutic meaning of TNF-induced hemorrhagic necrosis as a whole seems obvious. It almost certainly is responsible for rapid destruction of most of the center of the tumor by ischemia, thereby leaving a greatly reduced tumor burden for the host's immunologic defenses to cope with. Examination of the SA1 sarcoma undergoing a TNF-induced hemorrhagic reaction revealed that the rapid progressive darkening of most of the tumor as seen from its external side several hours after giving rTNF intravenously is caused by numerous petechial hemorrhages in the tumor's vascular bed. This intratumor hemorrhaging signifies irreversible damage to the tumor's vasculature and results over the next 24 h in death and necrosis of most of the tumor's center. These observations agree, therefore, with descriptions of tumor hemorrhagic necrosis caused by endotoxin, as published by Algire et al. (16) more than 40 years ago. These authors concluded that tumor necrosis is caused by a loss of blood supply to the tumor resulting from a functional collapse of most of the tumor's vasculature. However, they observed, in addition, that in almost all cases the outer rim of the tumor and the blood vessels therein survive the hemorrhagic reaction, and are capable of rapid regrowth. Consequently, the tumor rapidly reestablishes itself unless other mechanisms are brought into play to destroy tumor tissue surviving the hemorrhagic reaction. According to results obtained with rTNF and TNS presented here, and the results obtained with endotoxin published elsewhere (14, 17), complete regression of the outer rim of tumor tissue that survives the initial hemorrhagic reaction requires that the host be capable of generating and expressing an adequate level of concomitant antitumor immunity. Presumably, the greater the extent of hemorrhagic necrosis, the smaller the amount of surviving living tumor tissue that needs to be destroyed by host immunity, and the more likely it is that complete tumor regression will ensue.

This scheme of TNF-induced tumor regression implies that for TNF to initiate a therapeutic effect, the tumor needs to be adequately vascularized. It also implies that tumor blood vessels are more susceptible to TNF than other blood vessels. It has been demonstrated in this connection, that TNF can directly affect the physiology of vascular endothelial cells *in vitro* (20–22) and is cytotoxic for these cells under certain conditions if present in adequate concentration (21). It seems obvious that the vasculature of the tumor is more susceptible to a sublethal dose of TNF than the vasculature of normal tissues, because it is only in the tumor that hemorrhaging is noticeable. We failed to find any macroscopic evidence of hemorrhaging in the viscera of tumor-bearing mice treated with a therapeutic dose of rTNF or TNS. It is known, in keeping with this idea, that the vasculature of tumors is structurally abnormal (reviewed in reference 23), and this implies underlying physiological defects. Presumably, however, the extreme outer region of a tumor's vasculature is less abnormal, because this escapes destruction by the TNF-induced hemorrhagic reaction. It is even possible that differences among tumors in their response to TNF therapy might be a reflection of differences in the degree of abnormality of the endothelial cells of their blood vessels.

As shown by Tracy et al. (24), however, very high, uniformly lethal doses of

TNF (also called cachectin) cause localized hemorrhaging and ischemic destruction in the tissues at large, including those of the lungs and intestines. Additional symptoms of TNF/cachectin-induced physiological decompensation include hypotension, hemoconcentration, intravascular coagulation, and metabolic acidosis. Unfortunately, to cause extensive hemorrhagic necrosis and/or regression of the SA1 sarcoma, it was necessary to give the host doses of rTNF or TNS capable of causing these same symptoms to some degree. Others have experienced the same problem in attempting to cause regression of the Meth A fibrosarcoma with rTNF (18, 19).

On the other hand, and in keeping with the findings of Buetler et al. (25), these toxic symptoms failed to manifest themselves in SA1 sarcoma-bearing mice that were given anti-rTNF antibody intravenously before being given TNF. However, neutralization of toxicity went hand in hand with neutralization of the ability of TNF to cause tumor hemorrhagic necrosis and regression. The ability of this same antibody to inhibit endotoxin-induced hemorrhagic necrosis and regression of the SA1 sarcoma will be dealt with in the paper that follows (26).

Lastly, the results presented in Table III show that more (six times) anti-rTNF antibody was required to neutralize the cytotoxic activity of natural TNF (TNS) than was required to neutralize the immunogen (rTNF). Obviously, a number of possibilities could be offered to explain this. It is possible, for example, that the antibody has more affinity for rTNF than for natural TNF. Alternatively, natural TNF may be more therapeutically active than rTNF. With regard to the later possibility, studies are underway to establish the amount of natural TNF protein present in TNS, so that activities of the pure rTNF and natural TNF can be compared on a molar basis.

Summary

The ability of murine recombinant tumor necrosis factor (rTNF) and natural TNF in tumor-necrotizing serum (TNS) to cause regression of the SA1 sarcoma was investigated. We found that to cause regression of a 9-d SA1 sarcoma, near lethal quantities of rTNF and TNS had to be given to the host. However, even at these highly toxic doses, rTNF was not reliable at causing complete tumor regression. On the other hand, both types of TNF were reliable at causing a tumor hemorrhagic reaction that resulted in the destruction of >75% of the tumor's center in 24 h. The TNF-induced hemorrhagic reaction involved the development of numerous petechial hemorrhages in the tumor's vascular bed, which apparently resulted from destruction of the tumor's blood vessels. It was possible to follow the development of the hemorrhagic reaction against time after giving rTNF or TNS by measuring the intratumor extravasation of ⁵¹Cr-labeled syngeneic red cells. According to this method, TNF-induced intratumor hemorrhaging was in progress within 1 h of giving TNF and continued for about a 6-h period. However, the hemorrhagic reaction was greatly reduced and complete regression of the rim of the living tumor tissue that survived hemorrhagic necrosis failed to occur, if SA1 sarcoma was growing in T cell-deficient (TXB) mice. This indicates that the TNF-induced hemorrhagic reaction is partly dependent, and the tumor regression that follows is completely dependent on host immunocompetence. This suggests in turn, that rTNF does not directly

destroy SA1 tumor cells in vivo, even though it was shown that it can destroy SA1 tumor cells in vitro. This interpretation is supported by the additional findings that rTNF was no more therapeutic against a 3-d (3-mm) SA1 than against a 9-d (8-mm) SA1, and was no more therapeutic when injected directly into the tumor than when injected intravenously. Lastly it was possible to completely inhibit the ability of rTNF and TNS to cause tumor hemorrhagic necrosis and regression by infusing the host with a monospecific, polyvalent anti-rTNF antibody that neutralized the cytotoxic action of rTNF in vitro.

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