

THE ANTITUMOR FUNCTION OF TUMOR NECROSIS FACTOR (TNF)

II. Analysis of the Role of Endogenous TNF in Endotoxin-induced Hemorrhagic Necrosis and Regression of an Established Sarcoma

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Although there is no doubt that tumor necrosis factor (TNF) can cause extensive hemorrhagic necrosis of the centers of established murine tumors, convincing evidence that TNF can cause these same tumors to routinely undergo complete regression is lacking. The results presented in the preceding companion paper (1) support the contention of others (2, 3) that the therapeutic efficacy of parenterally injected rTNF, as judged by its ability to cause tumor regression, is unimpressive. It was shown with the SA1 sarcoma that, in spite of the fact that this tumor is highly sensitive to endotoxin-induced complete regression (4, 5), it does not always undergo regression in response to treatment with TNF, even when near lethal doses are given.

The companion paper presented results (1) in keeping with the view that TNF-induced tumor hemorrhagic necrosis is responsible for the rapid destruction of most of the center of the tumor, but that the subsequent regression of the surviving rim of living tumor tissue is dependent on the host being able to generate an underlying antitumor immune response. The results also suggest that TNF-induced hemorrhagic necrosis results from the destruction of most of the tumor's vasculature. The gross pattern of the TNF-induced hemorrhagic reaction within the SA-1 sarcoma is in keeping with descriptions of endotoxin-induced tumor hemorrhagic reactions published by Algire et al. over 40 years ago (6).

This was not unexpected, given the general assumption that TNF is the host molecule that mediates endotoxin-induced tumor hemorrhagic necrosis. However, this assumption is not well founded, because it is not based on the demonstration that TNF activity resides in the serum of tumor-bearing mice responding to endotoxin. Instead, it is based on the demonstration (7, 8) that TNF activity is present in tumor-necrotizing serum obtained from *Propionibacterium acnes*-treated, or BCG-infected mice responding to endotoxin. In other words, it is apparent that there is no formal evidence that TNF is involved in endotoxin-induced necrosis and regression of established murine tumors.

The purpose of this paper is to supply such evidence. It will show, on the one

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hand, that the endotoxin-induced hemorrhagic reaction that leads to ischemia and rapid destruction of most of the center of the SA1 sarcoma is associated with the intratumor production of TNF. It will show, on the other hand, that the endotoxin-induced hemorrhagic reaction and subsequent complete tumor regression can be inhibited by infusing the host with an adequate quantity of an antibody capable of neutralizing the antitumor activity of TNF. It will also be shown that, whereas endotoxin-induced regression of the ring of tumor tissue surviving the endotoxin-induced hemorrhagic reaction depends on the expression of an adequate level of antitumor immunity, the hemorrhagic reaction itself is not significantly dependent on host immunocompetence.

Materials and Methods

The procedures used in this study were the same as those used in the preceding paper (1), except for the procedures detailed below.

Adoptive Immunization. To prepare donor spleen cells, AB6F₁ donor mice bearing a 9-d SA1 sarcoma were killed by cervical dislocation, their spleens were removed, diced into pieces, and pushed through a 60-mesh stainless screen into PBS containing 1% FCS. The suspension was triturated to break up clumps, passed through surgical gauze to remove debris, washed in PBS, and resuspended in PBS for intravenous infusion. The recipients were TXB mice (1) bearing a 7-d SA1 sarcoma. They each received one spleen equivalent (1.5×10^8) of donor spleen cells. Donor mice bearing a 9-d SA1 sarcoma are known to be generating peak levels of concomitant antitumor immunity, whereas TXB recipient mice are not capable of generating a significant antitumor immune response (9).

Antibody Treatment. To determine whether donor spleen cells that transferred immunity were L3T4⁺ T cells, the spleen cells were treated at 2×10^7 cells/ml for 30 min at 10°C with a 1:5 dilution of anti-L3T4 mAb. The antibody was generated in vitro by the GK-1.5 hybridoma (American Type Culture Collection, Rockville, MD), as described elsewhere (10). The cells were then washed and incubated at 37°C for 1 h in the same volume of a 1:10 dilution of rabbit serum as a source of complement. Finally, they were washed and resuspended in PBS for intravenous infusion.

Anti-rTNF IgG. The polyvalent, monospecific, rabbit anti-rTNF IgG used in these studies was generated and purified from serum as described in the preceding companion paper (1). The neutralizing titer (U/ml) is defined as the reciprocal of the highest dilution of anti-rTNF IgG that when mixed with an equal volume of a preparation of TNF having 20 U of cytotoxic activity/ml, neutralizes 50% of the cytotoxic activity as determined in vitro on actinomycin D-treated L929B cells.

Results

Endotoxin-induced Tumor Regression Is Dependent on Antitumor Immunity. Because it was shown in the preceding companion paper (1) that the host needs to be immunocompetent for exogenous TNF to cause regression of its tumor, it was considered necessary to confirm first the results of earlier studies (9, 10) showing that endotoxin-induced regression of the SA1 sarcoma also depends on an antitumor immune response. This involved showing that endotoxin fails to cause regression of the SA1 sarcoma growing in immunoincompetent mice unless they were first infused with T cells from tumor-bearing immunocompetent donors.

Fig. 1 shows that, whereas intravenous injection of 25 µg of endotoxin caused complete regression of the SA1 sarcoma growing in immunocompetent mice, it failed to cause regression of the tumor growing in TXB mice. It can be seen in

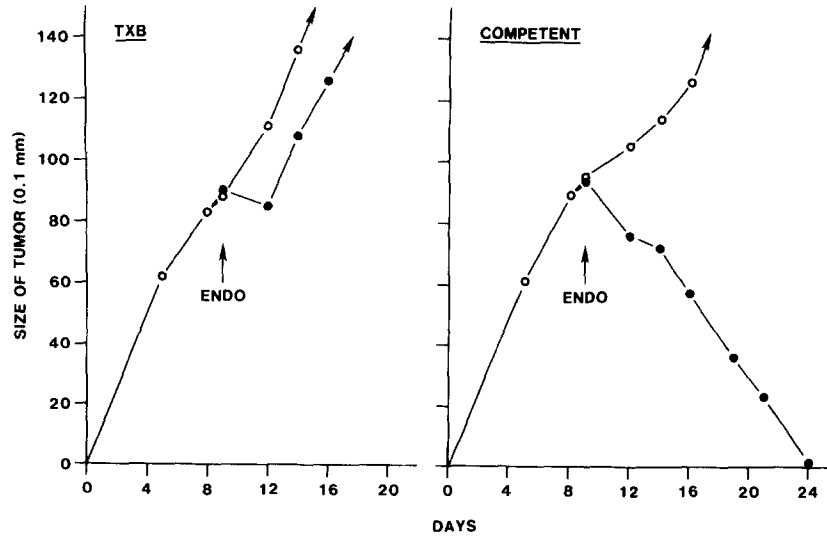


FIGURE 1. Evidence that endotoxin-induced regression of the SA1 sarcoma requires that the tumor be growing in an immunocompetent host. Intravenous injection of 25 μ g of endotoxin on day 9 of tumor growth caused complete regression of the tumor in immunocompetent (*right*), but not in TXB mice (*left*). Means of five mice per group.

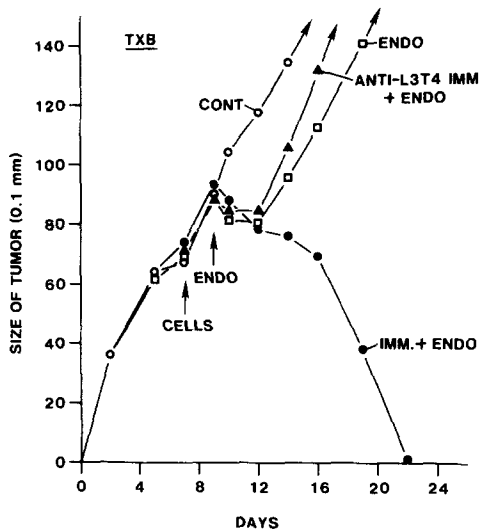


FIGURE 2. Evidence that the SA1 sarcoma in TXB mice can be primed to undergo regression in response to endotoxin therapy by infusing the TXB mice with splenic T cells from immunocompetent donor mice responding to a 9-d tumor (IMM + ENDO). The spleen cells (1.5×10^6) were infused on day 7 of tumor growth, and endotoxin was injected on day 9. The donor spleen cells that primed the recipient tumor for regression were functionally eliminated by treatment with anti-L3T4 antibody and complement (anti-L3T4 IMM + ENDO). Means of five mice per group.

Fig. 2, however, that it was possible to restore the capacity of TXB mice to cause complete regression of the SA-1 sarcoma in response to endotoxin treatment, provided the mice were infused 48 h earlier with spleen cells from immunocompetent donor mice responding to a 9-d tumor. Fig. 2 also shows that the donor cells that primed the recipient tumor for endotoxin-induced regression were L3T4⁺ T cells, as evidenced by their functional elimination by treatment with anti-L3T4 antibody and complement. Previous publications showed (9, 10) that

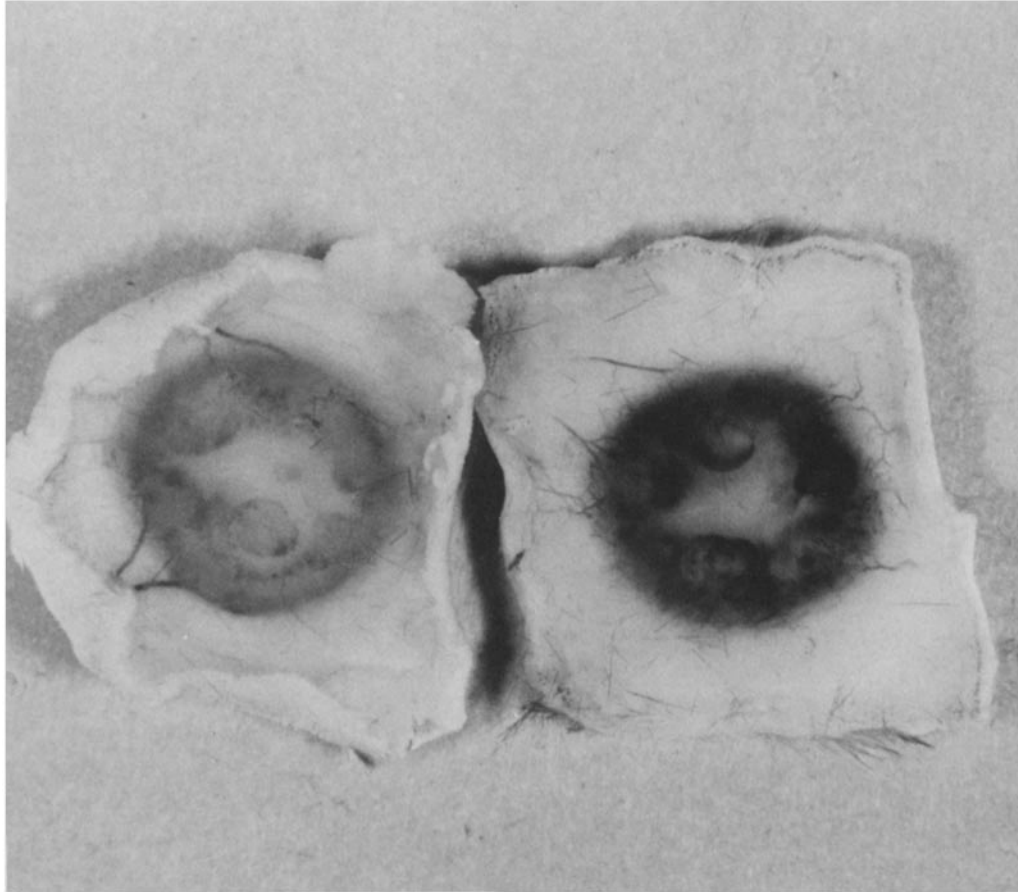


FIGURE 3. Excised tumor from a control host (*left*) and from a host that was given endotoxin 4 h earlier (*right*) photographed from the internal side while under water. The multiple hemorrhages in the vascular bed of the treated tumor are obvious.

these T cells are not susceptible to treatment with anti-Ly-2 antibody and complement, and that they are specific for the SA1 sarcoma.

Endotoxin-induced Hemorrhagic Necrosis, as Opposed to Regression, Is Less Dependent on Antitumor Immunity. It is apparent that endotoxin-induced complete destruction of the SA1 sarcoma is achieved by two separate mechanisms: (*a*) an initial hemorrhagic reaction that causes rapid necrosis of most of the center of the tumor within 24 h, and (*b*) a subsequent T cell-mediated immunological mechanism that destroys the ring of surviving tumor tissue during the next week or so. Fig. 3 shows the appearance of a 9-d SA1 sarcoma undergoing a hemorrhagic reaction in response to endotoxin given intravenously 4 h earlier. It is obvious from the photographs that endotoxin caused extensive hemorrhaging in the tumor's vascular bed during the 4-h period.

As was the case with TNF (1), it was possible to follow the rate and extent of intratumor hemorrhaging by measuring the intratumor extravasation and accumulation of ^{51}Cr -labeled syngeneic red cells. Fig. 4 shows the extent of the

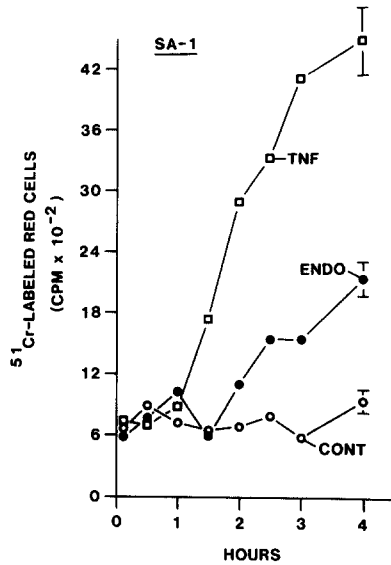


FIGURE 4. Endotoxin-induced intratumor hemorrhaging shown in Fig. 3, as measured by intratumor extravasation of ^{51}Cr -labeled syngeneic red cells that were infused intravenously 1 h before giving endotoxin. The hemorrhagic reaction did not commence until 90 min after giving endotoxin, and continued for the 4-h duration of the experiment. The hemorrhagic reaction caused by a therapeutic dose of endotoxin was much less intense than that caused by a therapeutic dose (2.5×10^5 U) of rTNF. Means counts per tumor of four tumors per time point \pm SD.

intratumor hemorrhagic reaction caused by the standard dose ($25 \mu\text{g}$) of endotoxin that is used to cause complete regression of the tumor in all mice. The hemorrhagic reaction caused by the most therapeutic dose of rTNF (2.5×10^5 U) is included for comparison. It can be seen that endotoxin caused progressive intratumor extravasation of ^{51}Cr -labeled red cells after a delay of ~ 1 h. However, extravasation of red cells seemed to start earlier, and to progress at a faster rate and to a much greater extent in 4 h in the tumors of mice given rTNF, even though, as shown in the preceding paper, (1) this dose of rTNF was less therapeutic than endotoxin in terms of its ability to cause tumor regression. Red cell extravasation did not continue to a significant extent beyond 4 h in this experiment.

With regard to the necessity of host immunocompetence for endotoxin to cause hemorrhagic necrosis of the SA1 sarcoma, Fig. 5 shows that intratumor extravasation of ^{51}Cr -labeled red cells was not substantially less if the tumor was growing in TXB mice. In this experiment it was not until the hemorrhagic reaction had proceeded for ~ 4 h that a lower level of red cell extravasation was seen. It needs to be stressed, however, that in another experiment this difference after 4 h was not observed. This is in keeping with the observation that there was no discernible visible difference between the extent of endotoxin-induced hemorrhagic necrosis in a tumor growing in normal mice and tumor growing in TXB mice. The observation that endotoxin-induced tumor hemorrhagic necrosis is not greatly decreased in immunodepressed mice is in keeping with visual observations made by others some years ago (4, 5, 11).

Endotoxin-induced Tumor Hemorrhagic Reaction Is Associated with T Cell-independent Production of Intratumor TNF. If TNF is responsible for mediating the antitumor effects of endotoxin, then TNF should be detected in the tumor after injection of a therapeutic dose of endotoxin. Table I shows that this was the case with endotoxin-treated mice bearing a 9-d SA1 sarcoma. It can be seen that 2 h

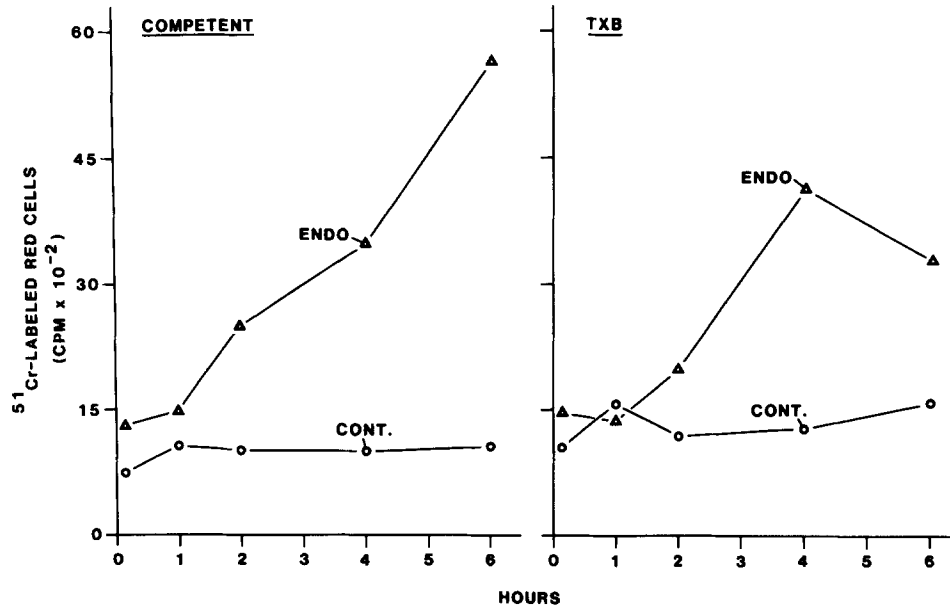


FIGURE 5. Endotoxin-induced intratumor hemorrhagic reaction, as measured by extravasation of ⁵¹Cr-labeled red cells, was not greatly reduced if the tumor was growing in TXB mice. The only difference between tumors growing in TXB mice (*right*) and immunocompetent mice (*left*) was that red cell extravasation did not progress beyond 4 h in the former. This difference was not always seen. Means counts per tumor of four tumors per time point.

after giving endotoxin intravenously, TNF-like activity, as measured by cytotoxicity against L929B cells *in vitro*, could be extracted from the tumor. This was the case, moreover, regardless of whether the tumor was growing in immunocompetent or TXB mice. It can also be seen in Table I that appreciable and similar levels of cytotoxic activity were present in the blood of tumor-bearing, as well as non-tumor-bearing, endotoxin-treated mice.

Evidence that the cytotoxic activity present in tumor extracts and in serum of endotoxin-treated mice was TNF is presented in Table II, where it can be seen that cytotoxic activity in all preparations was neutralized by anti-rTNF antiserum. Moreover, the neutralizing titers of the anti-rTNF serum for the cytotoxic activities of tumor extracts and serum were similar to those obtained for the cytotoxicity of natural TNF present in TNS. However, as also shown in Table II and in the preceding paper (1), the anti-rTNF antiserum was capable of neutralizing rTNF at much higher dilution.

The time course of appearance of TNF in the tumor, spleen, and serum in response to endotoxin is shown in Table III. It can be seen that TNF was present in all three compartments 1 h after giving endotoxin, and that it decreased progressively in quantity thereafter. However, it disappeared more slowly from the tumor, in that it was still present in measurable quantity in this, but not the other compartments, at 6 h.

The possibility that the TNF measurements presented above are not accurate representations of the amounts present in the host at the time of sacrifice, because of degradation and inactivation of the TNF during homogenization and

TABLE I
Endotoxin-induced TNF Levels in Sera and Tumors of Normal and TXB Tumor-bearing Mice

Host	Tumor	Preparation	Total TNF units*
Normal	None	Serum	<16
TXB	None	Serum	<16
Normal	None	Serum, 2 h after i.v. endotoxin	512
TXB	None	Serum, 2 h after i.v. endotoxin	1,024
Normal	9-d SA-1	Serum	<16
TXB	9-d SA-1	Serum	<16
Normal	9-d SA-1	Serum, 2 h after i.v. endotoxin	512
TXB	9-d SA-1	Serum, 2 h after i.v. endotoxin	768
Normal	9-d SA-1	Tumor homogenate [‡]	BDL [§]
TXB	9-d SA-1	Tumor homogenate [‡]	BDL [§]
Normal	9-d SA-1	Tumor homogenate, 2 h after i.v. endotoxin [‡]	384
TXB	9-d SA-1	Tumor homogenate, 2 h after i.v. endotoxin [‡]	256

* Total serum TNF assumes 1 ml serum/mouse. Total TNF/tumor = (homogenate vol × TNF units/ml)/number of tumors.

[‡] Tumor homogenates (10% wt/vol) were prepared by placing five tumors in PBS (pH 7.4) containing 10% (vol/vol) FCS. Tumor suspensions were homogenized on ice with a motorized blender. After homogenization, tissue debris was pelleted by centrifugation, and supernatants were assayed for TNF activity.

[§] TNF activity below detectable levels (BDL) at a 1/16 dilution of supernatant from a 10% wt/vol homogenate.

TABLE II
Identification of Endotoxin-induced Cytotoxic Activity as TNF

Host	Cytotoxic preparation	Anti-rMuTNF serum neutralizing titer*
—	rMuTNF	U/ml
Normal + <i>P. acnes</i> [‡]	Serum (TNS), 2 h after i.v. endotoxin	40,141
Normal	Serum, 2 h after i.v. endotoxin	6,554
Normal + SA-1	Serum, 2 h after i.v. endotoxin	6,144
TXB + SA-1	Serum, 2 h after i.v. endotoxin	6,144
Normal + SA-1	Tumor homogenate, 2 h after i.v. endotoxin	6,144
TXB + SA-1	Tumor homogenate, 2 h after i.v. endotoxin	8,192
TXB + SA-1	Tumor homogenate, 2 h after i.v. endotoxin	4,915

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

[‡] ICR mice treated intraperitoneally 14 d earlier with formalin-killed *P. acnes* is the standard way of priming mice for the production of endotoxin-induced serum-containing natural TNF (TNS).

extraction, is highly unlikely, in view of the results presented in Table IV. These results show that if known quantities of either rTNF or natural TNF were added to the homogenizing tubes just before homogenization, there was no loss of

TABLE III
TNF Levels in Tumor, Spleen, and Blood of Mice at Progressive Times After Intravenous Injection of Endotoxin

Time after endotoxin	Total activity in:		
	Tumor	Spleen	Serum
<i>h</i>			
0	BDL*	BDL*	<16
1	928	1,011	2,048
2	934	597	256
3	448	218	<16
6	229	BDL*	<16

* TNF activity below detectable levels (BDL) at a 1/16 dilution of supernatant from a 10% wt/vol homogenate.

TABLE IV
Stability of TNF during Physical Disruption and Processing of Splens and Tumors

Preparation (10% wt/vol)	Added TNF before disruption (U/ml)	TNF activity at times after disruption*		
		5 min	1 h	24 h
		<i>U/ml</i>		
Spleen	rMuTNF (2,888)	2,042	2,048	1,024
Spleen	TNS (1,625)	1,024	512	512
9-d SA-1	rMuTNF (1,733)	2,048	2,048	1,024
9-d SA-1	TNS (928)	512	1,024	512

* The preparations were maintained below 5°C during homogenization, centrifugation, and storage.

cytotoxic activity during homogenization and subsequent storage at 5°C for 24h.

Effect of Anti-rTNF Antibody on Endotoxin-induced Hemorrhagic Necrosis and Regression. The foregoing results are in keeping with the hypothesis that TNF is the host molecule responsible for mediating endotoxin-induced hemorrhagic necrosis and regression of the SA1 sarcoma, in that they show that TNF is present in the tumor during the endotoxin-induced hemorrhagic reaction. However, this evidence is correlative rather than causal. Convincing causal evidence would consist of showing that endotoxin-induced hemorrhagic necrosis and/or regression of the SA1 sarcoma can be inhibited by infusing the host with an antibody with a capacity to neutralize the in vitro and in vivo antitumor action of TNF. It was shown in the preceding companion paper (1) that the anti-rTNF antibody available in this laboratory is not only capable of completely neutralizing the in vitro cytotoxic activity of exogenous rTNF and natural TNF, but is also capable of inhibiting the ability of these TNF preparations to cause hemorrhagic necrosis and regression of the SA1 sarcoma in vivo.

However, in spite of its ability to neutralize the in vivo antitumor activity of exogenous TNF, Fig. 6 shows that this antibody was much less efficient at inhibiting the hemorrhagic reaction caused by endotoxin. It can be seen that

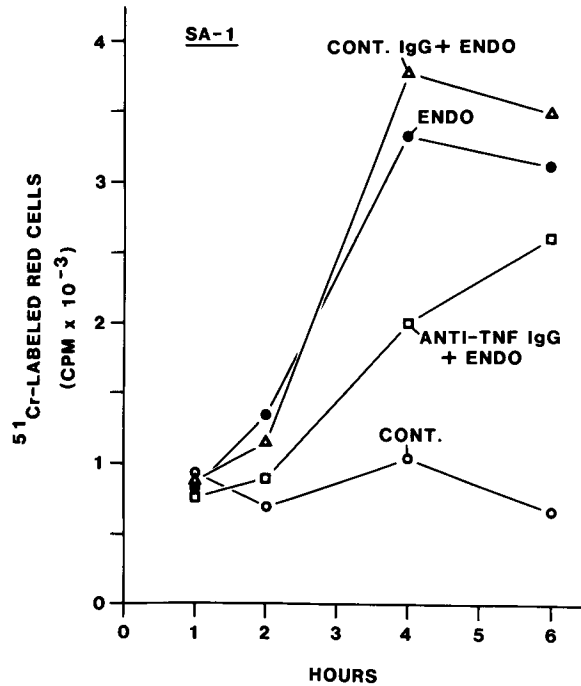


FIGURE 6. Partial inhibition of endotoxin-induced hemorrhagic reaction by intravenous injection 2 h earlier of 1.6×10^5 neutralizing units of anti-rTNF antibody. The tumor hemorrhagic reaction was induced on day 9 of tumor growth and measured in terms of the intratumor extravasation of ⁵¹Cr-labeled syngeneic red cells. Antibody treatment reduced the rate of intratumor hemorrhaging by ~50%. Mean counts per tumor with four tumors per group.

TABLE V

Anti-rMuTNF IgG and TNF Levels in Tumors, Spleens, and Blood of 9-d SA-1 Tumor-bearing Mice After Intravenous Infusion of Endotoxin and Anti-rMuTNF Neutralizing IgG

Time after infusion (0 h) of endotoxin and anti-rMuTNF IgG*	Recovered anti-rMuTNF IgG (total neutralizing units)			Recovered TNF units		
	Tumor	Spleen	Serum	Tumor	Spleen	Serum
5 min	533	403	52,000	—	—	<16
1 h	262	383	26,000	BDL	BDL	<16
2 h	471	389	26,000	BDL	BDL	<16
4 h	369	214	26,000	BDL	BDL	<16
6 h	911	437	13,000	BDL	BDL	<16
24 h	779	221	13,000	BDL	BDL	<16

* Intravenous injection of 34,000 neutralizing units of anti-rMuTNF IgG and 25 μg of endotoxin.

intravenous infusion of 1.6×10^5 neutralizing units of the antibody given 2 h before 25 μg of endotoxin could not completely inhibit intratumor hemorrhaging, as measured by intratumor extravasation of ⁵¹Cr-labeled red cells. Instead, it reduced the endotoxin-induced hemorrhagic reaction by <50%, even though the antibody must have been present in considerable excess. Table V shows that over a period of 24 h after infusing anti-rTNF antibody there was no TNF activity detectable in extracts of tumor and spleen, or in the serum of endotoxin-treated mice. On the contrary, it was possible to extract from the tumor more than enough free antibody to neutralize the amount of endogenous TNF known

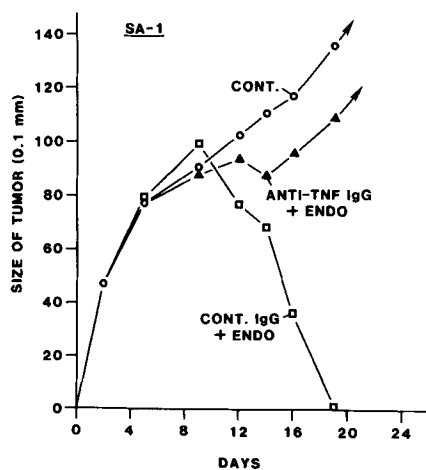


FIGURE 7. A dose of 1.6×10^5 neutralizing units of anti-rTNF antibody given intravenously inhibited the ability of $25 \mu\text{g}$ of endotoxin given 2 h later to cause complete regression of a 9-day SA1 sarcoma. Although complete regression failed to occur, there was some hemorrhagic necrosis and partial regression. An equal quantity of control IgG failed to influence the antitumor effects of endotoxin. Means of five mice per group.

to be present in the tumor according to the results shown in Tables I and III. These findings indicate, therefore, that a substantial degree of intratumor hemorrhaging occurred in the apparent absence of endogenous TNF. However, failure to detect TNF may have been due to its neutralization by antibody after homogenization.

On the other hand, the same quantity of anti-rTNF antibody (1.6×10^5 neutralizing units) was capable of inhibiting the ability of endotoxin to cause regression of the SA1 sarcoma in five of five mice. This is shown in Fig. 7, where it can also be seen that infusing the same quantity of control IgG had no effect on endotoxin-induced tumor regression. However, infusion of 1/5th or 1/50th the quantity of anti-rTNF antibody failed to completely prevent tumor regression in all mice tested, even when injected directly into the tumor at several sites (results not shown). Therefore, anti-rTNF antibody was not reliable at preventing endotoxin-induced tumor regression, unless it was infused in large quantity.

Discussion

On the basis of evidence that 2 h postendotoxin serum from *P. acnes*-treated or BCG-treated mice contains TNF, and is capable, on intravenous infusion, of causing hemorrhagic necrosis of the Meth A fibrosarcoma in syngeneic mice, it has been generally assumed (7, 8, 12) that TNF mediates endotoxin-induced hemorrhagic necrosis and regression of established murine tumors. It is somewhat surprising, however, that no attempt has been made to determine whether serum from endotoxin-treated, tumor-bearing mice themselves, possesses tumor-necrotizing activity, since this would represent much more relevant evidence that TNF is involved in the antitumor effects of endotoxin. Even if an attempt had been made, it is unlikely that postendotoxin serum from tumor-bearing mice would have proved to be capable of causing tumor hemorrhagic necrosis and regression of a recipient's tumor. Indeed, several attempts in this laboratory (results not shown) to demonstrate that 2 h postendotoxin serum from mice bearing the SA1 sarcoma can cause regression of this tumor in recipient mice were unsuccessful. According to the results presented here, this should have

been the case, because 2 h postendotoxin serum from mice bearing a 9-d SA1 sarcoma was shown to contain only $\sim 10^8$ cytotoxic units of TNF per milliliter. This amount of activity is two orders of magnitude less than the amount of TNS or rTNF that needs to be given to cause necrosis and regression of a 9-d SA-1 tumor. Therefore, endotoxin can cause tumor hemorrhagic necrosis and regression of the SA1 sarcoma, without causing the host to liberate highly toxic quantities of TNF into its circulation. Instead, endotoxin treatment appears to cause the production of therapeutic quantities of endogenous TNF in the tumor itself.

The results show that after giving a therapeutic dose of endotoxin to mice bearing a 9-d SA1 sarcoma, appreciable quantities of TNF are extractable from the tumor while it is undergoing a hemorrhagic reaction. The intratumor TNF was quantitated on the basis of its cytotoxic activity on L929B cells in culture, and identified as TNF on the basis of its susceptibility to neutralization by anti-rTNF antiserum. Moreover, because at any one time during the endotoxin-induced hemorrhagic reaction there was almost as much TNF in the tumor as there was in blood, it seems almost certain that intratumor TNF did not come from blood. This interpretation is supported by the additional finding that TNF could be detected for a longer period of time in the tumor (6 h) than in the blood (4 h) after giving endotoxin. It seems almost certain, therefore, that intratumor TNF is produced in the tumor itself, presumably by intratumor macrophages. It is known that macrophages are the primary source of TNF (reviewed in references 12, 13), and that they are present in murine tumors in large numbers (14). A histological examination of the SA1 sarcoma in this laboratory (unpublished observations) revealed the presence of large numbers of perivascular macrophages in the vascular bed of the tumor.

None of the foregoing discussion deals, however, with causal evidence that TNF mediates endotoxin-induced hemorrhagic necrosis and regression of the SA1 sarcoma. Causal evidence consisted of the demonstration that endotoxin-induced hemorrhagic necrosis and regression of the SA1 sarcoma could be inhibited by infusing the host with an anti-rTNF antibody with a demonstrated capacity to neutralize the ability of rTNF both to kill L929B cells *in vitro* and cause hemorrhagic necrosis of the SA1 *in vivo* (1). However, while the anti-rTNF antibody was capable of completely neutralizing the *in vivo* action of intravenously infused rTNF and natural TNF, it was incapable of completely inhibiting the ability of endotoxin to cause hemorrhagic necrosis of the SA1 sarcoma. Instead, it reduced the rate of development of the tumor hemorrhagic reaction by $\sim 50\%$, as measured by a 50% reduction in the rate of intratumor extravasation of ^{51}Cr -labeled red cells. This was the case, moreover, even though the quantity of anti-rTNF antibody given was large enough to result in antibody excess in the tumor for a sustained period of time. There are at least two possible reasons why anti-rTNF IgG failed to completely inhibit endotoxin-induced intratumor hemorrhaging. It is possible that TNF produced intratumorally mediated intratumor hemorrhaging before it could all be neutralized by the antibody. Alternatively, it is possible that endotoxin evokes the participation of a second TNF-independent mechanism that causes intratumor hemorrhaging. This would not be affected by the antibody. In either case, most of the evidence

supports the view that vascular endothelial cells, rather than tumor cells themselves, are the targets of endotoxin-induced antitumor mechanisms, and that it is the destruction of the tumor's vasculature that is the cause of tumor necrosis. This is in keeping with the interpretation of Algire et al. (6), who described endotoxin-induced tumor hemorrhagic necrosis 40 years ago. It is also in agreement with the observations made here and with those discussed in the preceding companion paper (1). Indeed, it was suggested more recently by others (3) that rTNF-induced necrosis of the Meth A fibrosarcoma is caused by damage to the tumor's vasculature, rather than by direct destruction of tumor cells.

It needs to be pointed out with regard to the tumor hemorrhagic reaction, moreover, that a therapeutic dose of endotoxin caused substantially less intratumor hemorrhaging than a therapeutic dose of rTNF or natural TNF, as measured by intratumor extravasation of ^{51}Cr -labeled red cells. In spite of this, however, endotoxin was much more reliable at causing tumor regression than TNF. It seems likely, therefore, that endotoxin-induced tumor regression depends on the participation of mediators in addition to TNF. It is known, for example, that endotoxin induces the production of interferons (15) and IL-1 (16), each of which might synergize with TNF to cause hemorrhagic necrosis and regression of established tumors. TNF is almost certainly an essential participant, however, because the tumor hemorrhagic reaction can be appreciably decreased, and tumor regression prevented by intravenous infusion of enough anti-rTNF antibody.

The additional essential participant in endotoxin-induced tumor regression is antitumor immunity, as evidenced by the demonstration here and elsewhere (4, 5) that endotoxin fails to cause regression, as opposed to hemorrhagic necrosis, of the SA1 sarcoma growing in immunoincompetent mice. More direct evidence of this was supplied by the finding that endotoxin can cause regression of the SA1 sarcoma growing in immunoincompetent mice provided they are first infused with L3T4⁺, tumor-sensitized T cells from concomitantly immune donor mice bearing a 9-d tumor. It was shown elsewhere (9, 10) that this immunological priming of the recipient tumor for endotoxin-induced regression with donor T cells is specific, and that it requires that the T cells be infused 48 h before endotoxin is given. This and other evidence (9) suggest that the therapeutic effect of endotoxin requires an adequate level of underlying antitumor immunity be acquired before endotoxin is given. Whether this immunity is augmented by mediators released in response to endotoxin treatment is not yet known. In the absence of such information, the simple interpretation of the results is that endotoxin facilitates the expression of an already acquired subtherapeutic level of antitumor immunity by causing TNF-mediated hemorrhagic necrosis of most of the tumor's center, thereby leaving only a thin rim of living tumor tissue for immunity to cope with.

Summary

In agreement with the results of previous studies (1), it was shown that intravenous injection of endotoxin into mice bearing 9-d SA1 sarcoma resulted in a tumor hemorrhagic reaction that rapidly caused necrosis of most of the center of the tumor, and then the complete regression of the rim of living tumor

tissue that survived the hemorrhagic reaction. The tumor hemorrhagic reaction was confined to the vascular bed of the tumor, and its rate and extent of development were measured in terms of the intratumor extravasation of ^{51}Cr -labeled syngeneic red cells. The development of the hemorrhagic reaction was associated with the presence in the tumor over a 6-h period of endogenous TNF that was measured in terms of its capacity to kill L929B cells *in vitro* and identified by its susceptibility to neutralization with a monospecific, polyvalent anti-rTNF antibody. The same antibody was capable *in vivo* of inhibiting the endotoxin-induced tumor hemorrhagic reaction by only ~50%, even when present in the tumor in excess. However, it was capable when given in the same quantity of inhibiting the ability of endotoxin to cause complete tumor regression. The fact that TNF was generated in the tumor during the tumor hemorrhagic reaction, and that infusion of a sufficient quantity of anti-rTNF antibody severely interfered with hemorrhagic necrosis and prevented tumor regression represents convincing evidence that TNF is an essential participant in endotoxin-induced regression of an established SA1 sarcoma. Moreover, because tumor regression, as opposed to hemorrhagic necrosis, failed to occur if the tumor was growing in immunoincompetent mice, but did so if the mice were infused with tumor-sensitized T cells, it can be concluded that an adequate level of T cell-mediated immunity is also an essential requirement for endotoxin-induced tumor regression. The participation of other endotoxin-induced mediators in tumor regression cannot be ruled out.

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