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Toxicity of Tumor Necrosis Factor Is Synergistic With γ-Interferon and Can Be Reduced With Cyclooxygenase Inhibitors

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In recent studies, we have demonstrated that recombinant human tumor necrosis factor (rH TNF), as a single agent, has only minimal therapeutic activity for the treatment of metastatic disease, but when combined with recombinant murine γ-interferon (rM γ-IFN), we observed significantly more therapeutic activity than when either agent was administered alone. However, this combination also resulted in increased toxicity. Thus, we undertook a systematic toxicologic study of rH TNF alone or in combination with rM γ-IFN. Briefly, the toxicity was similar to the generalized Shwartzman's reaction seen during endotoxin shock, with multifocal microthrombi and ischemic necrosis as sequelae. Lesions were observed in the lungs, liver, gastrointestinal tract (preferentially in the duodenum and cecum), testes or uterus, and bone marrow. Our results suggest that TNF (either directly administered or induced *in situ*) and its induction of arachidonic acid metabolites form one element of toxicity in this model. This conclusion is supported by studies revealing that the toxicity of rH TNF in combination with rM γ-IFN can be reduced by inhibitors of the cycloxygenase/lipoxygenase pathway. (Am J Pathol 1987, 128:410–425)

THE ADEQUATE evaluation of the immunomodulatory and therapeutic activities of cytokines was hampered until recently by the lack of sufficient purified materials. Recombinant DNA technology has made it possible to produce many of these cytokines in large quantities. The cytokine that became known as tumor necrosis factor (TNF) was first reported by Carswell et al.[1] Sera from endotoxin-treated rodents that had been sensitized with an immunopotentiator such as bacille Calmette–Guérin contained a substance that, when injected into mice with transplanted intradermal tumors, caused extensive hemorrhagic necrosis of the tumors.[2–6] TNF was also found in culture supernatants from macrophages treated with endotoxin.[7–9] Partially purified and recombinant preparations of TNF have been tested for direct cytotoxicity against murine and human cell lines *in vitro* and *in vivo*.[4,8–21] Tumor, but not normal, cell lines from both species are susceptible to the cytotoxic activity of murine and human TNF. Furthermore, both murine and human TNF are active against murine and human tumor transplants in normal and nude mice, respectively.[1,4,22–25] However, few preclinical therapeutic studies have been undertaken with recombinant, partially purified, or unpurified TNF, and most of these have been against intradermal tumors.

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In recent studies, we examined the therapeutic activity of recombinant human (rH) TNF for experimental and spontaneous metastases.[26] Significant therapeutic activity was observed following intravenous but not intraperitoneal administration of rH TNF. In contrast, significantly increased therapeutic activity was observed with the combination of recombinant murine γ-interferon (rM γ-IFN-γ) and rH TNF as compared with rH TNF alone or rM γ-IFN administered under suboptimal conditions. In these studies, we noted marked toxicity with combined doses of rH TNF and rM γ-IFN, which were nontoxic when administered separately. We therefore undertook systematic toxicity studies to better understand the underlying mechanisms of toxicity. These studies suggest that there is a common mechanism of toxicity for TNF and agents that induce TNF *in situ*. In addition, our finding that toxicity can be reduced with cyclooxygenase/lipoxygenase inhibitors and therefore imply a role for arachidonic acid metabolites.

Materials and Methods

Animals

Specific pathogen-free male C57BL/6N mice (H-2^b), 4 weeks of age, were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility.

Tumors

These studies used the metastatic melanoma variant B16-BL6 from the B16 melanoma,[27] which arose spontaneously in a C57BL/6N (H-2^b) mouse. This cell line was maintained as a monolayer in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, 2-fold-concentrated vitamin solution, glutamine, sodium pyruvate, and nonessential amino acids. The cell lines were free of *Mycoplasma* and pathogenic murine viruses.[28]

Agents

rM γ-IFN (1.3×10^7 U/mg) and rH TNF (5×10^7 U/mg) were generously provided by Genentech (S. San Francisco, Calif). All media, salt solutions, and agents (<0.01 ng/ml) were endotoxin-negative, as determined by the Limulus lysate assay (<0.1 ng/ml).

Therapy of Established Metastases

Experimental lung metastases were established in 8-week-old C57BL/6 mice by the intravenous injection of 5×10^4 *in vitro*-propagated B16-BL6 melanoma cells in 0.2 ml calcium-magnesium-free Hanks' balanced salt solution (HBSS). The schedule for therapy of metastases varied and is described within the text. Therapy was continued for 4 weeks, and mice that had survived 1 week after tumor challenge were killed and underwent necropsy. The determination of therapeutic efficacy was based on the number of pulmonary metastases.

We also evaluated the therapeutic efficacy of recombinant cytokines against spontaneous metastases derived from B16-BL6 melanoma. B16-BL6 melanoma cells (5×10^4 in 0.05 ml calcium-magnesium-free HBSS) were injected into the posterior footpads of 8-week-old syngeneic mice. When the primary tumor reached a diameter of 0.8 to 1 cm, the tumor-bearing leg was resected at midfemur to include the popliteal lymph node. Therapy was initiated 24 hours later by means of various protocols and was continued for 4 weeks. Animals underwent necropsy 1 week after the last injection, and the number of lung metastases in each group was determined.

Necropsy

For studies on the sequential development of toxicity, mice were killed with CO_2 at different time points, and complete necropsies were performed (5 mice per group). The following tissues were fixed in 10% neutral buffered formalin for microscopic studies: lung, liver, spleen, kidney, uterus or testis, femur, and the entire gastrointestinal tract. Any other tissues that grossly appeared to be involved were also prepared for microscopic examination. The osseous tissues were decalcified before tissue processing; routine paraffin sections were cut at 5 μ and stained with hematoxylin and eosin.

Cytostasis Assay

The cytostatic properties of rH TNF and rM γ-IFN were assessed after the coculture of 10,000 tumor cells and various doses of the agents in 96-well plates. After a 24-hour incubation at 37 C, the wells were pulsed with 1 μCi ^{3}H-thymidine and incubated an additional 24 hours. The wells were washed with warm medium, and the cells were trypsinized and harvested with a MASH harvester.[16] The incorporation of ^{3}H-thymidine was determined by means of a beta scintillation counter. We used the following formula to determine the percentage of cytostasis:

$$\% \text{ of cytotoxicity} = 100 \times \frac{\text{experimental cpm}}{\text{control cpm}}$$

Results

Therapy of Experimental and Spontaneous Metastases With rM γ-IFN, rH TNF, or Both

The results of treating spontaneous metastases 3 times per week with intravenous injections of rH TNF are shown in Table 1. The animals received 500,000, 50,000, 5000, or 500 units of rH TNF. When rH TNF was administered by intravenous injection, 500,000 U/animal was somewhat greater than the maximum tolerated dose such that the median survival time was reduced from >29 days (for the metastasis bearing animals treated with rH TNF) to 17 days (for excipient-treated animals with metastasis). Significant therapeutic activity was observed when 50,000 or 5000 U/animal of rH TNF was administered by intravenous injection (Table 1). In contrast, rH TNF over a 3-log dose range had no significant activity when administered by the intraperitoneal route (Table 2). The difference in the therapeutic activity of TNF by intraperitoneal as opposed to intravenous administration appears to be associated with the different pharmacokinetics of these routes of administration. After the intravenous administration, much higher serum levels are achieved than after intraperitoneal administration (Figure 1).[26] Although the T-alpha half-lives are very similar for these two routes of administration, the biologically effective dose is much lower after intraperitoneal administration, ie, concentration over time. Thus, presumably these higher levels of serum activity result in the increased therapeutic activity. It should be noted that the prior injection of γ-IFN has no effect on absorption of TNF on the pharmacokinetics of TNF after either intravenous or intraperitoneal administration. Unlike rH TNF, daily intraperitoneal injections of rM γ-IFN at 50,000 U/animal, as did polyinosinic:polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose (Poly ICLC), had significant therapeutic activity. Interestingly, when 50,000 U/animal of rH TNF was admixed with rM γ-IFN and administered daily by intraperitoneal injection for 4 weeks, a significant increase in therapeutic activity was noted, as compared with the activity of rM γ-IFN alone ($P = 0.01$). This increase in therapeutic activity was suggested by the *in vitro* cytostasis studies previously reported.[11,12,17,20,22,25,29-32] Therefore, it appears that at least some of the therapeutic activity of these cytokines is associated with their cytostatic properties. However, this is unlikely to be the complete mechanism of activity, because low levels (approximately 10 U/ml) of serum TNF are observed after the intraperitoneal administration of 1 μg of rH TNF.[26] This level,

Table 1—Treatment of Spontaneous Metastases with rH TNF Administered by Intravenous Injection

Biological response modifiers	Dose/animal	Metastases		P*
		Median	Range	
Saline	—	11	16–27	—
Poly-ICLC	10 μg	1	0–4	0.000
rH TNF	500,000 U	0	0–65	0.027
rH TNF	50,000 U	0	0–36	0.034
rH TNF	5,000 U	1	0–36	0.020
rH TNF	500 U	25	0–65	0.496

B16-BL6 tumor cells were injected into the left or right posterior footpad of each mouse. When the tumors reached a 0.9 cm diameter, the tumor-bearing limb was resected at midfemur to include the popliteal lymph node. Therapy was initiated 1 day later with three weekly intravenous injections for 4 weeks. Necropsies were performed 1 day after the last injection. (n = 10 mice).

The median survival time for all groups was 29 days except for the animals receiving 500,000 U/animal of rH TNF, which had a mean survival time of 17 days due to TNF toxicity.

*Probability of no difference in the number of nodules in comparison with mice that received saline control, as determined by means of the Mann–Whitney U-test.

Table 2—Treatment of Experimental Metastases With rH TNF and rM γ-IFN

Biological response modifiers	Dose/animal	Route	Schedule	Metastases		P*
				Median	Range	
Saline	—	Intraperitoneal	daily	55	0–> 300	—
Poly ICLC	10 μg	Intravenous	3x weekly	3	0–17	0.0012
rH TNF	500,000 U	Intraperitoneal	daily	26	0–81	0.07
rH TNF	50,000 U	Intraperitoneal	daily	29	5–141	0.35
rH TNF	5000 U	Intraperitoneal	daily	29	0–257	0.16
rH TNF	50,000 U	Intravenous	daily	24	6–88	0.041
rM γ-IFN	50,000 U	Intraperitoneal	daily	7	0–54	0.003
rH TNF/ rM γ-IFN	50,000 U 50,000 U	Intraperitoneal	daily	1	0–5	0.0009†

C57BL/6 mice were given intravenous injections of 5×10^4 B16-BL6 tumor cells, and immunotherapy was initiated 2 days later. Therapy consisted of the injection of each biologic response modifier for 4 consecutive weeks. Necropsies were performed 35 days following tumor challenge, and the extent of experimental metastasis was determined with the aid of a dissecting microscope (n = 10/mice).

*Probability of no difference in the number of nodules in comparison with mice that received the saline control, determined by means of the Mann–Whitney U-test.

†Probability of no difference in the number of nodules in comparison with mice that received γ-IFN alone ($P < 0.01$) determined by means of the Mann–Whitney U-test.

although relatively constant after intraperitoneal administration, is insufficient *in vitro* to produce any cytostatic or cytotoxic activity for B16-BL6 cells. Thus, the therapeutic activity achieved with intraperitoneal injections of rH TNF is associated with either its immunomodulatory or coagulative inducing properties. In contrast, intravenous administration of 50,000 units of rH TNF produced TNF levels greater than 10,000 U/ml, which is sufficient to kill over 90% of B16-BL6 tumor cells *in vitro* (results not shown). However, this high level of serum rH TNF activity was transient[26] and appears not to have been maintained long enough to produce significant cytostatic activity against B16-BL6 tumor cells. It is likely, therefore, that the immunomodulation- and perhaps coagulation-inducing properties of rH TNF also have a role in its therapeutic activity.[26,33-39]

Survival Curves of Animals Receiving rM γ-IFN and rH TNF

To systematically address the toxicity of rH TNF along or with rM γ-IFN was examined in normal non-tumor-bearing mice. The mean survival time of mice not bearing tumors is approximately 6 hours following the intravenous injection of 500,000 units of rH TNF with 50,000 units of rM γ-IFN, 72 hours for animals receiving daily injections of 250,000 U/animal of rH TNF plus 50,000 U/animal of rM γ-IFN, and 96 hours following the injection of 50,000 U/animal of rH TNF with 50,000 U of rM γ-IFN. Mice receiving 5000 U/animal of rH TNF plus 50,000 U/animal of rM γ-IFN survived four daily injections (Figure 2). In contrast, mice without tumor burdens survived the daily administration (4 days) of 1,050,000 U/animal of rH TNF or > 300,000 U/animal of rM γ-IFN when each was injected as a single agent. However, as shown in Table 1, mice with a preexisting tumor burden have increased sensitivity to rH TNF, such that toxicity is observed when doses of 500,000–1,050,000 units are administered chronically.

Histopathology of TNF Toxicity

To gain insight into the mechanism of toxicity of rH TNF in the presence or absence of rM γ-IFN, systematic histopathology studies were undertaken. The histopathology following TNF or TNF/γ-IFN administration is similar to disseminated intravascular coagulation or the generalized Shwartzman's reaction, which is seen clinically as part of endotoxin shock. Twenty-four hours after a single injection of rH TNF, histopathologic lesions were most common in mice receiving 500,000 U/animal, followed by those receiving 250,000 U/animal. Occasional lesions were present in mice receiving doses as low as 50,000 U/animal, a dose that gave significant therapeutic efficacy for spontaneous metastases. Lesions generally consisted of combinations of focal to multifocal degeneration necrosis, thrombosis, and occasionally hemorrhage. Foci of hepatocellular degeneration necrosis were present throughout the parenchyma of the liver (Figure 3), although they typically occurred just beneath or were contiguous with the capsule. These foci ranged in size from a diameter of three or four

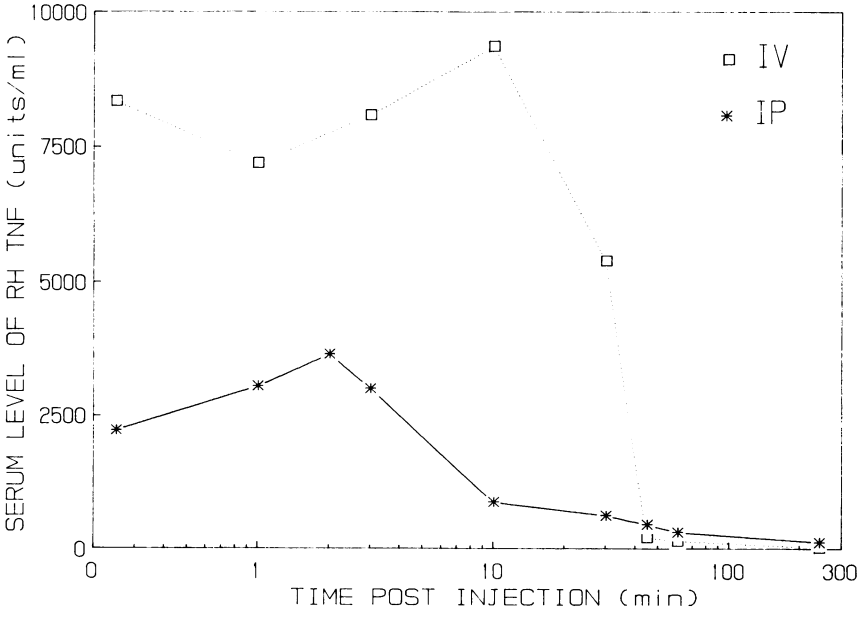

Figure 1—Serum pharmacokinetics of rH TNF following either intravenous or intraperitoneal administration.

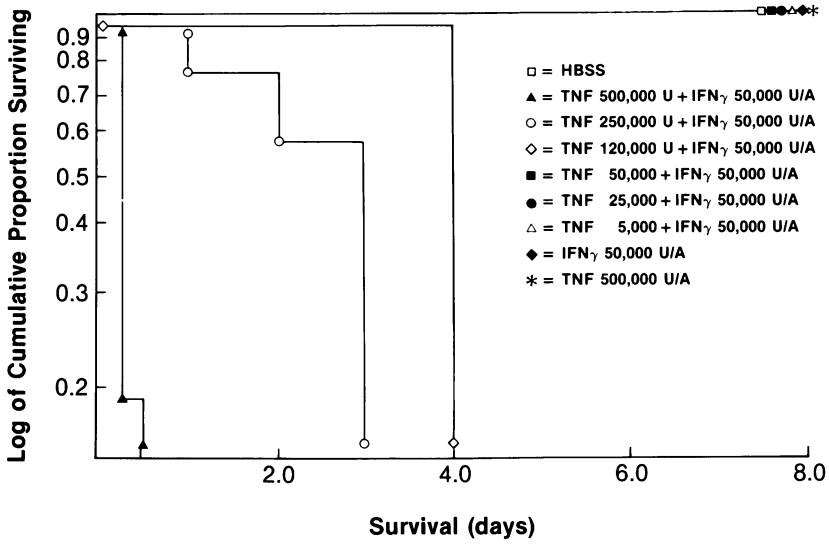

Figure 2—Kaplan–Meyer survival curves of mice that received four daily intravenous injections of various doses of rH TNF in conjunction with rM γ-IFN (50,000 U/animal) or either agent alone. The Kruskal–Wallis analysis was used for determining a significant effect on survival (n = 10).

hepatocytes to one or two hepatic lobules. Microthrombi were occasionally present in the vicinity of necrotic foci, but inflammatory cells were absent. In the bone marrow and uterus there was mild to moderate multifocal necrosis, usually accompanied by microthrombosis and hemorrhage (Figure 4). Multifocal necrosis of granulosa cells in the ovary was associated with minimal vascular endothelial lesions (Figure 5). In the gastrointestinal tract, lesions were most common in the duodenum and cecum but occurred at other sites. These lesions were usually characterized by necrosis of individual crypt epithelial cells, unaccompanied by vascular change (Figure 6), although hemorrhagic necrosis, microthrombosis, and transmural edema also occurred. Microthrombi were occasionally present in vessels within the spleen and lung (Figure 7) but were not associated with histologically obvious secondary changes. By 96 hours following a single injection of rH TNF, microthrombotic as well as many degenerative and necrotic changes, largely in the liver, were replaced by focal, perivascular accumulations of mononuclear and neutrophilic inflammatory cells (Figure 8). Uterine and ovarian necrosis and some intestinal hemorrhagic necrosis were still present by 96 hours.

Because of the toxicity at 500,000 U/animal of rH

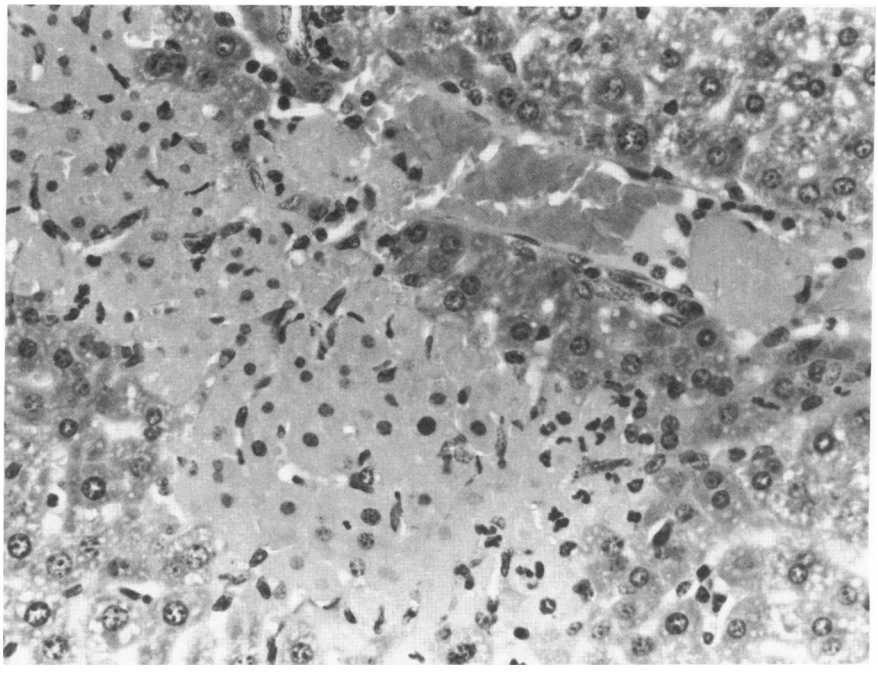

Figure 3—Focus of hepatocellular degeneration and necrosis adjacent to a partially thrombosed vein (*arrow*). The mouse had received 10 μg of rH TNF 24 hours earlier. (×240)

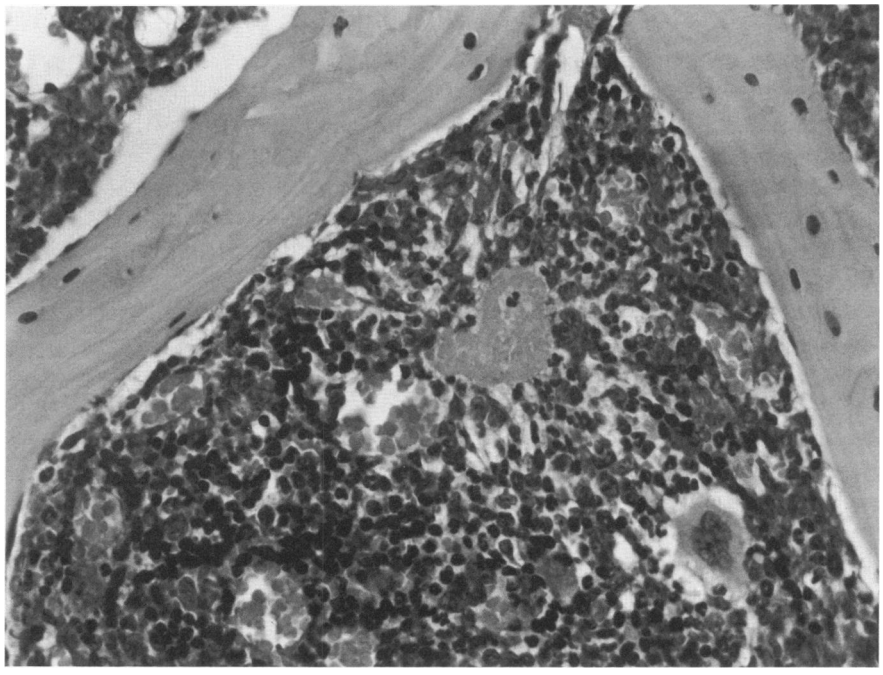

Figure 4—Venous thrombus in bone marrow from a mouse that had received 10 μg of rH TNF 24 hours earlier. (×240)

TNF, the subacute toxicity studies were based on four daily intravenous injections of rH TNF at 250,000, 125,000, 50,000, 25,000, and 5000 U/animal. In these studies, toxicity was observed again at 250,000, 125,000, and 50,000 U/animal (as defined by histologic changes) but not at lower doses. Twenty-four hours after the fourth injection of TNF, there was multifocal necrosis of the bone marrow and marked extramedullary myelopoiesis in the spleen and liver (Figure 9). *In vitro* studies with bone marrow stem cell assays have also revealed direct effects on stem cell proliferation.[40–43] Vascular endothelial cells were hypertrophied, and leukocytes were frequently adherent. There were scattered thrombi in the liver and lung, but lesions were fewer and more subtle than those observed in mice receiving a single injection of 500,000 U/animal. By 96 hours after the fourth injection of rH TNF, most of the lesions had resolved, with

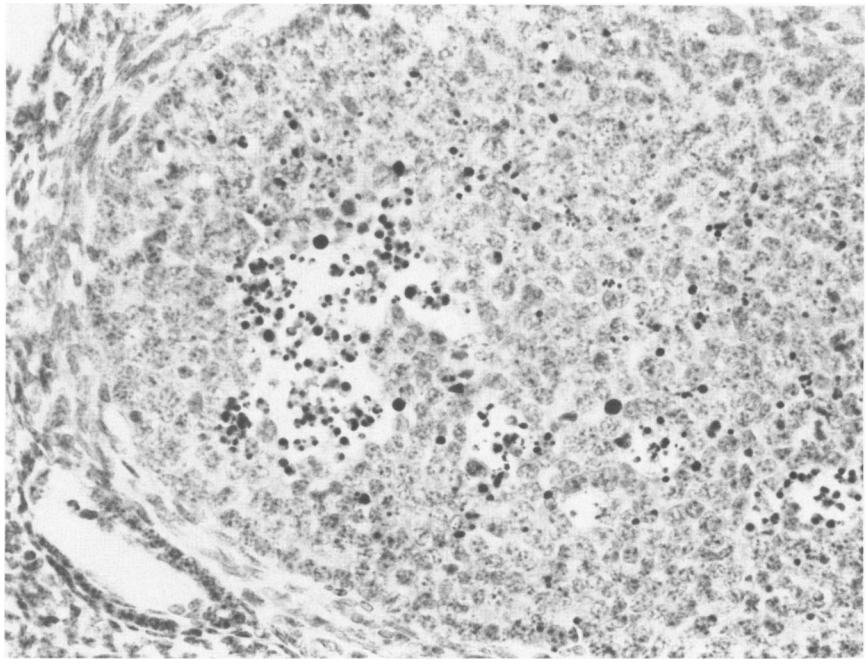

Figure 5—Necrotic granulosa cells in the follicle of an ovary. The mouse had received 10 μg of rH TNF 24 hours earlier. (×240)

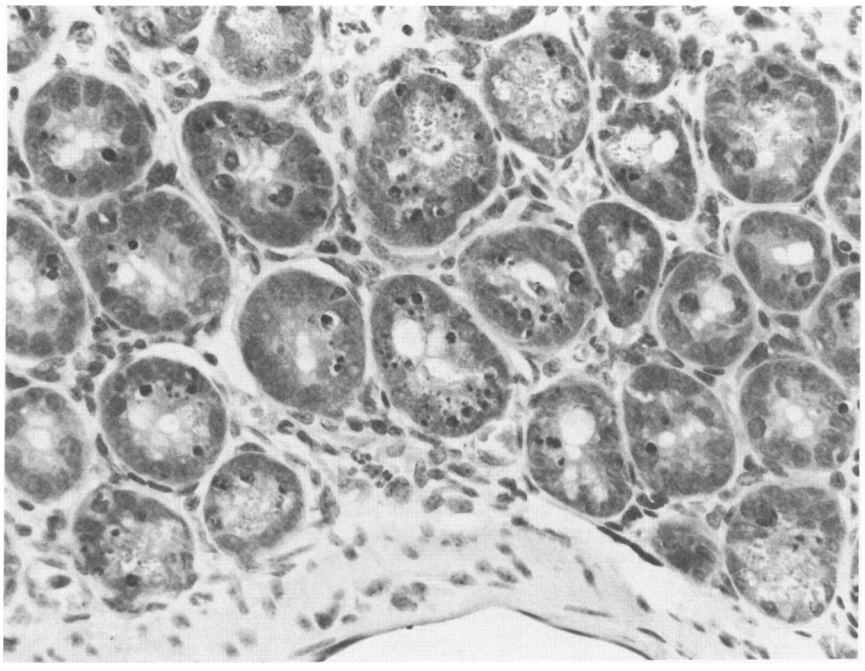

Figure 6—Individual epithelial cells lining the crypts of the duodenum are necrotic. The mouse had received 10 μg of rH TNF 24 hours earlier. (×240)

medullary and extramedullary myleopoiesis remaining. Tail veins of many of the animals receiving 4 injections of TNF were thrombosed.

Histopathology of Combined rH TNF and rM γ-IFN

The intravenous injection of 500,000 U of rH TNF with 50,000 U/animal of rM γ-IFN resulted in 100% mortality within 7 hours. One of 5 animals receiving 250,000 U/animal of rH TNF in conjunction with rM γ-IFN was dead by this time. Thus, at 500,000 U/animal, the histopathologic lesions were less pronounced than those observed with rH TNF alone, presumably because lesions had insufficient time to progress. The principal histologic appearance in animals dying acutely was congestion of the spleen, bone marrow, small intestine (especially the duodenum), cecum, and lung. Midzonal hepatocytes had cytoplasmic va-

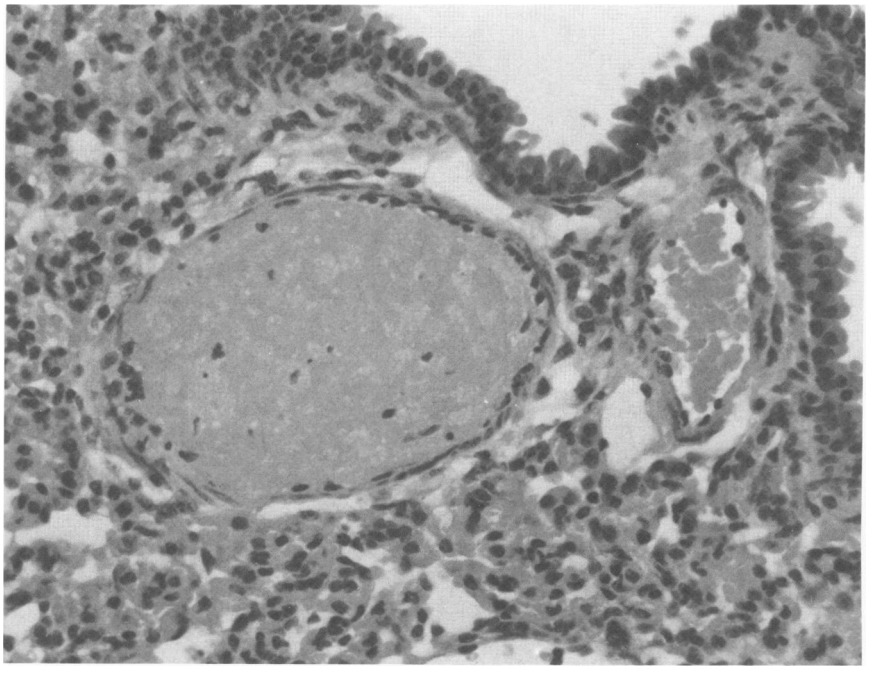

Figure 7—Venous thrombus in lung of a mouse given 10 μg of rH TNF 24 hours earlier. (×240)

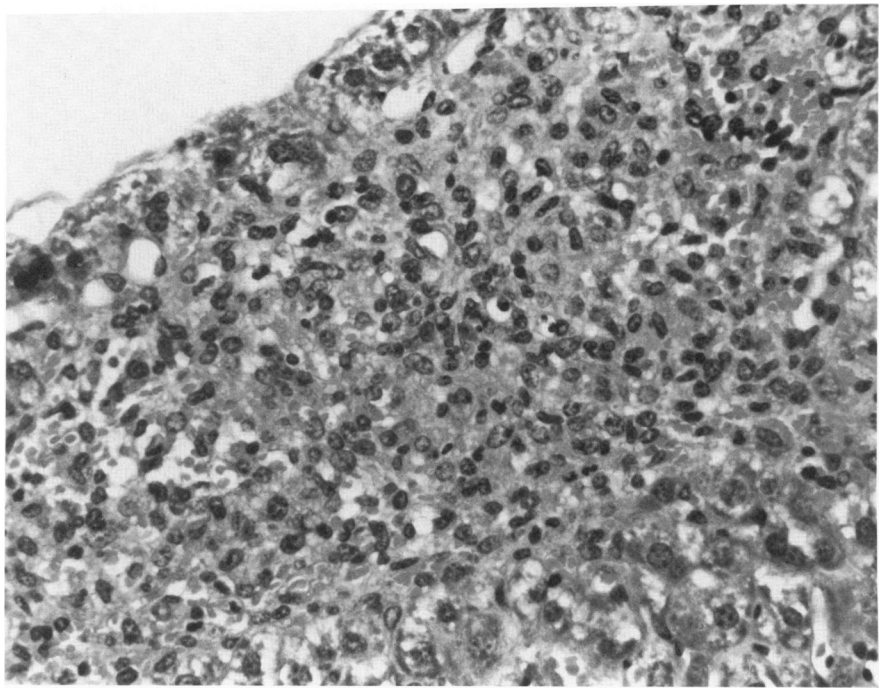

Figure 8—Ninety-six hours after a single 5-μg injection of rH TNF, foci of coagulative necrosis in the liver are replaced by accumulations of mononuclear inflammatory cells. (×240)

cuolation with few microthrombi scattered in the same region (Figure 10). Mice that received less than 500,000 U of rH TNF in combination with rM IFN-γ had lesions similar to those just described. In addition, these mice had marginated mononuclear and neutrophilic inflammatory cells and hypertrophied endothelium in many tissues, including lung, liver, and gastrointestinal tract (Figure 11). Necrosis of intestinal crypt epithelial cells, uterine hemorrhage, focal hepatic necrosis, and microthrombi in multiple sites were observed at doses as low as 5000 units of rH TNF in combination with 50,000 U/animal of rM γ-IFN, although not in every animal. Mice examined 96 hours after a single combined dose of rH TNF (50,000 U/animal) and rM γ-IFN had marked myelopoiesis in the bone marrow and at hepatic and splenic

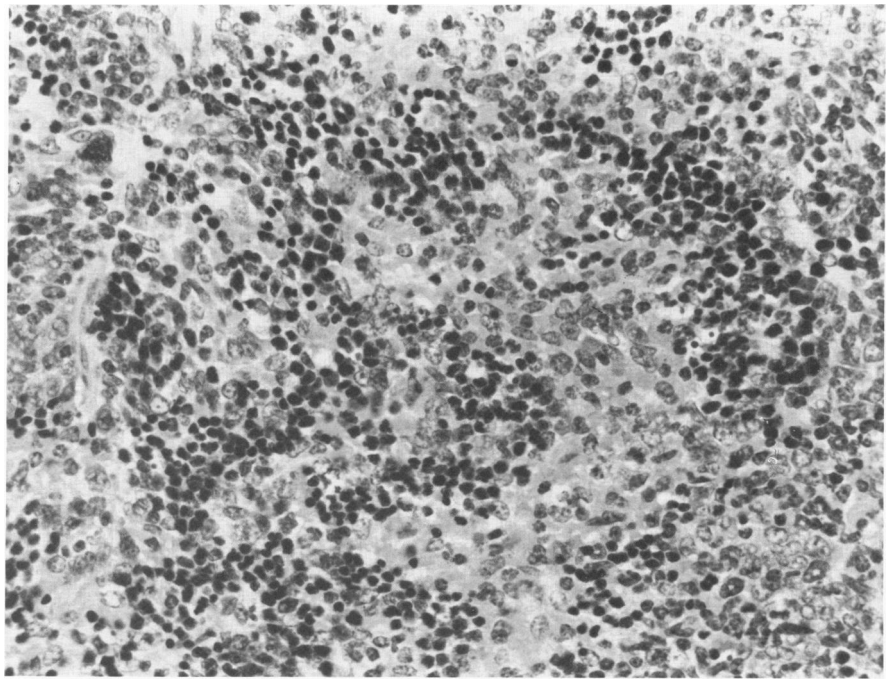

Figure 9—Dark-staining cells represent myeloproliferative elements in the spleen. The mouse had received a single 5-μg injection of rH TNF 96 hours earlier. (×240)

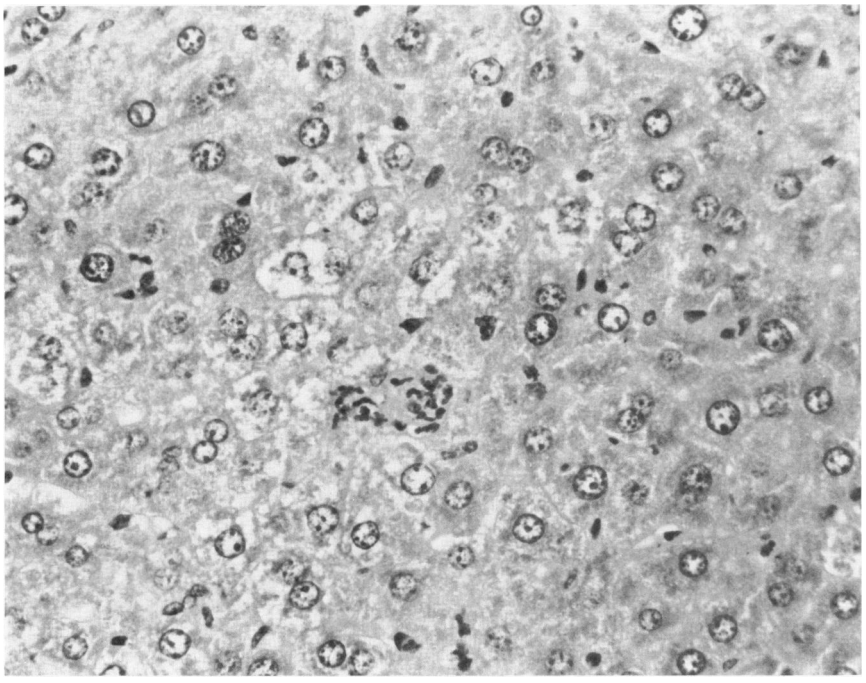

Figure 10—Midzonal focus of hepatocellular degeneration surrounding a thrombosed vein (*arrow*). The mouse had received 5 μg of rH TNF combined with 50,000 units of γ-IFN 24 hours before necropsy. (×360)

sites (Figure 12). These myelopoietic sites were composed predominantly of immature granulocytic elements.

To quantitate the extramedullary myelopoiesis, the number of myelopoietic foci in 10 randomly selected 1-sq mM areas of liver were quantitated from each mouse. Mice given an injection 96 hours previously of a single dose of rH TNF (50,000 U/animal) had 2–5 hepatic myelopoietic foci per 10-sq mm area. The livers of mice injected 24 hours earlier generally lacked myelopoietic foci, although solitary foci were occasionally present. Mice receiving four injections of rH TNF (50,000 U/animal) had 4–11 myelopoietic foci per 10-sq mm area of liver 96 hours after the last injection. Similarly, there were 9–11 hepatic myelopoietic foci per 10-sq mm surface area in mice that received a single injection of rH TNF and rM γ-IFN 96 hours earlier. Mice given combinations of rH TNF

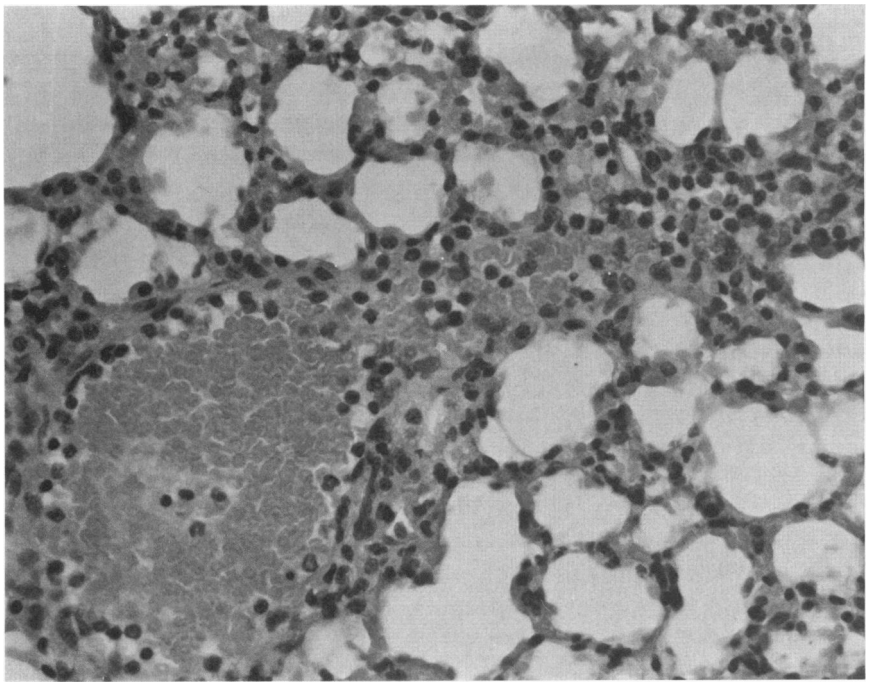

Figure 11—Congestion, endothelial cell hypertrophy, and margination of leukocytes in a pulmonary vein. The mouse had received 10 μg of rH TNF combined with 50,000 units of γ-IFN 24 hours before necropsy. (×240)

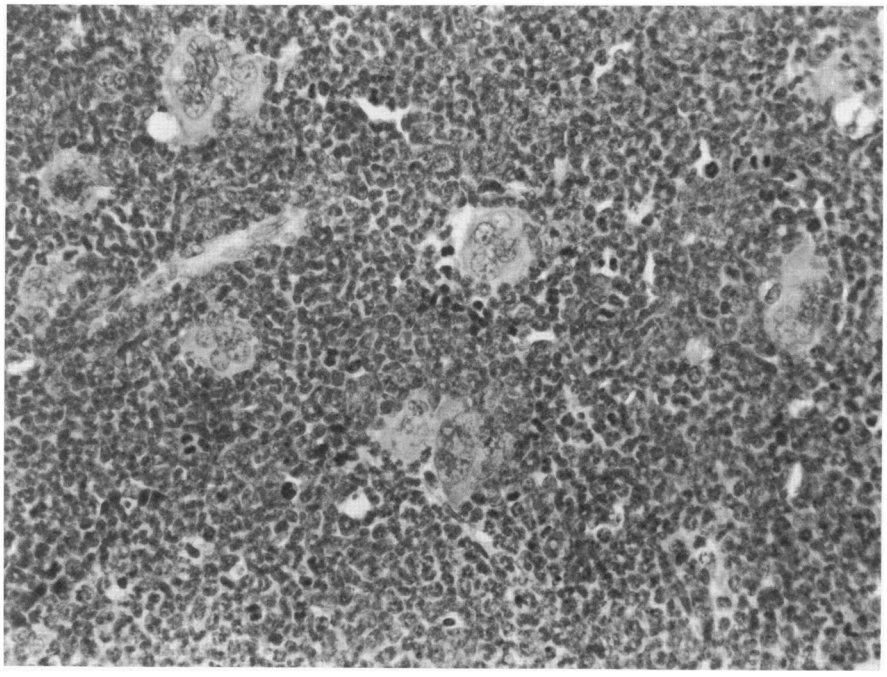

Figure 12—Bone marrow hyperplasia in a mouse given a single injection of 5 μg rH TNF combined with 50,000 U γ-IFN 96 hours earlier. Hyperplastic myeloid elements surround several megakaryocytes. (×240)

(50,000 U/animal) and rM γ-IFN and evaluated 24 hours later, were similar to those given rH TNF alone, in that they generally lacked extramedullary myelopoietic foci. Mice given four injections of combined rH TNF and rM γ-IFN and examined 96 hours later had 6–11 myelopoietic foci per 10-sq mm of liver surface. Figure 13 demonstrates myelopoietic foci in a section of liver similar to those counted to document the extent of myelopoiesis discussed above.

The subacute toxicity studies included 250,000, 125,000, 50,000, 25,000, and 5000 U/animal of rH TNF in addition to the rM γ-IFN. However, due to the toxicity, a full series of four injections were not administered to animals in the 250,000-unit group (which received two injections) or the 125,000-unit group (which received three injections); animals in the other dose groups received all four injections. Mice receiving 50,000 units of rH TNF (plus rM

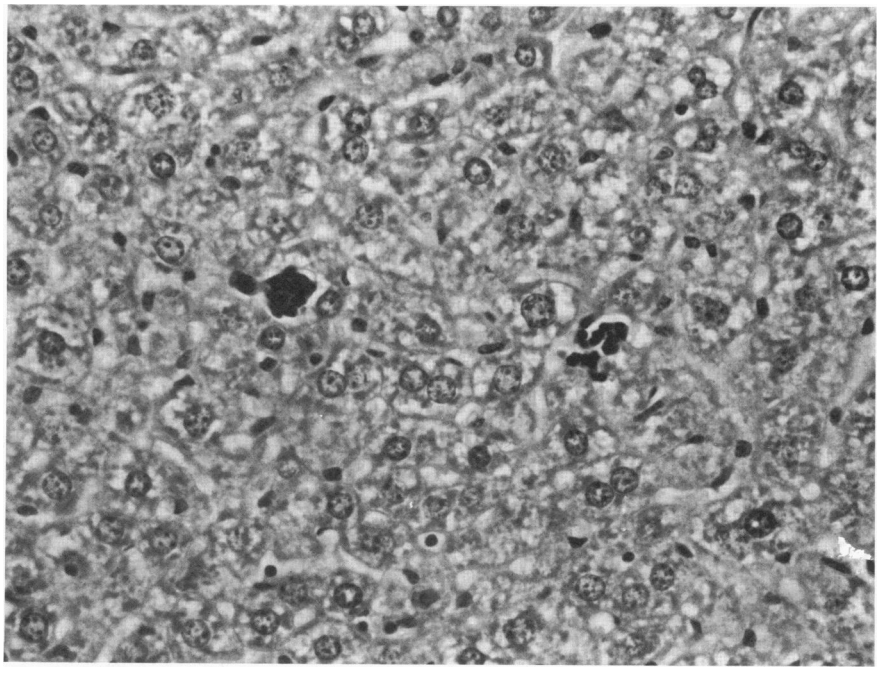

Figure 13—Two typical darkly staining myelopoietic foci in the liver from a mouse given 5 μg rH TNF combined with 50,000 U γ-IFN 96 hours earlier. (×240)

γ-IFN) were moribund after the fourth injection and underwent necropsy shortly thereafter. Other necropsies were done 24 hours after the last injection. Additional necropsies were performed 96 hours after the last injection for mice receiving 25,000 or 5000 U/animal of rH TNF in combination with 50,000 U/animal of γ-IFN.

The results of necropsies revealed that 24 hours after the last injection, or when animals were moribund, there were marked, multisystemic lesions. Grossly, the duodenum was distended and a mottled red. The lungs, liver, intestines, and uterine horns had multiple hemorrhagic foci on serosal surfaces. The hemorrhagic necrosis and edema were of sufficient severity to be grossly visible (Figure 14). Histologically, there was widespread congestion that was particularly obvious in the spleen, liver, lung, gastrointestinal tract, and uterus. There were midzonal and subcapsular microthrombi with foci of degeneration and necrosis in the liver. Cryptal epithelial cells in the cecum, duodenum, and, less frequently, stomach were necrotic. Foci of necrosis and thrombosis were common in bone marrow. There was focal degeneration and necrosis of seminiferous tubules in many sections of testicle. Microthrombi and occasional hemorrhage occurred in some sections of uterus and lung. In addition, the endothelium in many vessels was hypertrophied and had neutrophilic and mononuclear cells marginated to the surface.

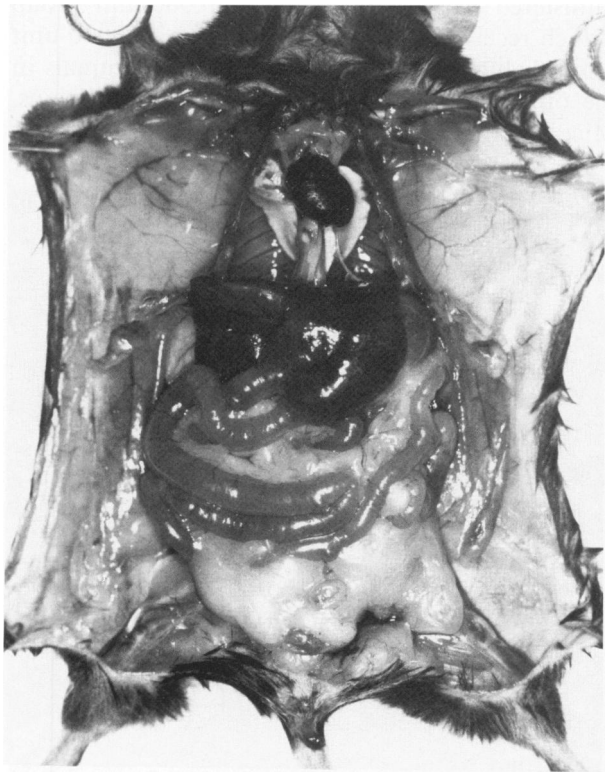

Figure 14—Gastrointestinal hyperemia and hemorrhage in a mouse that had received an intravenous injection of rH TNF (500,000 U/animal) and rM γ-IFN (50,000 U/animal).

Mice that received 25,000 or 5000 U/animal of rH TNF with rM γ-IFN and were necropsied 96 hours after the last injection had fewer lesions than those examined at 24 hours. Scattered thrombi were still present in bone marrow, liver, and lung, although some thrombi had been endothelialized (Figure 15). Necrotic foci persisted in the bone marrow, but foci in the liver consisted primarily of degenerative cells with accumulations of neutrophils and mononuclear cells. Myelopoietic foci were common in the bone marrow, spleen, and liver. Histologic lesions were not present in the kidneys or hearts of any mice.

Reduction or Prevention of Toxicity

Based on the suggestion of ischemic necrosis and the occurrence of microthrombi in several organ systems, we hypothesized that some of the toxicity might be associated with activation of the coagulation pathway and platelet aggregation. We therefore examined the survival of mice receiving a lethal injection of rH TNF (500,000 U/animal) with rM γ-IFN (50,000 U/animal) with and without the administration of aspirin (Figure 16). In these studies, we found that a single injection of 25 mg/kg of aspirin prior to the injection of rH TNF and rM γ-IFN prolonged survival past the acute phase of 6–8 hours in untreated mice, although most of the animals had died within 24 hours. In contrast, the injection of 25 mg/kg of aspirin twice a day at 4-hour intervals resulted in a significant increase in survival (50%) (Figure 16).

In other studies (results not shown) we undertook a histologic evaluation of animals receiving rH TNF and rM γ-IFN in the presence or absence of aspirin at 25 mg/kg injected 30 minutes before and 4 hours after the rH TNF and rM γ-IFN. In these studies, a significant depression in gastrointestinal track was observed such that virtually no lesions were observed anywhere within the lower or upper gastrointestinal track. However, the pulmonary congestion, hepatic microthrombi, and focal necrosis were retained. This suggests, therefore, that the effect of the aspirin as monitored by prolongation of survival is apparently associated with a decrease in gastrointestinal track lesions as opposed to hepatic or pulmonary pathology. This may be due to the number of activated macrophages in the lung and liver as compared with the intestines.

In other studies, indomethacin in the drinking water at 5×10^{-5} M resulted in a significant increase in survival following a single injection of the cytokine

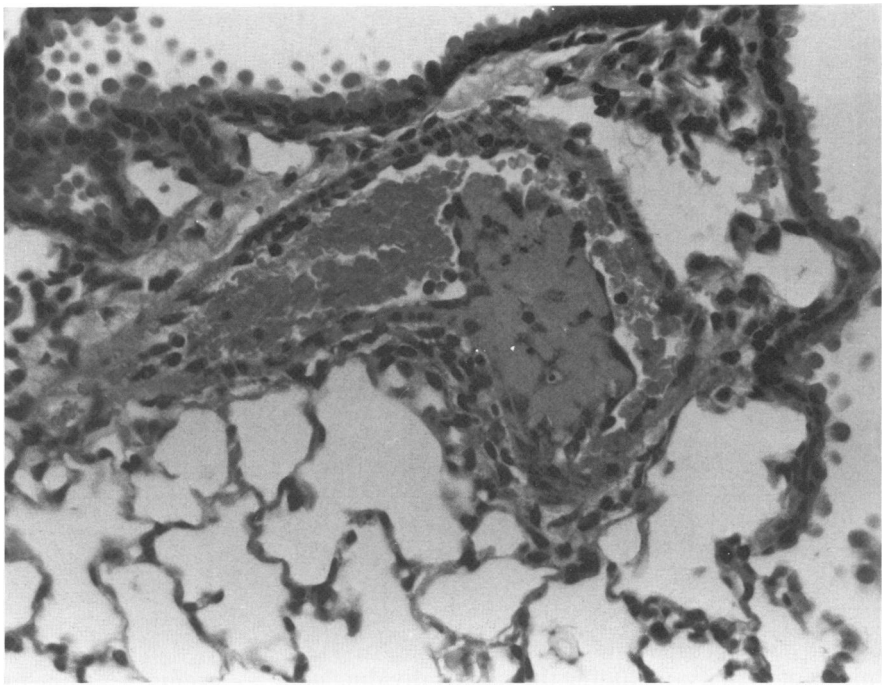

Figure 15—Endothelialized arteriolar thrombus in the lung of a mouse that had received daily injections of rH TNF combined with 50,000 U of γ-IFN for 4 days and had undergone necropsy 96 hours later. (×240)

combination (Figure 17). These doses of aspirin or indomethacin were utilized on the basis of the ability of these agents to significantly depress coagulation times. This dose of aspirin, 1 hour after injection, prolonged tail bleed clotting times to greater than 30 minutes, compared with 45 seconds for normal mice. This suggests that an effective dose of these cyclooxygenase inhibitors was provided. The twice-daily administration of 50 mg/kg of aspirin resulted in the greatest survival in mice injected with rH TNF plus rM γ-IFN. Unfortunately, 50 mg/kg of aspirin in conjunction with indomethacin or higher doses of indomethacin (10^{-4} M) or aspirin (100 mg/kg) were toxic. In addition to the loss of platelet aggregation associated with arachidonic acid metabolites, other mechanisms of coagulation, including fibrin-mediated coagulation and macrophage/endothelial cell-derived platelet-aggregating factor or procoagulant activity, could be part of the pathogenesis. However, heparin at 16 U/animal had no significant effect on rH TNF/rM γ-IFN toxicity. In addition to the toxicity to bone marrow cells associated with ischemic necrosis, rH TNF has a direct antiproliferative effect on stem cells (results not shown)[40–43] (ie, TNF has direct mecha-

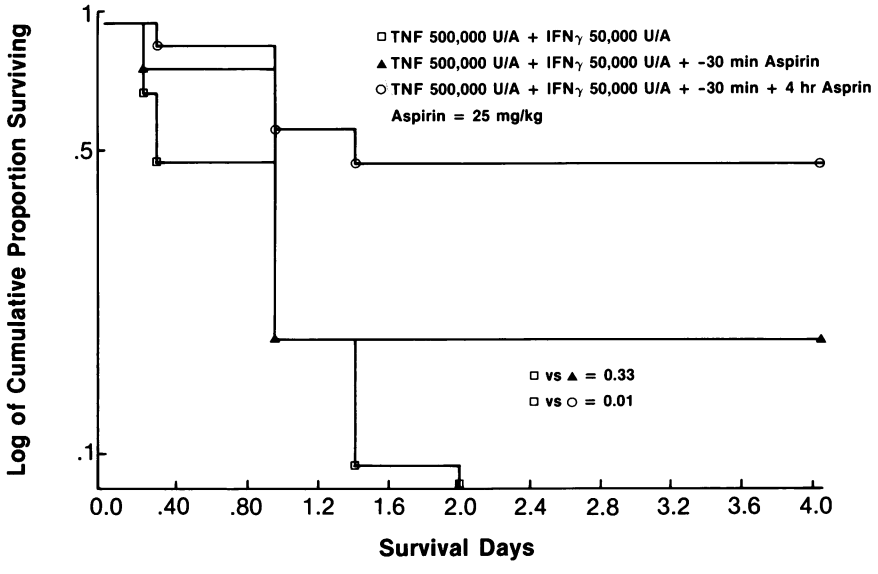

Figure 16—Kaplan–Meyer survival curve of mice that received 500,000 U/animal of rH TNF and 50,000 U of rM γ-IFN. Groups of mice also received one 25 mg/kg injection of aspirin either 30 minutes before the cytokine or 30 minutes before and 4 hours after cytokine administration. The Kruskal–Wallis test was used for statistical analysis.

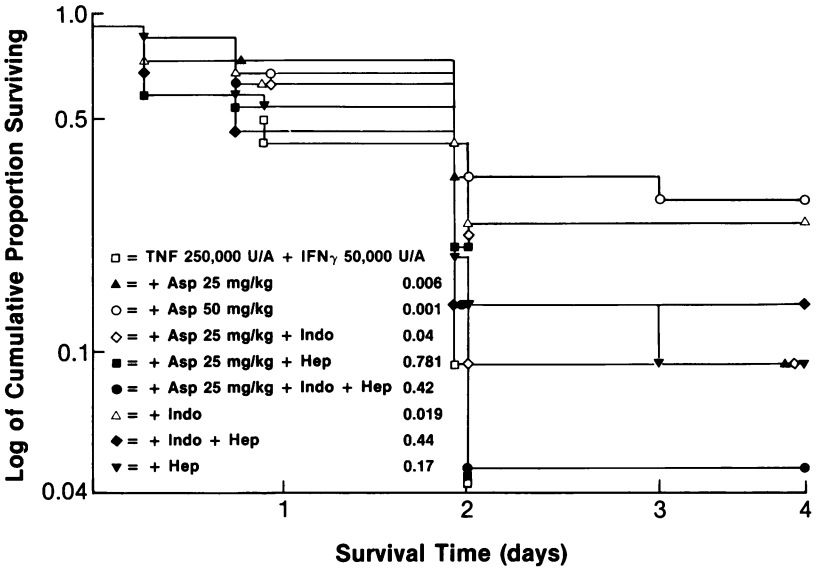

Figure 17—Kaplan–Meyer survival curves of mice that received 250,000 U/animal of rH TNF and 50,000 U of rM γ-IFN. Groups of mice also received aspirin (*Asp.*), twice daily at either 25 or 50 mg/kg; indomethacin (*Indo*) at 5×10^{-5} M in the drinking water beginning 24 hours prior to cytokine administration; heparin (*Hep*) at 16 μg/animal; or various combinations of these agents. Statistical analyses were done with the Kruskal–Wallis test.

nisms of toxicity, including the rapidly dividing bone marrow cells and presumably intestinal crypt cells, which are not associated with ischemic necrosis). Nonetheless, some of the toxicity associated with rH TNF in combination with rM γ-IFN appears to be secondary to microthrombi and a subsequent ischemic necrosis, and this mechanism of toxicity can be reduced in part by cyclooxygenase/lipoxygenase inhibitors such as aspirin or indomethacin.

Discussion

The most challenging aspect of cancer treatment is the control of metastases that are present at primary tumor diagnosis.[28,44,45] RH TNF was expected to have therapeutic activity, in no small part because of its name and the dramatic response observed with intradermal Meth A tumors. Unfortunately, most patients do not present with a highly antigenic intradermal tumor. Thus, it should not be too surprising that clinical trials with rH TNF have produced very limited responses and only with cutaneous lesions, although it is optimistic to expect therapeutic responses in a Phase I trial setting. In our studies of rH TNF in rodent models of metastatic disease, it has had only slight therapeutic activity[26] most notably for spontaneous metastases. Therefore, it was encouraging to note that there was synergistic cytotoxicity/cytostasis against human and murine tumors when rM γ-IFN was added to rH TNF.[11,12,17,20,25,26,29–32] Although this increased activity was not noted for all tumors and, indeed, some tumors were refractory to both agents,[11,26] some tumors that were refractory to either agent alone became responsive to the combination of agents.[11,26] Thus, the additive therapeutic activity observed against both subcutaneous (results not shown) and experimental-spontaneous metastases suggests therapeutic adjuvant activity for rH TNF. Unfortunately, this increase in therapeutic activity was paralleled by an increase in toxicity. The histologic lesions most consistently observed with rH TNF alone or in combination with rM γ-IFN involved the vasculature. These lesions varied from endothelial hypertrophy, frequently associated with marginated inflammatory cells, to thrombosis, edema, and hemorrhage. Many of the degenerative and necrotic lesions could be associated with thrombi or were otherwise compatible with ischemic lesions. In some instances, such as with necrotic crypt epithelial cells or necrotic testicular or ovarian cells, an association with thrombi was not clearly established. However, this could be due to thrombi out of the plane of the histologic section. Alternatively, because these are all rapidly dividing cells, they could be direct targets for TNF-mediated cytotoxicity,[11] as they are for most conventional chemotherapeutic agents.

TNF and γ-IFN, singly and synergistically, cause specific alterations in the morphology and behavior of endothelial cells *in vitro*.[36] These changes include exposure of substrata and loss of stainable fibronectin matrix. In addition, endothelial cells cultured in the presence of recombinant TNF have enhanced tissue factor procoagulant activity and an attenuated anticoagulant protein C pathway.[34] These changes occurring *in vivo* could promote blood coagulation and

thrombus formation. Aspirin (by inhibiting platelet aggregation and adhesion) and, subsequently, thrombosis, appears to diminish this phase of TNF-mediated toxicity. It has also been demonstrated *in vitro* that recombinant TNF stimulates the adherence of neutrophils to endothelium.[46] This is in substantial agreement with our histologic observations, which indicated that neutrophils and other leukocytes were marginated to normal and hypertrophied endothelium. Neutrophils are activated by recombinant TNF and γ-IFN singly and synergistically,[35] and this, coupled with their close proximity, could mediate endothelial cell injury. Recombinant TNF also stimulates release of collagenase and prostaglandin E_2 from fibroblasts and other cells,[47] which could contribute to PMN and platelet activation and result in the vascular injury observed histologically.

The extramedullary myeloproliferation observed following administration of rH TNF alone or in combination with rM γ-IFN may be in response to the necrotic and depressive effects of TNF on bone marrow. However, recombinant TNF also may have a direct growth-enhancing effect, as demonstrated with some nonmyeloid cell lines,[11] or may induce production/secretion of GM-CSF.[48]

The histopathology associated with the injection of rH TNF is reminiscent of other immune modulators, many, if not all, of which may induce TNF, suggesting that there may be a common mechanism of toxicity involving TNF. Because microemboli are a common feature of TNF toxicity, the observation that prostaglandin inhibitors such as aspirin and indomethacin significantly reduced toxicity suggests that the toxicity may be platelet-associated. It remains to be seen whether the decrease in coagulative parameters and toxicity is associated with a depression in therapeutic activity. The observation that a reduction of toxicity is associated with aspirin administration, regardless of its effect on therapeutic activity, will provide insight into the potential mechanisms of therapeutic activity for TNF. It is apparent that only part of the therapeutic activity of rH TNF is associated with direct cytostatic/cytotoxic activity. Thus, the therapeutic activity of rH TNF may be partially associated with its immunomodulatory properties or its direct or indirect effects on coagulation. Future studies on the effect of hypercoagulation on the therapeutic activity of TNF in combination with γ-IFN for both systemic and intradermal neoplastic disease (although the two types of studies may produce disparate results) should provide additional information concerning the mechanism of therapeutic activity. Furthermore, these preliminary results with aspirin suggest that a wide variety of other inhibitors and antagonists of coagulation–platelet aggregation may be able to reduce significantly the toxicity not only of TNF but also of other immunomodulatory agents, particularly those that induce TNF.

Bacterial endotoxin or lipopolysaccharide (LPS) is highly toxic to most mammals, most notably humans. When administered intravenously, a "shock" state termed endotoxin shock is induced which involves a multiorgan system failure. Fatality is generally associated with injury involving the lungs, kidneys, and gastrointestinal track "necrotizing enterocolitis."[40] This syndrome occasionally occurs in the course of invasive gram-negative infections and is associated with a high mortality. Research studies have suggested that endotoxin shock may be associated with the production of TNF, because passive immunization against TNF protects mice against the lethal effects of endotoxin.[49] Similarly, rats treated with TNF have been shown to have characteristic pulmonary and small bowel lesions reminiscent of endotoxin shock.[50] A number of studies suggest that platelet activating factor may have an important role in at least the ischemic bowl necrosis associated with endotoxin shock.[48,51] Indeed, leukotrienes have also been shown to have a role in the ischemic bowl necrosis. In these studies, treatment with indomethacin has not been shown to decrease the toxicity.[48,51] It appears, therefore, that TNF may have a role in endotoxin shock, although the mechanism of platelet involvement has yet to be completely clarified. Additional studies with platelet-aggregating factor antagonists and other antagonists of leukotriene and prostaglandin production are needed for full understanding of the mechanism of tissue injury.

In summary, the systematic preclinical study of immunomodulatory, immunotherapeutic, and toxicologic parameters of cytokines may provide data that will facilitate their utilization in the clinic. It remains to be determined whether such an approach will aid the transition of an agent such as rH TNF into clinical studies.

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