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Exacerbation of toxic effects by endotoxin contamination of recombinant human tumor necrosis factor

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Summary. The toxic effects of endotoxin-free human recombinant tumor necrosis factor (rH-TNF), shown to contain < 50 pg endotoxin/mg rH-TNF, were investigated and compared with those of rH-TNF and endotoxin coadministered at 4–400 ng endotoxin/mg rH-TNF in female Sprague-Dawley rats. The mean lethal dose of 5.9 mg/kg rH-TNF found for the endotoxin-free rH-TNF was far higher than that attributed to rH-TNF by other investigators. Coadministration with endotoxin derived from *E. Coli, Salmonella abortus equi,* or *Serratia marcescens* reduced the apparent mean lethal dose of rH-TNF in correspondence to the endotoxin concentration, with a value of 0.7 mg/kg rH-TNF observed at 1600 ng, 757 ng, and 5260 ng endotoxin/mg rH-TNF, respectively. Coadministration also resulted in more severe histopathologic and physicochemical effects than rH-TNF alone. Histopathologic abnormalities observed only in coadministration included interlobular edema and hemorrhage of the pancreas and, most remarkably, splenomegaly, which was not observed with rH-TNF alone even at lethal doses. The results indicate that particular care in determining endotoxin contamination is essential in any consideration of TNF toxicity.

Introduction

Tumor necrosis factor (TNF) is a cytokine produced mainly by macrophages whose wide range of biologic activities includes the modulation of adipocyte metabolism and induction of fever. Its most prominent feature is its ability to lyse tumor cells in vitro and induce hemorrhagic necrosis of certain tumors in vivo (from which its name was derived). Recombinant DNA techniques have enabled the production of large quantities of human TNF containing only trace amounts of contaminants, and its applicability to cancer therapy is now under investigation in phase I and II trials [3, 10].

A series of pioneering studies on TNF toxicity was begun in 1985 by Cerami and co-workers. Beutler et al. [2] reported that passive immunization resulted in reduced mortality in mice injected with lethal doses of endotoxin and suggested that TNF is an important mediator of the lethal effect of endotoxins. Following the injection of their own preparation of human recombinant TNF (rH-TNF) in rats, Tracey et al. [16] demonstrated the occurrence of many deleterious effects commonly associated with endotoxins and reported the mean lethal dose of rH-TNF to be 0.7 mg/kg. Reports by the same and other investigators [8, 12, 27] have also described acute toxic effects in mice and dogs following administration of TNF.

However, in reviewing the reports by Cerami and colleagues [2, 16], we noted that the endotoxin content of their rH-TNF preparations, described as < 0.4 μg/mg protein as assessed by *Limulus* amebocyte lysate tests, was apparently quite high. The threshold of endotoxin detection by the *Limulus* amebocyte assay is generally about 100 pg/ml, which translates to about 50 pg endotoxin/mg TNF at the TNF concentration of 2 mg/ml used in their studies. Moreover, in rH-TNF preparations used here and elsewhere, the endotoxin content is generally < 50 pg/mg protein.

A preliminary study on the lethality of our rH-TNF preparation in rats resulted in no deaths following the infusion of 2 mg/kg rH-TNF. We therefore conducted the present study to determine whether rH-TNF preparations containing only trace amounts of endotoxin (< 50 pg/mg protein) would produce in rats the toxic features reported by Cerami and colleagues, whether the addition of endotoxin to this preparation would alter these toxic effects, and how much additional endotoxin would be required to produce the severe toxic effects these authors described.

Materials and methods

Materials. rH-TNF was kindly provided by Asahi Chemical Ind. Co. Ltd. (Tokyo, Japan). Its specific activity, determined by L-M cell cytotoxicity assay, was 2.3×10^6 units/mg protein [9, 14]. Its endotoxin content, determined by *Limulus* amebocyte lysate assay, was < 50 pg/mg TNF. Infusion solutions were prepared by diluting rH-TNF with endotoxin-free vehicle solution (0.1% gelatin phosphate-buffered saline containing < 0.1 ng endotoxin/ml) or with vehicle solution containing endotoxin.

Endotoxin. Bacterial endotoxic lipopolysaccharides (LPS) from *E. coli* (0127:B8), *Salmonella abortus equi,* and *Serratia marcescens* were obtained from Difco Laboratories (Detroit, Mich). All endotoxins were dissolved and diluted with endotoxin-free saline. The endotoxin content in the infusion solutions was determined by *Limulus* amebocyte

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lysate assay (USP Bacterial Endotoxin Test, colorimetric method; Pyrodick, Seikagaku Kogyo Co, Ltd., Tokyo, Japan); 1 ng endotoxin/ml was equivalent to 1.1 Endotoxin units/ml based on the USP Endotoxin Reference Standard.

Animals. In assessing the acute toxicity of TNF, we attempted to follow as closely as possible the experimental procedures described by Tracey et al. [16]. We were particularly careful to exclude any conditions that might modify the pathophysiological state of the animals. Female Sprague-Dawley rats (specific pathogen-free, 9 weeks old) were obtained from Charles River Japan Inc. (Siga, Japan). They were maintained under observation on commercial pellets and water ad libitum in an air-conditioned room with a barrier system for 2 weeks to preclude the influence of latent infection on the results of the experiments and allow the animals to recover from stress caused by the change in environment. At the end of the observation period all rats were healthy, with body weights ranging from 210 to 250 g.

Administration of rH-TNF. The rH-TNF preparation (total volume, 0.5 ml) was infused into the rats via the tail vein in various doses over 5 min. Infusion was carried out at a constant rate with autoinfusion pumps (KN-201, Natusme Seisakusho Co., Tokyo, Japan). The control rats received 0.5 ml vehicle solution in the same manner.

Determination of mean lethal dose (LD_{50}) of rH-TNF. The LD_{50} was determined for both rH-TNF alone and rH-TNF with endotoxin at various doses. The animals were infused with the rH-TNF preparations and observed for 7 days. The LD_{50} was determined from the mortality rate at the end of the observation period according to the method of Behrens and Karber [1].

Histopathologic examinations. A preparation containing 0.5 or 2 mg/kg rH-TNF with trace amounts (< 50 pg) or 40, 200, or 400 ng endotoxin (*E. coli* 0127:B8)/mg rH-TNF was injected into rats separated into eight groups of six rats each. A preparation without rH-TNF containing trace amounts or 80, 400, or 800 ng/kg endotoxin was injected into rats separated into four groups of six rats each, the first group of which served as control. Furthermore, the effect of rH-TNF alone was investigated by the injection of a preparation containing 8 mg/kg rH-TNF in one group of rats ($n = 6$).

Autopsies were immediately carried out on all rats succumbing within 6 h after injection. Survivors were sacrificed 6 h after injection for histopathologic examinations. The kidneys, lungs, spleen, and thymus were weighed, and tissues from the gastrointestinal tract (GI), thyroid, pancreas, liver, spleen, thymus, kidneys, lungs, heart, adrenal, bone marrow, and brain were subjected to routine histopathologic examination.

Experiments on physicochemical changes. A preparation with 0.5 mg/kg rH-TNF and trace amounts or 40 or 200 ng endotoxin (*E. coli* 0127:B8)/mg rH-TNF or without rH-TNF and trace amounts or 20 or 100 ng/kg endotoxin was injected into rats separated into six groups of six rats each. No higher dose of rH-TNF was used, as mortality at doses $\geqslant 2$ mg/kg rH-TNF with endotoxin contamination had been observed in groups earlier in the study, thus precluding full-group evaluation of physicochemical changes.

At 1, 3, and 5 h after injection, the rats were anesthetized with ethyl ether and blood was collected from the abdominal aorta. The hematologic parameters examined included erythrocyte, leukocyte, and platelet counts, hemoglobin content, and hematocrit value. The blood chemical parameters examined included total protein, albumin, lactic dehydrogenase (LDH), creatine phosphokinase (CPK), glutanic oxalacetic transaminase (GOT), GPT, urea nitrogen, free fatty acid, total cholesterol, triglyceride, glucose, phospholipid, lactic acid, and sodium and potassium ions.

Results

Acute toxicity

rH-TNF without endotoxin. In all of the 45 rats shown in Table 1, which received uncontaminated rH-TNF at doses $\geqslant 1$ mg/kg, suppressed movement and tachypnea occurred soon after infusion; diarrhea was also observed in almost all of them. Bloody diarrhea was observed in four of the eight rats given 2 mg/kg rH-TNF, and even more frequently in rats given the two higher doses. However, none of the animals receiving doses of $\leqslant 2$ mg/kg died during the 7-day observation period. In the group receiving doses of 4 mg/kg, 4 of 15 rats died; 10 of 15 rats receiving doses of 8 mg/kg succumbed to rH-TNF infusion. All of these deaths occurred between 6 h and 48 h after infusion. Based on these results, the mean lethal dose was 5.9 mg/kg rH-TNF.

rH-TNF with E. coli endotoxin. The acute toxicity of the preparations containing both rH-TNF and endotoxin (*E. coli* 0127:B8 lipopolysaccharide) was assessed; the doses and results are shown in Table 2. The preparations contained rH-TNF at the appropriate concentrations for infusion of 0.5 or 2 mg/kg, with endotoxin added at 0, 4, 40, or 400 ng/mg rH-TNF. The preparations with no additional endotoxin and thus an endotoxin content of < 50 pg/mg rH-TNF were taken as controls. The rats were observed for 7 days following the infusion. None of the rats receiving rH-TNF preparations without endotoxin contamination died. Among the rats receiving rH-TNF at 0.5 mg/kg, only one death was recorded, in the group receiving preparation contaminated with endotoxin at 400 ng/mg rH-TNF. Mortality was higher in the groups receiving contaminated rH-TNF at 2 mg/kg rH-TNF, with death occurring in three of six rats receiving preparation contaminated with endotoxin at 400 ng/mg rH-TNF.

Influence of endotoxin contamination on LD_{50}. To examine the degree of endotoxin contamination sufficient to effect a reduction in the LD_{50} of rH-TNF to 0.7 mg/kg, the LD_{50} reported by Tracey et al. [16], preparations containing 0.7 mg/kg rH-TNF and endotoxin derived from *E. coli* 0127:B8, *Salmonella abortus equi,* or *Serratia marcescens* at 0, 100, 400, 1600, or 6400 ng/mg rH-TNF were infused and the recipient rats were observed for 7 days, as shown in Table 3. In three additional groups, each composed of seven rats receiving infusions of 4480 ng endotoxin in vehicle solution (the same as the highest dose given with rH-TNF) to assess the effect of each endotoxin without rH-TNF, no mortality or other toxic effect was observed.

Table 1. Mortality in rats following rH-TNF infusion

rH-TNF (mg/kg)	Number of deaths						Mortality
	0 h	4 h	8 h	1 day	2 days	3–7 days	
0	0	0	0	0	0	0	0/8
1	0	0	0	0	0	0	0/8
2	0	0	0	0	0	0	0/8
4	0	0	1	2	1	0	4/14
8	0	1	3	4	2	0	10/15

Determined by 7 days of observation following infusion of endotoxin-free rH-TNF or 0.5 ml vehicle solution (0.1% gelatin phosphate-buffered saline); values shown indicate the LD_{50} of 5.9 mg/kg rH-TNF as calculated by the method of Behrens and Karber [1]. No deaths occurred during the subsequent 7-day observation period

Table 2. Mortality in rats following infusion of rH-TNF with contamination by *E. coli* 0127 : B8 (endotoxin observation period as indicated in Table 1)

rH-TNF (mg/kg)	Endotoxin content (ng/mg rH-TNF)	Number of deaths					Mortality
		0 h	4 h	8 h	1 day	2–7 days	
0.5	0	0	0	0	0	0	0/6
	4	0	0	0	0	0	0/6
	40	0	0	0	0	0	0/6
	400	0	0	1	0	0	1/6
2	0	0	0	0	0	0	0/6
	4	0	0	0	1	0	1/6
	40	0	0	1	0	0	1/6
	400	0	0	3	0	0	3/6

Table 3. Mortality in rats following infusion of rH-TNF with contamination by endotoxin derived from *E. coli* 0127 : B8, *Salmonella abortus equi*, or *Serratia marcescens* (observation period as indicated in Table 1). Similar infusion of each endotoxin alone in seven rats at 4480 ng/kg (equivalent to 6400 ng/mg rH-TNF) resulted in no deaths

rH-TNF (mg/kg)	Endotoxin content (ng/mg rH-TNF)	Endotoxin		
		E.coli 0127 : B8	*S.abortus equi*	*S.marcescens*
0.7	0	1/14	–	–
	100	1/14	2/14	0/14
	400	2/14	3/14	1/14
	1600	7/14	10/14	1/14
	6400	14/14	14/14	8/14

Each of the endotoxins effectively reduced the LD_{50} of rH-TNF. Based on these results, the estimated concentrations effecting an LD_{50} of 0.7 mg/kg rH-TNF were 1600, 757, and 5260 ng/mg rH-TNF for the endotoxins from *E. coli* 0127:B8, *Salmonella abortus equi*, and *Serratia marcescens*, respectively. At the same dose of uncontaminated rH-TNF, only 1 of 14 rats died.

Histopathologic changes

Histopathologic examination revealed no significant changes in the control group receiving only the vehicle solution. The group receiving endotoxin alone at a dose of 800 ng/kg also showed no pathologic findings except hypertrophic changes of Kupffer's cells in the liver.

Several histopathologic abnormalities that appeared to be dose-dependent were noted in the rats receiving rH-TNF alone. Mucosal hyperemia and/or hemorrhage invariably occurred in rats receiving rH-TNF at $\geqslant 0.5$ mg/kg. In the groups receiving 2 or 8 mg/kg rH-TNF, hemorrhage in the large intestinte was observed in all cases; however, no necrotic changes in the GI tract were observed in the latter. Punctate bleeding and hydropic changes in the thyroid were frequently noted in these groups and were also dose-dependent. No significant changes in the lungs were observed except one case of punctate bleeding in a limited area. The kidneys were also apparently unaffected even at the highest rH-TNF dose, apart from slight congestive changes in a few cases.

Endotoxin had no significant effect when coadministered at a concentration of 40 ng/mg rH-TNF with 0.5 mg/kg rH-TNF, but at higher concentrations it resulted in more severe histopathologic changes than rH-TNF alone, the severity increasing with endotoxin content. Endotoxin-contaminated rH-TNF also induced several abnormalities that were not observed with rH-TNF alone, even at doses of 8 mg/kg; these included splenic congestion and hemorrhage, with a decrease in lymphocytes at the white pulp, and interlobular edema and hemorrhage in the pancreas. Among the groups receiving 2 mg/kg rH-TNF, the final mean spleen weight was 0.44 ± 0.02 g with no endotoxin contamination, 0.60 ± 0.07 g with 200 ng endotoxin/mg rH-TNF, and 0.84 ± 0.07 g with 400 ng endotoxin/mg rH-TNF, as shown in Fig. 1. Thrombus for-

Table 5. Blood chemistry following infusion of rH-TNF with or without contamination by *E. coli* endotoxin

Dosage		GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	Total protein (g/dl)	Albumin (g/dl)	Urea nitrogen (mg/dl)
rH-TNF (mg/kg)	Endotoxin (ng/mg TNF)						
0	0	100.9± 12.4	32.2± 5.1	300.5±119.8	7.89±0.17	3.83±0.06	11.9±1.4
0	200[a]	98.8± 21.4	34.1± 5.4	331.7±177.8	8.01±0.52	3.87±0.12	14.9±1.8**
0.5	0	155.9± 61.3	74.1±29.9**	496.0±267.5	7.34±0.29**	3.55±0.09**	18.5±3.4**
0.5	40	192.9±133.0	90.1±63.6	745.2±386.5*	7.10±0.37**	3.46±0.20**	22.0±4.1**
0.5	200	235.3±91.0**	87.0±26.6**	1366.3±375.8 **,****	6.76±0.34**,****	3.34±0.15**,***	27.6±1.8**,****

Determination of blood chemistry was carried out 1, 3 and 5 h after injection. For brevity, the mean ± SE for each group of six rats at 3 h after infusion is shown. Statistical significance: * ($P<0.05$) or ** ($P<0.01$) in comparisons with vehicle solution; *** ($P<0.05$) or **** ($P<0.01$) in comparisons with rH-TNF alone

[a] Equivalent to endotoxin dose at 200 ng/mg rH-TNF

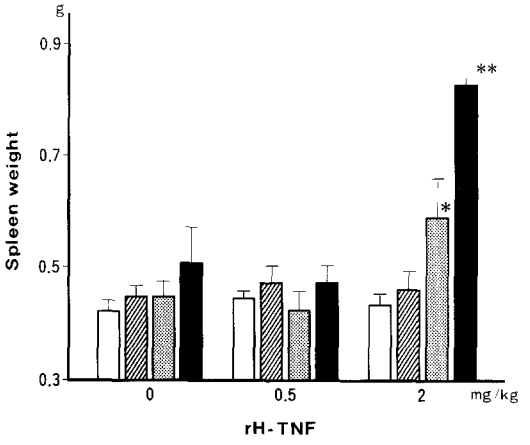

Fig. 1. Spleen weight following infusion of rH-TNF with or without contamination by *E. coli* endotoxin, determined 6 h after infusion or immediately after death. Infusion of endotoxin alone (shown at 0 mg rH-TNF/kg) was carried out at doses equivalent to those in the 2-mg TNF solution. Each column and bar represents the mean ±SE for six animals. Endotoxin contamination: *open column,* none; *hatched column,* 40 ng/mg TNF; *dotted column,* 200 ng/mg TNF; *closed column,* 400 ng/mg TNF. Statistical significance in comparison with the control (rH-TNF 0, endotoxin 0): * ($P<0.05$) or ** ($P<0.01$)

mation in various tissues was also frequently observed with the contamination, but only rarely in those animals receiving rH-TNF alone. The main trends for the findings are summarized in Table 4.

Physicochemical changes

As measured at 1, 3, and 5 h after infusion of rH-TNF alone, a gradual decrease in blood volume, total protein, and albumin was observed together with an increase in hematocrit value, which may be attributable to the extravasation of plasma into tissues. The GOT, GPT, LDH, and urea nitrogen levels gradually increased. The peripheral leukocyte count had dropped 1 h after infusion but returned to the original level at 5 h. The pCO_2 value had dropped at 1 h; it remained fairly constant thereafter, perhaps due to hyperventilation. CPK levels had risen at 1 h after infusion but returned to the original level at 3 h. Endotoxin contamination of rH-TNF preparations generally exacerbated these changes in a dose-dependent manner. Between the groups receiving rH-TNF alone and those receiving rH-TNF contaminated with 200 ng endotoxin/mg rH-TNF, statistically significant differences were noted in hematocrit, total protein, LDH, and urea nitrogen values (Fig. 2 and Table 5).

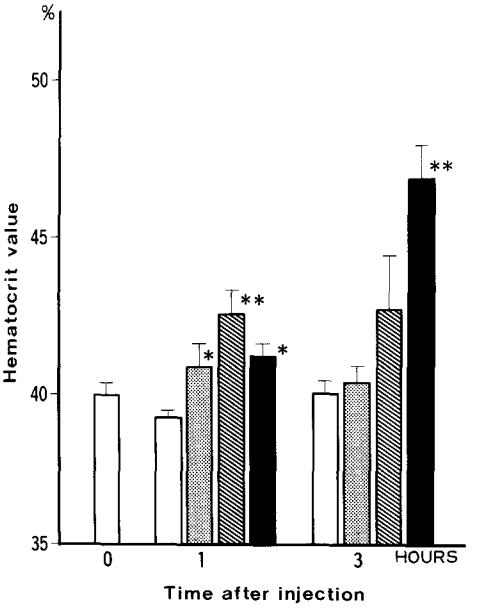

Fig. 2. Hematocrit following infusion of rH-TNF with or without contamination by E. coli endotoxin, determined 1 and 3 h after infusion. Each column and bar represents the mean ±SE for six animals. *Open column,* control (vehicle solution only); *dotted* column, rH-TNF without endotoxin; *hatched column,* rH-TNF with 40 ng endotoxin/mg TNF; and *closed column,* rH-TNF with 400 ng endotoxin/mg TNF. Endotoxin alone at doses equivalent to those at 200 ng endotoxin/mg TNF had no significant effect. Statistical significance: * ($P<0.05$); ** ($P<0.01$) in comparisons with the vehicle solution; ## ($P<0.01$) in comparisons with rH-TNF alone

Table 4. Histopathologic changes following infusion of rH-TNF with or without contamination by *E. coli* endotoxin

Organ and finding	rH-TNF (2 mg/kg)																		rH-TNF (8 mg/kg)					
	Endotoxin contents (ng/mg rH-TNF)																							
	0						200						400											
Animal number (d:dead animal)	1	2	3	4	5	6	1	2 d	3 d	4 d	5 d	6	1 d	2 d	3 d	4 d	5	6	1	2	3	4	5	6
Spleen																								
Decrease in lymphocytes at the white pulp	−	−	−	−	+	+	++	++	+	−	−	−	++++	+++	−	+	++	+	++	−	+	−	++	
Congestion/ hemorrhage	+	−	−	−	−	−	−	+++++		+++++		−	+++++++		++	+++++		−	−	−	−	−	−	+
Fibrin thrombi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Stomach																								
Congestion	−	±	−	−	−	−	−	±	±	−	+	−	±	+	+	+	−	−	−	−	−	−	−	−
Hemorrhage	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−
Fibrin thrombi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Jejunum																								
Congestion	−	−	−	−	−	−	−	+	+	+	−	−	−	+	±	+	−	−	−	−	−	−	−	−
Ileum																								
Congestion	±	−	±	−	−	−	−	±	+	+	+	−	±	+	±	+	−	−	−	−	−	−	−	−
Hemorrhage	−	−	−	−	−	−	−	−	±	+++	−	−	−	±	±	−	−	−	−	−	−	−	−	−
Cecum																								
Hemorrhage	+++++	++	++++++++		+	++	++	++++++++++		+	++++++++++													
Edema	++	+	++	++++++++++		+	+	+	++++++++++		+	−	++	++++++++++		+	+	+	++	++	++			
Erosin	−	−	−	++	−	−	−	+	−	−	−	−	−	−	+	+++++		−	−	−	−	++	+	−
Fibrin thrombi	+	−	−	+	−	−	−	+	−	−	−	+	−	−	+	−	++	+	+	+	−	−	−	−
Colon																								
Hemorrhage	+	−	+	+	−	−	±	+	+	−	−	−	±	±	++	±	−	NP	++	+	+	+	±	−
Congestion	+	−	+	−	−	−	±	+	+	−	−	−	+	±	−	−	−	NP	++	−	−	−	+	+
Fibrin thrombi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	NP	+	+	−	−	−	−
Rectum																								
Hemorrhage	+	−	++	+	−	++	+	++	+	++++		−	−	±	−	+++++		+	−	−	+	+	−	−
Congestion	+	−	−	+	−	−	−	−	±	−	−	−	±	±	±	−	−	+	−	±	−	−	+	+
Edema	−	−	+	−	−	+	−	−	−	+++	+	−	−	−	−	++	+	+	−	−	−	−	−	++
Pancreas																								
Interlobular edema	−	−	−	−	+	+	+	+	+	++	+	++	+	+	−	−	++++		−	−	−	−	−	−
Hemorrhage	−	−	−	−	−	−	−	−	±	+	−	−	±	−	−	+++	−	++	−	−	−	−	−	−
Fibrin thrombi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
Thyroid																								
Hemorrhage	++	±	++	++	++	+	++	++	++	+++++		++	++	++++++++++			++	−	−	++	++	++	++	
Fibrin microthrombi	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mesenteric lymph node																								
Blood resorption	−	−	−	−	−	−	+	+	+	−	−	−	−	−	±	−	−	−	−	−	±	−	−	−
Hemorrhage	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Congestion	−	−	−	−	−	−	−	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−

Histopathologic examinations were carried out immediately after death or 6 h after infusion. Control groups receiving vehicle solution alone or endotoxin without rH-TNF showed no significant abnormalities. Findings are presented as: −, normal; ±, slight; +, mild; ++, moderate; +++, severe; NP, no preparation given

Discussion

In the present study, we first attempted accurately to determine the acute toxicity of rH-TNF in rats. The LD_{50} value of our rH-TNF preparation was found to be 5.9 mg/kg, far higher than the value of 0.7 mg reported by Tracey et al. under similar experimental conditions. This difference may be of clinical importance, since therapeutic doses are often estimated from acute toxicity tests in animal models. Although these results are not directly applicable to the determination of therapeutic doses, they clearly imply that rH-TNF can more safely be given than has commonly been presumed on the basis of earlier studies.

Uncontaminated rH-TNF induced various histopathologic changes in rats in a dose-dependent manner. Lesions were mostly confined to the GI tract, apart from hemorrhage in the thyroid and adrenals. Our findings are in good accord with those of Remick et al. [12], who found no alterations in organs of mice injected with rH-TNF except in the bowels.

None of the renal distension, necrotic lesions of the bowel, or pulmonary hemorrhage previously reported by

Tracey et al. [16] were induced by our rH-TNF preparations, even at the dose at which two-thirds of the animals succumbed. In light of the observed physicochemical changes and microscopically unimpaired internal organs, we suggest that the lethal effect of rH-TNF is largely attributable to circulation collapse due to the extravasation of plasma into tissues and intestinal lumens.

The only major difference between the experiment of Tracey et al. [16] and our own for the determination of the LD_{50} of rH-TNF appeared to be the degree of endotoxin contamination in the rH-TNF preparations. To investigate this difference, we added endotoxin derived from *E. coli* to our rH-TNF preparation and assessed its effect on toxicity. The results suggested a dose-dependent increase in mortality; at a dose of 2 mg/kg rH-TNF, contamination at 400 ng endotoxin/mg rH-TNF resulted in approximately 50% mortality, whereas no deaths were observed in the absence of endotoxin contamination. For the *E. coli* and two other endotoxins, we found that the apparent LD_{50} value of rH-TNF was effectively reduced to 0.7 mg/kg by endotoxin at 757–5260 ng endotoxin/mg rH-TNF. The findings thus suggest that the low LD_{50} values in the earlier studies may largely, if not entirely, be attributed to a comparatively high endotoxin content.

Endotoxin also appeared to exacerbate the histopathologic and physicochemical abnormalities induced by rH-TNF. In addition, two other effects specific to endotoxin-contaminated rH-TNF were observed. The spleen weight increased significantly in the animals receiving contaminated rH-TNF but not in those receiving rH-TNF alone, even at the highest concentration (8 mg/kg). Prominent interlobular edema and hemorrhage in the pancreas were also observed only with endotoxin contamination. In contrast to the findings of Tracey et al. [16], however, no significant changes were observed in lungs and kidneys, even with the highest dose of contaminated rH-TNF, at which $>50\%$ of the recipients rats succumbed. The reason for this discrepancy remains to be resolved. An apparent enhancement of rH-TNF toxicity by endotoxin has also been noted by Bloksma and Hofhuis [4]. However, to the best of our knowledge, our report is the first to demonstrate both qualitative and quantitative differences between the effects of pure rH-TNF and those of the combination of rH-TNF and endotoxin.

Endotoxin given in vivo is known to induce a group of proteins now referred to as Biological Response Modifiers (BRMs), which include TNF and interleukins. These BRMs regulate the production and potentiate the effects of one another in a highly complex network of bioregulation, one result of which being that endotoxin-associated effects generally cannot be predicted from the presence or activity of any one BRM alone but can be explained only by the participation of multiple factors [11]. In this light, we suggest that endotoxin in conjunction with rH-TNF induces the production of certain factors that modify the effect of rH-TNF, thus leading to the observed reduction in LD_{50} and different histopathologic findings. Interferons and interleukin-1 are reportedly synergistic to TNF [6, 15], and the possibility of their involvement in these effects is now under investigation.

The pioneering work of Cerami and co-workers [2, 16] has established that TNF plays an important role in endotoxin-induced shock. However, this appears to have led to a widespread belief that TNF is responsible for most, if not all, of the deleterious effects associated with endotoxin. For several reasons, this would appear to be an oversimplification, as it neglects a variety of BRMs and other factors induced by endotoxin. First, it may be noted that endotoxin-induced production of TNF in substantial amounts has been observed only in well-planned experiments that included appropriate priming procedures [5, 7]. Ordinarily, even lethal doses of endotoxin fail to induce the production of rH-TNF in an amount sufficient for the latter alone to cause severe toxicity. Second, in infectious diseases and septic shock, TNF is either undetectable or present only in amounts for below the levels observed in cancer patients treated with rH-TNF infusion, who nevertheless manifest no major rH-TNF side effects [13, 19]. Finally, our present findings demonstrate that even high doses of rH-TNF fail to result in many of the features of endotoxin-induced shock and that the copresence of endotoxin is necessary for the occurrence of a rapid lethal effect. We suggest that some factors other than rH-TNF, acting either independently or in conjunction with the latter, play a pivotal role as mediators of the deleterious effects of endotoxin.

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