TOLERANCE TO TUMOR NECROSIS FACTOR IN RATS AND THE RELATIONSHIP TO ENDOTOXIN TOLERANCE AND TOXICITY

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The development of endotoxin tolerance by daily administration of nonlethal doses of endotoxin is well described in humans and experimental animals (1). Tolerance can be induced to the lethal (2), metabolic (3), and pyrogenic (4) effects of endotoxin. The mechanism of endotoxin tolerance is unclear, but recent studies suggest a prominent role of modulation of macrophage function. Macrophages from endotoxin tolerant animals exhibit a decreased production of prostaglandins (5) as well as decreased production of lipoprotein-lipase suppressor activity (6).

Another recent series of investigations has convincingly demonstrated that TNF, a monokine secreted by macrophages in response to endotoxin and other stimuli, plays a prominent role in the toxic and lethal effects of septicemia and endotoxin (7–10). Intravenous administration of human rTNF to rats (8) and dogs (9) simulates the hemodynamic, metabolic, and hormonal derangements of septic shock. Passive immunization of mice with polyvalent anti-TNF antibodies before administration of a lethal dose of endotoxin significantly improves survival (10), and treatment with mAbs to TNF in baboons prevents death from a lethal injection of bacteria (11).

Our laboratory (12) and others (13) have noted tolerance to the anorectic effects of TNF with repetitive administration. Because of the prominent role of TNF in endotoxin toxicity and the suggestion of tolerance to repeated doses of TNF similar to endotoxin tolerance, we evaluated the phenomenon of TNF tolerance in rats and the interrelationship between endotoxin and TNF tolerance.

Materials and Methods

Animals. Male Fischer 344 rats (150–200 g) were individually housed in a light- and humidity-controlled environment with a 12-h light/dark cycle. All rats were fed C-21 (ICN Pharmaceuticals Inc., Cleveland, OH), a casein-based semi-synthetic diet that is non-scatterable and allows accurate measurement of food intake (14).

Reagents. Human rTNF was the generous gift of the Cetus Corp., Emeryville, CA, and had a sp act of 10^7 U/mg as measured in the L929 bioassay and an endotoxin level of 30 pg/2.5 × 10^6 U as measured by a standard limulus assay (15). TNF was reconstituted with PBS/0.5% BSA immediately before administration to rats in all experiments. LPS (*Esche-*

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richia coli 0127:R8; Sigma Chemical Co., St. Louis, MO) was reconstituted with sterile normal saline on the day of use. In neutralizing antibody experiments, a highly purified preparation of polyvalent rabbit anti-recombinant human TNF IgG antibody (Endogen, Boston, MA) was used.

Nitrogen Balance Studies. In nitrogen balance studies, the rats were housed individually in metabolic cages allowing daily collection of both urine and stool. Urine was collected in a container with 1 ml of concentrated sulphuric acid. Urine nitrogen was quantitated by chemoluminescence (Antek Instruments Inc., Houston, TX). Aliquots of stool were analyzed in a similar fashion. Nitrogen intake was calculated from the daily food intake of C-21, which has a known nitrogen content of 40.6 mg nitrogen per g C-21.

ELISA for Anti-TNF Antibodies. Human rTNF was diluted to 10 μg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and 100 μl/well was plated on 96-well Immulon microelisa plates (Dynatech Laboratories, Inc., Alexandria, VA) and kept at 4°C for 16 h. The plates were washed three times with PBS/0.05% Tween-20 and blocked for 60 min with PBS/1% BSA/0.05% Tween-20 blocking solution. The plates were washed, and 200 μl of serial dilutions of rat sera was added for 120 min. Each sample was assayed in triplicate. The plates were washed again, and 200 μl of a 1:1,000 dilution of goat anti-rat IgG alkaline phosphatase (Sigma Chemical Co.) or a 1:800 dilution of sheep anti-rat IgM alkaline phosphatase (Serotic, Indianapolis, IN) was added to each well for 120 min. The plates were washed, and 175 μl of 1 mg/ml of *p*-nitrophenyl phosphate (Sigma Chemical Co.) was added. After 45 min, the reaction was stopped with 50 μl of 3 M NaOH. The plates were counted at 405 nm in a microelisa reader (Dynatech Laboratories, Inc.). Sera from rats immunized by intradermal injections of TNF in Freund's complete adjuvant were analyzed in each assay as positive controls.

L929 TNF Bioassay. A standard cytotoxicity assay for TNF activity using a TNF-sensitive L929 cell line was performed (16). Briefly, 100 μl of 2.5×10^5 cells/ml suspension of L929 cells in RPMI/10% FCS complete media was plated on 96-well plates (Costar Data Packaging Corp., Cambridge, MA) and cultured overnight to establish a monolayer. On parallel plates, serial dilution of sera samples were made in complete media with 1.5 μg/ml actinomycin-D (Merck Sharpe & Dohme, West Point, PA) and 100 μl of this solution was transferred to plates with the L929 cells giving a final concentration of 0.75 μg/ml of actinomycin-D. After 18 h, the surviving cells were fixed, stained with 0.5% crystal violet (Sigma Chemical Co.), and the plates were counted in a titertek at 590 nm. 1 U of activity was defined as the reciprocal of the dilution required to produce a 50% decrease in absorbance relative to control cells exposed to actinomycin alone. Each sample was assayed in triplicate. In assays using a neutralizing antibody, samples were mixed with a 1:1 vol of a 1:100 dilution of polyvalent anti-human TNF antibody for 2 h at 4°C before analysis in the L929 bioassay. These assays were performed in parallel with samples treated with control nonimmune rabbit serum (Endogen).

Pathology. In pathologic studies, rats were anesthetized with intraperitoneal pentobarbital, and a segment of small intestine immediately proximal to the cecum and the liver was quickly excised and placed in 10% formaldehyde. The lungs were then excised and irrigated gently by tracheal cannulation with 5 ml of 10% formaldehyde and then placed in a vial with 10% formaldehyde. Standard hematoxylin and eosin slides were prepared (American Histo Labs, Rockville, MD), and the slides were read in the blinded fashion by a pathologist (M. J. Merino).

Statistics. Data are presented as mean ± SEM unless otherwise stated. Parametric results are analyzed by independent student's *t* test and nonparametric by Wilcoxon rank sum test. Survival comparisons are made by Fisher's exact test.

Results

The metabolic and nutritional effects of repeated doses of TNF were evaluated in Fischer 344 rats. Human rTNF administered twice daily intraperitoneally at a dose of 100 μg/kg led to a decrease in daily food intake from 11.5 to 3.7 g (a 68% decrease), a decrease in daily body weight change from 4.6 to −6.0 g per day (Fig.

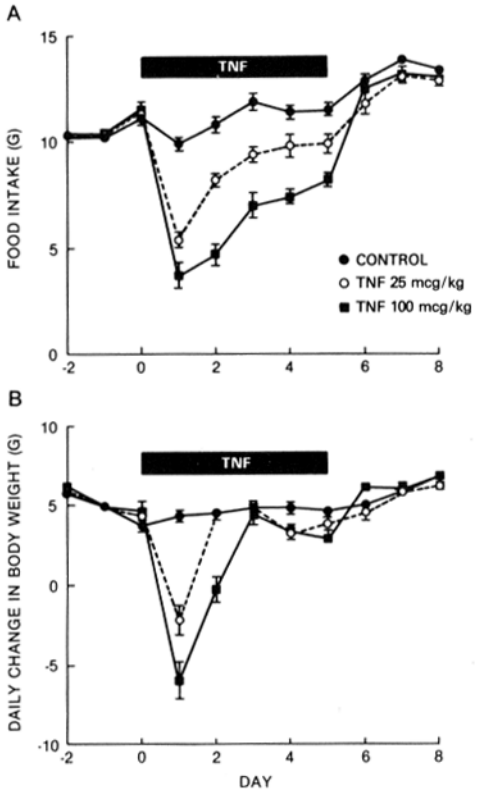

FIGURE 1. Fischer 344 rats (150–175 g) were individually housed and fed a diet of C-21. After a period of standardization of food intake and body weight, rats were randomized to receive either twice daily intraperitoneal carrier buffer (n = 20) or TNF twice daily at a dose of 25 μg/kg (n = 18) or 100 μg/kg (n = 17). The treatment period was 5 consecutive days. Daily food intake (*A*) and body weight change (*B*) were measured. Data are mean ± SE.

1, *A* and *B*), and negative daily nitrogen balance from 221 mg/d to −24 mg/d (Table I). However, continued twice daily administration of TNF at the same dose led to a reversal of these nutritional alterations. By the third day of treatment, the food intake doubled from 3.7 to 7.0 g, and the daily nitrogen balance and the daily change in body weight became positive at 96 mg of nitrogen per day and 4.4 g per day, respectively, despite continued TNF administration. Further treatment with TNF at the same dose led to continued recovery of food intake and nitrogen balance (Fig. 1 and Table I).

The initial alterations in food intake and body weight change were dose dependent, and the rate of recovery was also proportional to the dose of TNF administered. For example, at low-dose TNF (25 μg/kg twice daily) food intake decreased to 5.4 g during the first day, but increased to 8.2 g during the second day (a 50% recovery). In the high-dose TNF (100 μg/kg twice daily) group, food intake for the first 2 d was 3.7 and 4.7 g (a 27% recovery). Similarly body weight change in the low-dose group dropped initially to a loss of 2.2 g for the first day, but recovered to control levels of a 4.4 g per day gain in weight by the second day. In the high-dose group, the rats were still losing weight at −0.6 g per day during the second day. A tolerant state in terms of food intake and body weight change (defined as levels not different from control rats) was reached by 2–3 d of treatment in the low-dose group, but was not reached at 5 d of treatment in high-dose TNF group (Fig. 1).

TABLE I

Nitrogen Balance in Rats Treated with TNF for 5 Consecutive Days vs. Control

Day-of TNF treatment	Nitrogen balance (N/day)	
	Control	Cachectin
	mg	
1	213 ± 60	−23 ± 26*
2	206 ± 34	−26 ± 21*
3	319 ± 32	96 ± 30*
4	256 ± 32	150 ± 36*
5	273 ± 31	207 ± 20‡

10 growing male Fischer 344 rats (150–175 g) were housed individually in metabolic cages. The TNF group received human TNF (100 μg/kg, i.p.) twice daily (n = 5), while control rats had saline injections (n = 5). Animals were fed a C-21 diet. Urines were collected and analyzed as described in Materials and Methods. Nitrogen balance was recorded as nitrogen in from food intake minus nitrogen excreted in urine. Stool nitrogen was determined in selected rats and found to contribute <5% to total daily nitrogen loss and was not included in the calculations.

* $p < 0.05$ vs. control.

‡ $p < 0.05$ vs. TNF during days 1 and 2.

This observed tolerance to the anorectic effects of human TNF in rats also protected these animals against a subsequent challenge with a lethal dose of intravenous TNF (Table II). Rats receiving twice daily TNF at the high dose of 100 μg/kg for 5 d had a 100% survival when challenged 2 d later with an intravenous dose of TNF that killed 50% of control rats ($p < 0.05$). Tolerance to TNF toxicity also protected rats from a lethal dose of endotoxin. Rats pretreated with high-dose TNF had an 88% survival when challenged 2 d later with intravenous endotoxin compared with

TABLE II

Survival in TNF or Endotoxin-tolerant Rats Challenged with Intravenous TNF or Endotoxin

Tolerizing agent	Challenge agent	72-h survival
		%
Control (saline)	TNF	4/8 (50)
TNF	TNF	8/8 (100)*
LPS	TNF	9/9 (100)*
Control (saline)	LPS	1/16 (6)
TNF	LPS	14/16 (88)‡
LPS	LPS	10/10 (100)‡

Rats were tolerized to TNF by twice daily intraperitoneal injections of 100 μg/kg TNF for 5 consecutive days. Rats were tolerized to LPS by daily injection of 1 mg/kg LPS for 4 consecutive days. Control rats in each group received twice daily injections of carrier buffer. Rats were challenged with either 10 mg/kg LPS or 200 μg/kg TNF i.v. by tail vein injection 2 d after the final tolerization day. Survival was followed for 72 h.

* $p < 0.05$ vs. control/TNF challenge.

‡ $p < 0.01$ vs. control/LPS challenge.

TABLE III

Determination of Dose Effect, Length of Tolerization, and Duration of Tolerance Effect in TNF-tolerant Rats Challenged with Intraperitoneal Endotoxin

Days of TNF treatment	Day of endotoxin challenge	72-h survival		
			TNF	
		Control	25 μg/kg	100 μg/kg
		%	%	%
3	5	22/50 (44)	6/10 (60)	34/40 (85)*
5	9	6/18 (33)	3/9 (33)	9/9 (100)*‡
5	21	10/16 (62)	7/9 (78)	5/8 (62)

The two treatment groups received human TNF at doses of 25 μg/kg or 100 μg/kg i.p. twice daily for the number of days indicated in the left hand column. The control group received an injection of buffer intraperitoneally twice daily. Daily food intake and body weight were measured throughout the experiment to document tolerance. At the day indicated in the table (with day 1 being the first day of TNF treatment), rats received a single LD_{50} dose of 5 mg/kg endotoxin i.p. Survival is reported at 72 h after endotoxin challenge.

* $p < 0.005$ vs. control.

‡ $p < 0.01$ vs. 25 μg/kg dose.

a 6% survival in control rats challenged with the same endotoxin dose ($p < 0.01$). The interrelationship between TNF and endotoxin tolerance was further supported by the observation that endotoxin-tolerant animals are also protected against an LD_{50} dose of TNF (100% survival vs. 50% survival, $p < 0.05$) (Table II).

Characteristics of the TNF-induced endotoxin tolerant state were examined by varying the duration of TNF pretreatment, the dose of TNF used, and the timing of subsequent endotoxin challenge (Table III). TNF-induced tolerization to endotoxin is dose dependent as low-dose (25 μg/kg) twice daily TNF injections failed to provide protection against an LD_{50} dose of endotoxin in two different tolerization regimens in which a dose of 100 μg/kg was efficacious. A tolerization time period of 3 d of twice daily 100 μg/kg injections was able to protect rats against a lethal dose of endotoxin given 2 d later. Duration of TNF-induced tolerance to endotoxin was limited. Rats were protected against endotoxin challenge 2 d (Table II and III) and 4 d (Table III) after the last day of TNF tolerization, but the protective effects were not seen at 16 d after tolerization. In the latter instance, survival to endotoxin challenge was equal in both TNF-pretreated and control groups (Table III).

The protective effects of high-dose TNF pretreatment or TNF tolerance on survival to lethal doses of endotoxin also translated to clear improvement of histopathologic alterations of endotoxemia. The histologic changes present in control animals 8 h after high-dose endotoxin are partially or completely reversed in rats that were tolerant to TNF. The severe pneumonitis and edema in the lungs (Fig. 2, *a* and *b*), the hepatic necrosis (Fig. 2, *c* and *d*), and the ischemic necrosis of the small bowel (Fig. 2, *e* and *f*) are all improved dramatically in TNF-pretreated rats. Normalization of the severe histopathologic changes that occur after a single lethal dose of TNF to control animals were also observed in rats who were tolerant to TNF (data not shown).

One possible explanation for the mechanism of TNF tolerance is that it is an artifact of alterations in the pharmacokinetics of TNF after the multiple injections

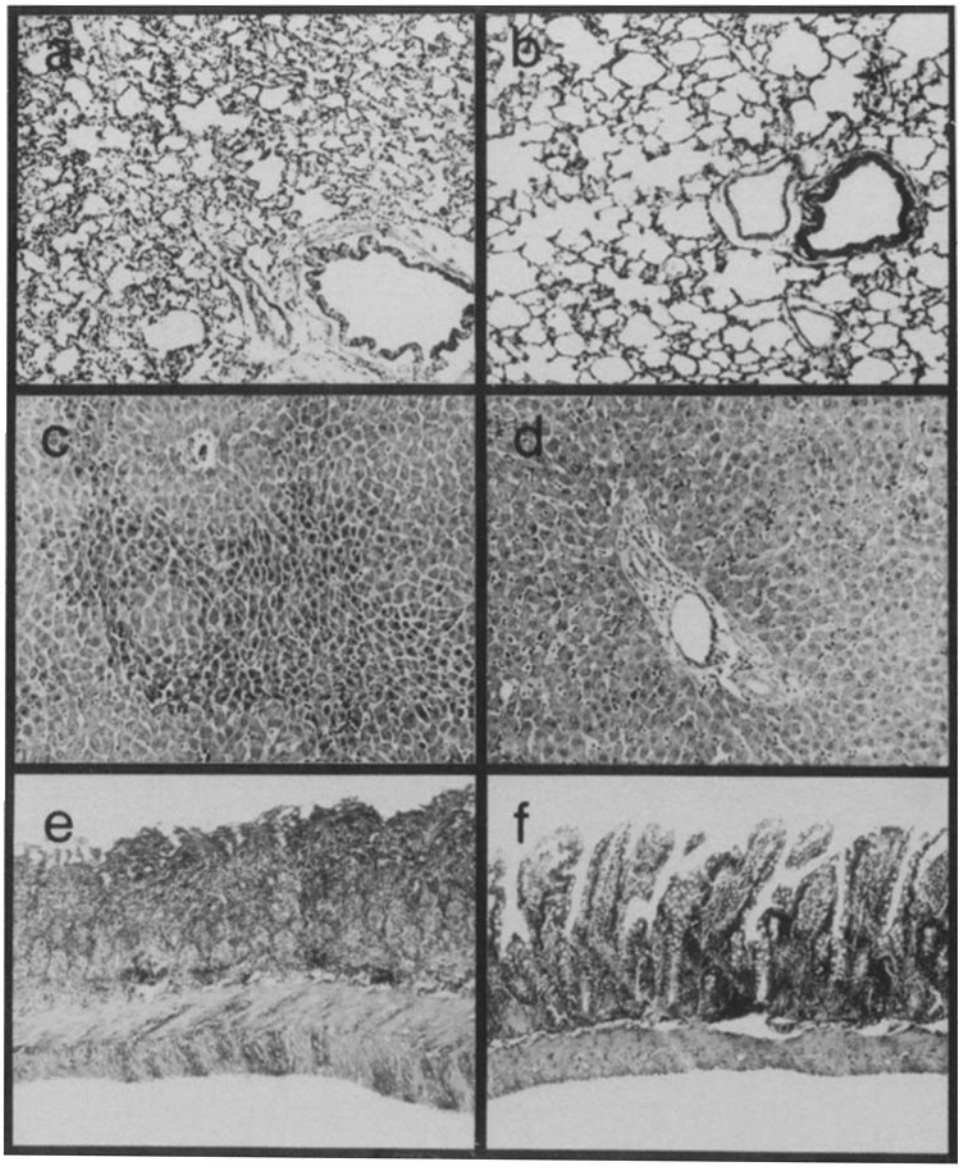

FIGURE 2. Photomicrographs of H & E slides from specimens of a rat made tolerant to TNF (*b*, *d*, and *f*) by twice daily 100 μg/kg i.p. injection of TNF or a control rat (*a*, *c*, and *e*). Specimens were obtained as described in the text 8 h after intravenous injection of 10 mg/kg of endotoxin. Lung control (× 65) (*a*), TNF tolerant (*b*); liver control (× 80) (*c*), TNF tolerant (*d*); ileum control (× 35) (*e*) and TNF tolerant (*f*).

during the tolerizing period. Repeated intraperitoneal injections of TNF might alter the absorption of subsequent intraperitoneal administration of TNF, or it might alter clearance or degradation of intravenously injected TNF. To evaluate these hypotheses, circulating TNF activity was assayed in TNF-tolerant and control rats. The level of TNF activity in the sera after intraperitoneal injection of 200 μg/kg of TNF

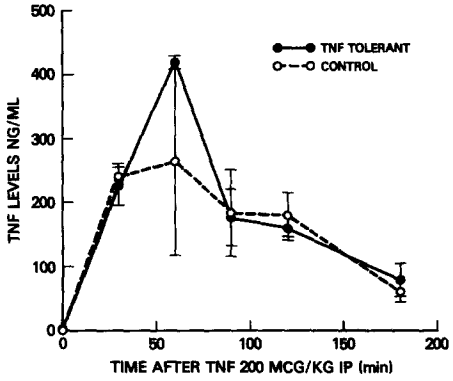

FIGURE 3. TNF tolerant rats (n = 6) were treated twice daily TNF at 100 μg/kg i.p. for 5 d. Control rats (n = 6) received saline injections. 1 d after the tolerization period, all rats received 200 μg/kg TNF i.p. Serial blood samples were assayed for TNF activity in the L929 bioassay at the times shown. A standard curve using rTNF was run with each assay to convert U/ml to ng/ml. Data are given as mean ± SE. There are no differences in TNF levels between TNF-tolerant and control rats at any time point.

in TNF-tolerant rats was not significantly different from the level in control rats (Fig. 3). Also, peak levels and clearance after intravenous injection of 200 μg/kg of TNF were not different between the two groups (Fig. 4). In both experiments, there was no residual circulating TNF activity in the TNF-pretreated groups at time zero, before the injection of 200 μg/kg of TNF. Parallel assays with the sample pretreated with a neutralizing antibody to human TNF at a 1:200 dilution eliminated all measurable activity (levels <10 pg/ml in the presence of the neutralizing antibody, data not shown) at every data point in both tolerant and control animals, confirming that the bioassays were measuring the injected human rTNF.

In another group of rats, the magnitude and time course of the humoral immune response to the intraperitoneal TNF tolerization regimen was evaluated. Rats tolerized to TNF at 100 μg/kg, twice daily for 5 d, had blood drawn on day 9 or 4 d after the last tolerization dose. A second group of rats tolerized in a similar manner had blood drawn on day 21 or 16 d after the last tolerization dose. Rats treated in this manner were shown to be refractory to a lethal dose of TNF at day 9 but not at day 21 (Table III). Titers of circulating anti-human TNF IgM and IgG antibodies were measured. At day 9, there was essentially no measurable circulating IgG or IgM to human TNF (Table IV). By day 21, low titers of IgG antibodies were present, but dilutions of only 1:25 gave <1/2 maximal absorbance in the ELISA.

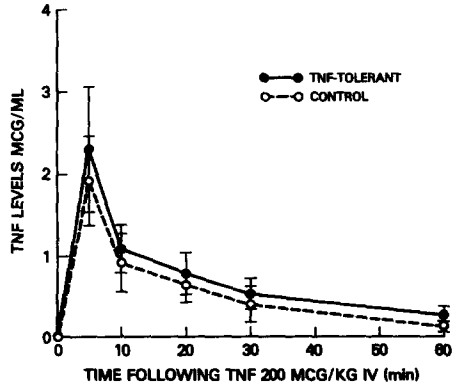

FIGURE 4. TNF-tolerant rats and control rats were treated identical to the experiment in Fig. 3 except the 200 μg/kg dose of TNF was given intravenously. The TNF activity was determined by the L929 bioassay. n = 6 in both groups. Data are as mean ± SE. There are no differences in TNF levels between TNF-tolerant and control rats at any time point.

TABLE IV

Levels of IgM or IgG Rat Anti-human TNF Antibodies after Tolerization of Rats to TNF

Day of sample	Antibody class	OD			
		TNF tolerant		Control	
		1:25	1:100	1:25	1:100
9	IgM	0.11 ± 0.06	0.05 ± 0.02	0.06 ± 0.06	0.03 ± 0.02
9	IgG	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.02	0.07 ± 0.02
21	IgG	0.85 ± 0.66*	0.57 ± 0.76*	0.10 ± 0.01	0.04 ± 0.01

F344 rats were tolerized to TNF by twice daily intraperitoneal injections of 100 μg/kg TNF for 5 consecutive days (days 1-5). On days 9 and 21, blood was obtained by retro-orbital puncture, and sera were assayed for anti-TNF antibodies. The data shown are the mean absorbance ± SE for the ELISA as described in Materials and Methods. $n = 10$ for each group.

* $p < 0.05$ vs. control.

Rats immunized with TNF in FCA and boosted three times with TNF in IFA were used as a positive control for all ELISA. Titers of >1:12,400 were present in these immunized rats. Therefore, human rTNF is antigenic for rats, but administration by repeated intraperitoneal injections provokes only a minimal response. The presence of low levels of anti-human TNF antibodies does not correlate with the TNF tolerant-induced resistance to a lethal dose of endotoxin.

Discussion

Repeated intraperitoneal administration of twice daily nonlethal doses of recombinant human cachectin/TNF for 3-5 d to rats makes these animals refractory to the usual toxic effects of this monokine. The initial alteration of nutritional parameters including food intake, body weight change, and nitrogen balance all normalize despite continued administration of the same dose of TNF. Similarly, the lethality of TNF is abrogated by TNF pretreatment with nonlethal doses. This TNF-tolerant condition also clearly translates into protection against the toxicity and lethality of endotoxin. TNF-induced tolerance to endotoxin can develop with only 3 d of TNF pretreatment, is dependent upon the dose of TNF used, and is present for at least 2-4 d after pretreatment, but dissipates by 2 wk.

The mechanism of this TNF tolerance is unclear. One explanation for the early recovery of nutritional parameters is that repetitive intraperitoneal injections of TNF alters absorbance of subsequent doses so that the actual delivered dose of TNF decreases during the tolerization period. However, as can be seen in Fig. 3, the blood levels of TNF activity after 200 μg/kg of TNF are equivalent in TNF-tolerant and control animals. Also, the tolerant state is not due to rapid clearance or degradation of TNF as the peak bioactivity in tolerant rats is not lower nor is the TNF cleared faster than in control rats after injection of the same intravenous dose (Fig. 4). Therefore, the TNF-tolerant animals demonstrate no toxicity to TNF despite equivalent levels of circulating TNF bioactivity.

Another possible mechanism for TNF tolerization is a humoral immune response. Passive immunization with anti-TNF antibodies has been shown to be protective against endogenous TNF in endotoxemia (10) and bacteremia (11). However, several factors argue against the tolerance phenomenon described in the present study as

an active immunization of rats to TNF. First, the time course of the response with improved nutritional parameters by 2 d is much faster than would be expected for a primary immune response. Second, levels of circulating anti-TNF antibodies are unmeasurable at the time when rats are TNF tolerant, and are present at low titers when the TNF tolerance has dissipated (Table IV). Finally, the effects of TNF across species barriers (17), and particularly the ability of anti-TNF antibodies to recognize xenogenic TNF, is low (18), so that antibodies to injected human TNF may afford minimal protection against endogenous rat TNF.

Therefore, the mechanism of TNF tolerance does not appear to be an artifact of TNF delivery or a primary humoral immune response. Possible alternative explanations include down regulation of number or affinity of specific cell surface receptors that are known to be present on a variety of normal cells (19) that may be direct targets of TNF toxicity. Also, the cellular immune responses to TNF may be altered to a refractory condition by repeated TNF stimulation.

The relationship of TNF tolerance to the well-described phenomenon of endotoxin tolerance is a new observation, and its mechanism is not clearly defined. In the rat, the endotoxin-tolerant condition protects against a lethal TNF challenge as well as a lethal endotoxin challenge, and similarly a TNF-tolerant condition protects against both TNF and endotoxin. The mechanism of endotoxin tolerance is thought to involve a decreased responsiveness of host macrophages to endotoxin stimuli [1, 5]. As macrophages express specific TNF receptors (20) and are thought to be the predominant source of endogenous TNF (7), modulation of macrophage activity may explain both endotoxin and TNF tolerance. However, the hypothesis that endotoxin-tolerant macrophages are functionally depleted in terms of monokine secretion and other responses to stimuli does not explain why endotoxin-tolerant rats are resistant to a lethal dose of TNF unless TNF toxicity is mediated primarily by a secondary response via the macrophages.

TNF tolerance may have relevance to two areas of clinical medicine. It is well recognized that TNF produces a wide range of effects on several different cell types (7, 21), but when present in excess amounts in endotoxemia or bacteremia, it clearly leads to severe toxicity and death (10, 11). Also, TNF may play a role in the pathogenesis of cancer cachexia (22). Therefore, any mechanism to modulate the toxic effects of TNF, such as induction of tolerance, may potentially improve survival and outcome in both sepsis and cancer cachexia. Another clinically relevant application of TNF tolerance is in the potential use of TNF as an anti-neoplastic agent. In animal studies, TNF has had profound anti-tumor effects in animal models (23), and several centers have administered rTNF as an experimental therapeutic agent to tumors often involving multiple dosing schedules (24). If the tolerance phenomenon observed in rats also occurs in humans, a full understanding of the kinetics and degree of tolerance may alter dosing schedules to optimize therapeutic efficacy and minimize toxicity. Further studies are necessary to find the precise mechanism of TNF tolerance and to define the application of TNF tolerance to sepsis, cancer cachexia, and anti-neoplastic treatment.

Summary

Treatment of rats with recombinant human TNF initially causes a marked decrease in food intake, a loss of body weight, and a negative nitrogen balance. These alterations normalize with continued twice daily intraperitoneal injections of the same

dose. Rats tolerized to TNF in this manner are refractory to a lethal dose of TNF. Also, TNF-pretreated and -tolerized rats have prolonged survival and reversed histopathologic changes after injection of a lethal dose of endotoxin compared with control animals. The TNF-tolerant state is dependent on the dose of TNF used and the length of TNF pretreatment. TNF-induced tolerance is relatively short lived, being present 2–4 d after TNF pretreatment and dissipating by 2 wk. Rats made tolerant to endotoxin are also tolerant to a lethal dose of TNF. A bidirectional crossreacting tolerance exists between TNF and endotoxin. The mechanism of TNF tolerance is unclear, but it does not appear to be due to a humoral immune response or a perturbation of the uptake and clearance of injected TNF.

Received for publication 2 March 1988.

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