

# PRETREATMENT WITH RECOMBINANT MURINE TUMOR NECROSIS FACTOR $\alpha$ /CACHECTIN AND MURINE INTERLEUKIN 1 $\alpha$ PROTECTS MICE FROM LETHAL BACTERIAL INFECTION

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Tumor necrosis factor  $\alpha$ /cachectin (TNF/C) is a well-characterized peptide (1) that may, through its diverse effects on cells, mediate many normal host functions, including tissue remodeling and the mobilization of energy reserves required by the infected host (2). In contrast to such beneficial effects, TNF/C has also been shown to have adverse effects on the animal host. It is a principal mediator of the toxic effects of LPS and an important mediator in murine cerebral malaria (3-5). Because of its toxic effects, TNF/C is generally considered to be an abnormal and deleterious response that plays little if any role in host defense against bacterial infection and thus has been considered as a target for therapeutic intervention to ameliorate host toxic responses (4, 6). Based on these considerations, one might expect that animals that are unable to produce TNF/C in response to bacterial endotoxin would be more resistant to lethal infection than animals that are able to produce TNF/C.

The C3H/HeJ mouse is unable to produce TNF/C directly in response to LPS, whereas its congenic pair, the C3H/HeN mouse, does (7). Therefore, we reasoned that the C3H/HeJ mouse would be more resistant than the C3H/HeN mouse to lethal infection with virulent Gram-negative bacteria capable of causing bacteremic infection and septic shock. In this study we show, however, that the C3H/HeJ mouse is more susceptible to lethal infection with K1-encapsulated *Escherichia coli* than is the C3H/HeN mouse, and that pretreatment of C3H/HeJ mice with a combination of recombinant murine TNF/C and recombinant murine IL-1 $\alpha$  protects these mice from infection with an inoculum of >20 LD<sub>50</sub>.

## Materials and Methods

*E. coli* 018:K1:H7, strain Bort, is an isolate retrieved from the spinal fluid of a neonate with meningitis. It is insensitive to lysis by rough LPS-specific phages (8) and, as a smooth organism, produces O-side chains as shown by SDS-PAGE. We selected a K1<sup>-</sup> mutant of this strain as a clone resistant to lytic K1 phages, as recently described (8).

C3H/HeJ mice were obtained from The Jackson Laboratories, Bar Harbor, ME. C3H/HeN mice were obtained from the Small Animal Section, National Institutes of Health, Bethesda, MD. Both strains of mice, all females, were used between 9 and 12 wk of age. LD<sub>50</sub> deter-

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minations were performed by randomly assigning six mice per group, with each given 10-fold dilutions of bacterial suspensions in volumes of 0.1 ml intraperitoneally. LPS was made by the hot phenol water extraction method. Animals were followed for up to 30 d before terminating the experiment; however, deaths rarely occurred after day 7.

The C3H/HeJ mice were given a single intraperitoneal injection of recombinant murine TNF/C- $\alpha$  (lot no. B8120;  $4 \times 10^7$  U/mg sp act; Genzyme, Boston, MA) and/or murine rIL-1 $\alpha$  ( $10^6$  U/mg sp act; kindly provided by Dr. Peter Lomedico, Hoffman-LaRoche, Inc., Nutley, NJ) just before challenge with *E. coli*. Dilution of the recombinant cytokines was made in pyrogen-free normal saline. There was 0.12 EU/ml of endotoxin contamination in the rTNF and <0.125 EU/ml in the rIL-1 as measured in the limulus assay.

TNF/C activity was assessed by the colorimetric determination of cytotoxicity to L 929 cells, as previously described (9, 10). For preparation of a standard curve, murine rTNF/C was added to normal mouse serum at concentrations of 400 ng/ml to 5.12 pg/ml. The serum was obtained from CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) and kept at  $-20^\circ\text{C}$  until use. The standards and test sera were then heat inactivated at  $56^\circ\text{C}$  for 30 min before being assayed for TNF/C. TNF/C was not inactivated by the heating, which concurs with data provided by the manufacturer. A standard curve was constructed by graphing optical density versus concentration of TNF/C in the standards. 50% cytotoxicity (i.e., 1 U of TNF/C activity) was observed with  $\sim 25$  pg of the standard TNF/C.

Production of TNF/C in C3H mice was accomplished by priming mice with 100  $\mu\text{l}$  of a 1:10 dilution of BCG injected intraperitoneally (kindly supplied by Dr. Carol Nacy, WRAIR, which she obtained from the Trudeau Institute, Saranac Lake, NY). 7–10 d later mice were given a nonlethal, intravenous dose of LPS prepared from the Bort strain of *E. coli* and bled 2 h later. Murine serum was kept at  $-70^\circ\text{C}$  until assayed for TNF/C.

## Results

In comparing the LD<sub>50</sub> of this clinical isolate for the two strains of mice, Bort was highly virulent to the C3H/HeJ mouse in which TNF/C production is blocked (Table I). Less than 10 organisms were required to kill these mice. In contrast, mice that produce TNF/C in response to LPS (C3H/HeN) required  $10^3$  times more bacteria to achieve lethal infection. This occurred in spite of the fact that purified LPS from this *E. coli* strain was 10-fold less potent in killing the C3H/HeJ mice (Table I). With the less virulent K1<sup>-</sup> mutant these differences in host infection doses were not seen. These findings suggest that TNF/C, in contrast to being solely a deleterious factor in host infection, may participate in stimulating important host defense mechanisms against infection with virulent organisms, as has been previously speculated (2).

To test this hypothesis, we pretreated C3H/HeJ mice with either normal saline or rTNF/C. In addition, because of previous reports of synergistic activity with combinations of IL-1 and TNF/C (11, 12), we also treated mice with IL-1 alone and in

TABLE I  
Susceptibility of LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ Mice to *E. coli* O18:K1:H7, Strain Bort, and Its LPS

	LD <sub>50</sub> *	
	C3H/HeN	C3H/HeJ
O18 LPS400	400 $\mu\text{g}$	3,750 $\mu\text{g}$
<i>E. coli</i> O18:K1 <sup>+</sup>	$10^4$ CFU	$<10^1$ CFU
<i>E. coli</i> O18:K1 <sup>-</sup>	$10^7$ CFU	$10^7$ CFU

\* Intraperitoneal challenge.

combination with TNF/C immediately before challenge with the virulent *E. coli*. All saline-treated mice died after infection with as little as 19 CFU. For challenges of 85 and 193 CFU, TNF/C alone (with doses of 1 or 10  $\mu\text{g}/\text{kg}$ ) and IL-1 (with doses from 500 to 5,000 U per mouse) provided transient protection at day 2, but this effect was lost by day 3 (Table II). In contrast, mice pretreated with a combination of 1  $\mu\text{g}/\text{kg}$  TNF/C and 500 U of IL-1 were significantly protected from lethal infection. This combination provided transient protection at  $>100$  LD<sub>50</sub> (2/5 mice challenged with 1,930 CFU alive at day 2 vs. 0/5 challenged with 19 CFU [data not shown]), and 80% protection at  $>20$  LD<sub>50</sub> at day 7 (Table II). Mice alive at day 7 remained healthy and active through day 30, at which time the experiment was terminated. Under these experimental conditions TNF/C and IL-1 were unable, however, to protect fully the C3H/HeJ mouse against  $10^4$  organisms, which is the LD<sub>50</sub> for the C3H/HeN mouse (Table I). The combination of TNF/C at 1  $\mu\text{g}/\text{kg}$  and IL-1 at 1,000 U was a less effective combination, with transient protection observed only in Exp. 1, with a bacterial challenge of 85 CFU. Culture of both blood and liver revealed recovery of strain Bort ( $10^7$  CFU/g tissue and per milliliter blood at 48 h), which indicated that infection resulting from the challenge was the cause of death in these animals. Neither death nor symptoms of sepsis (lethargy, piloerection, diarrhea) were noted after IL-1 administration up to 10,000 U.

A nonlethal dose of LPS (10  $\mu\text{g}$ ) given intravenously to BCG-primed C3H/HeN mice induced detectable levels of circulating TNF/C within 2 h. Levels of 300–3,000 pg/ml serum were detected in all mice so challenged. In contrast, no circulating TNF/C was detected in C3H/HeJ mice similarly given 10  $\mu\text{g}$  LPS. TNF/C was detected in C3H/HeJ mice, however, when given  $>400$   $\mu\text{g}$  of LPS intravenously. Four C3H/HeN

TABLE II  
*Survival of C3H/HeJ Mice Pre-treated Concurrently with Mouse  $\tau$ TNF- $\alpha$ ,  $\tau$ IL-1 $\alpha$  Either Alone or in Combination vs. Untreated Controls Challenge Intraperitoneally with Atleast 10 LD<sub>50</sub> of *E. coli* O18:K1:H7*

Pretreatment	Exp. 1*		Exp. 2*	
	Day 2	Day 7	Day 2	Day 7
a. None	2/7 <sup>†</sup>	0/7	0/5	0/5
b. TNF (1 $\mu\text{g}/\text{kg}$ )	4/7	1/7	3/5	0/5
c. TNF (10 $\mu\text{g}/\text{kg}$ )	5/5	0/5	1/2	0/2
d. IL-1 (500 U)	7/7	0/7	0/3	0/3
e. IL-1 (1,000 U)	6/7	1/7	1/3	0/3
f. IL-1 (500 U) + TNF (1 $\mu\text{g}/\text{kg}$ )	7/7	7/7 <sup>§</sup>	6/6	5/6 <sup>§</sup>
g. IL-1 (1,000 U) + TNF (1 $\mu\text{g}/\text{kg}$ )	4/4	0/4	0/6	0/6

\* Bacterial challenge in Exp. 1 was 85 CFU/mouse ( $\sim 10$  LD<sub>50</sub>) and in Exp. 2 was 193 CFU ( $\sim 20$  LD<sub>50</sub>). In Exp. 2 a control group of five mice received 19 CFU and all died (not shown).

<sup>†</sup> Values shown are the survivors per total number in original group tested at days 2 and 7 after bacterial challenge.

<sup>§</sup> Data were tested for statistical significance in the Kruskal-Wallis test for rank sums and groups were analyzed for two-tailed multiple comparisons by the method of Dunn (28).  $p < 0.020$ , Exp. 1: *f* vs. *e*; Exp. 2: *f* vs. *g*;  $p < 0.005$ , Exp. 1: *f* vs. *a, b*; Exp. 2: *f* vs. *a*;  $p < 0.00005$ , Exp. 1: *f* vs. *a*. All other comparisons did not achieve statistical significance.

mice that had not been previously primed with BCG were given 10 LD<sub>50</sub> of strain Bort via the intraperitoneal route. At 20–22 h all four mice appeared moribund (extreme lethargy, tachypnea, piloerection, and closed eyes) and had  $\sim 10^8$  CFU/ml circulating in their serum. None of these mice had detectable TNF/C in their serum. Four C3H/HeJ mice were also given 10 LD<sub>50</sub> of strain Bort intraperitoneally. These mice appeared moribund 29–44 h after bacterial challenge and also had  $\sim 10^8$  CFU/ml circulating in their serum. As in the case of the C3H/HeN mice, the C3H/HeJ mice had no detectable TNF/C in their serum. In these assays, the serum from the BCG-primed C3H/HeN mice that were given LPS served as positive controls for the presence of TNF/C.

### Discussion

TNF/C is considered to be the principal mediator of endotoxic shock (2, 3). When given to animals in quantities found to be produced endogenously in response to endotoxin, rTNF/C has been shown to produce shock and death, which is similar to that seen after bacteremia with Gram-negative bacilli (2–4). Furthermore, antibody against TNF/C can block the effects of endotoxin administered in vivo (6). In some animal models these anti-TNF/C antibodies, when administered before infection, can block the lethal effects of Gram-negative bacteremia induced by the injection of large numbers of bacteria (13). Therefore, antibodies and pharmacologic agents directed against TNF/C have become attractive candidates for prevention of septic shock in humans (4, 6). In this study we show that TNF/C in conjunction with IL-1 at low doses has a significant protective role in invasive infections that require bacterial replication and translocation in the host.

When a relatively avirulent mutant of *E. coli* was tested in the two mouse strains, there were no differences in LD<sub>50</sub> (Table I). Therefore, the defense mechanisms in which TNF/C may be involved appear less important for organisms of relatively low virulence and which may be easily killed by non-cytokine-mediated host defenses. In this situation TNF/C may manifest itself as a deleterious mediator; the use of anti-TNF/C antisera in animal models infected with these types of bacteria would be expected to show a protective effect. Such models, however, may be more an experimental expression of endotoxemia than of natural infection. For example, in the baboon shock model (13), anti-TNF/C mAb given prophylactically protected against septic shock induced with  $10^{11}$  *E. coli* per kilogram. Although the virulence properties of this *E. coli* are not specified beyond its serotype (086:B7), the large challenge inoculum implies that the organism has relatively low virulence. In our studies, we have shown that after an inoculum of as little as 19–85 CFU into the C3H/HeJ mouse (i.e., an inoculum of *E. coli* that is  $>10^7$ -fold less per weight of animal), the parental K1-encapsulated *E. coli* replicates and disseminates through the host where it achieves a level of  $10^7$  CFU/g of liver just before the death of the mouse. The virulence properties of bacteria that require the host to respond with cytokine-mediated host defenses remain to be defined.

The observation that TNF/C and IL-1 provided significant protection only when used in combination suggests there may be a synergism between their individual activities. Alternatively, these cytokines may be more efficient together in inducing the release of other mediators, such as has been shown in the induction of CSFs (11). One species of CSF, granulocyte/macrophage activating factor (GM-CSF), like

TNF/C (14), is also a potent activator of phagocytes (15). There have now been numerous studies demonstrating that rIL-1, rIL-2, and IFN- $\beta$ , when given alone, protect animals from lethal infection by intracellular bacteria (e.g., listeria) and by bacteria not usually considered to be controlled by the cellular immune system (e.g., *Klebsiella spp.*, *Pseudomonas aeruginosa*) (16–18). In these studies, the participation of other unknown cytokines or mediators is inferred since, in the case of IL-1 and 2, there is neither a direct bactericidal effect on the organism nor on the activation of phagocytes (16). In our study we used a strain of mouse (C3H/HeJ) that is less able to produce both TNF/C and IL-1 in response to LPS than is the C3H/HeN mouse (10  $\mu$ g of LPS induced detectable TNF/C in the latter mouse versus >400  $\mu$ g LPS in the C3H/HeJ mouse). Our finding that the combination of cytokines, at appropriate doses, protects the C3H/HeJ mouse from lethal infection is consistent with the hypothesis that TNF/C, a potent activator of phagocytes, participates in the normal IL-1- or IL-2-mediated protection. An important role for TNF/C in host resistance to infection with *Listeria monocytogenes* was suggested by the enhancement of lethal infection after pretreatment with anti-TNF/C antibody (19).

Our finding in separate experiments that doubling the dose of IL-1 abrogates the protective effect of a combination dose demonstrates in vivo that, to achieve protection during natural infection, there must be controlled regulation of these cytokines. This is consistent with past observations of TNF/C (7). In cell culture TNF/C and IL-1 are each able to induce more IL-1 (20, 21). In addition, other cytokines that are induced by TNF/C may in turn induce more TNF/C (22). As little as 200 ng/g or 5  $\mu$ g of TNF/C has been shown to adversely affect rodents (3, 23) and a combination of these cytokines does cause tissue damage (24). Consequently, our observation that the mice treated with the low doses of TNF/C and IL-1 remain healthy and active demonstrates that such toxic levels of TNF/C were not achieved and that these positive feedback loops were not activated.

Our results suggest that the host response mediated by TNF/C and IL-1 to LPS may be a potent stimulus to protective mechanisms against infection with highly virulent, invasive organisms capable of causing bacteremia and septic shock in man. Increases in TNF/C may be a normal response to bacterial invasion. Even with the induction of a nonlethal elevation of circulating TNF/C in BCG-primed mice, host regulatory mechanisms appeared to mitigate the potential adverse effects of this cytokine. In this light, TNF/C-mediated septic shock may be due to abnormal regulation of a normal response. The data that moribund mice with high levels of bacteria circulating in the blood do not have detectable TNF/C differs from the findings of circulating TNF/C close to death during meningococcal infection in man (25) and during murine cerebral malaria (5). These data suggest that if an abnormal TNF/C response is an important component of septic shock in C3H mice, the kinetics of its release and regulation are different from these latter infections.

Previous investigations have suggested that TNF/C-mediated responses may be important in host defenses against parasitic infection (26, 27), and that the combination of TNF/C and IL-1 protects against radiation-induced death (23). The deleterious, potentially fatal side effects of TNF/C are well recognized. Nonetheless, our experimental findings now reveal a need for caution before use of anti-TNF/C agents, which when used in a prophylactic mode might interfere with important TNF/C host-stimulated defenses.

### Summary

Tumor necrosis factor/cachectin (TNF/C) is the principal mediator of bacterial endotoxin-induced shock and death. We found that the C3H/HeJ mouse, which is less able to produce TNF/C in response to endotoxin, has a 1,000-fold greater susceptibility to lethal infection with *Escherichia coli* than the TNF-responsive congenic mouse, C3H/HeN. This surprising finding suggested that this lethal peptide may also be involved in host protection. To test this hypothesis we pretreated the C3H/HeJ mouse with a combination of recombinant murine TNF/C- $\alpha$  and IL-1 $\alpha$ . This combination protected these mice against an intraperitoneal bacterial challenge of >20 LD<sub>50</sub>s (nearly  $2 \times 10^2$  CFU) that grew to a level of >10<sup>7</sup> CFU/ml of blood and per gram of liver in untreated mice. This suggests a significant role for these cytokines in host defenses against invasive infections that require bacterial replication within the host. These protective mechanisms may not be important for less virulent organisms. These findings may have important implications for the proposed use of anti-TNF/C agents in the treatment of septic shock.

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