Enhancement of Resistance to Infections by Endotoxin-Induced Serum Factor from *Mycobacterium bovis* BCG-Infected Mice

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Necrosis of a variety of transplanted murine tumors can be induced by serum from *Mycobacterium bovis* BCG-treated mice challenged with a lethal dose of endotoxin. Results reported here show that the tumor necrosis serum (TNS) enhances resistance to infections, protecting mice against two types of challenges, either with *Klebsiella pneumoniae* or with the intracellular parasite *Listeria monocytogenes*. Moreover, TNS activity was demonstrated in animals which are refractory to lipopolysaccharide and very susceptible to infections, such as 8-day-old mice and adult C3H/He mice. Protection passively transferred by TNS was not related to antibodies, since it was not decreased by absorption with homologous organisms.

Two weeks after administration of *Mycobacterium bovis* BCG, animals become much more resistant to heterologous bacterial infections (3, 9). By that time, which coincides with a marked hepatosplenomegaly, their susceptibility to the toxic effect of endotoxin is considerably increased (2, 18, 33) and concomitantly lipopolysaccharide (LPS) becomes more cytotoxic in vitro toward macrophages (28). Administration of a lethal dose of LPS in mice or rabbits which have had their macrophages first activated by BCG induces the release of a factor in their serum which has been called tumor necrosis serum (TNS) since it mimics the necrotic actions of endotoxin on a variety of transplanted mouse tumors (5, 21). In vitro, TNS is directly cytotoxic for tumor cells, whereas freshly cultured embryo fibroblasts are not injured (5, 12). Several lines of evidence indicate that the active principle of TNS cannot be ascribed to LPS itself (12, 17).

The purpose of this investigation was to test the ability of TNS to increase the resistance of mice to bacterial infections by passive transfer. In many cases, a very small amount of endotoxin can effectively protect mice against a bacterial challenge (6). Therefore, it is difficult under usual conditions to rule out the role of some undegraded residual LPS in TNS as a major contributing factor capable of modifying resistance. For this reason, the activity of TNS was evaluated both in adult C3H/He mice and in newborn mice. The C3H/HeJ strain is known to be refractory to virtually all LPS-mediated effects (8, 11, 29, 32). Endotoxins are unable to enhance (6) or decrease (M. A. Parant, F. J.

Parant, and L. Chedid, unpublished data) non-specific resistance of this LPS low-responder subline to bacterial infections. Moreover, other immunostimulants are unable to enhance the He strain's resistance to *Klebsiella* infection (24). It was reported previously that even in LPS-responsive strains, 8-day-old mice cannot be fully protected by endotoxin against infectious challenges (25). Data reported here establish that TNS clearly protects adult C3H/He and even newborn mice challenged by either *Klebsiella* or *Listeria*. It is also demonstrated that this passive protection is not related to the presence of antibodies.

MATERIALS AND METHODS

Animals. Two-month-old Swiss mice were used to prepare TNS and to evaluate LPS toxicity after adrenalectomy. Infection experiments were performed either in 8-day-old (C57Bl/6 $\times$ AKR) F_1 hybrids raised at the Pasteur Institute or in 5- to 6-week-old C3H/He or C3HeB/Fe mice. The C3H sublines, which originated from Jackson Laboratories (Bar Harbor, Maine), were obtained from the Centre National de la Recherche Scientifique (Orléans, France). Previous experiments have shown that the C3H/He Orl subline has retained its unresponsiveness to LPS (6). The following abbreviations are used in this paper: eB for C3HeB/Fe Orl; He for C3H/He Orl; and B6AK for (C57Bl/6 $\times$ AKR) F_1 hybrids.

Preparation of TNS. Mice were infected intravenously with 2×10^7 viable BCG organisms (*M. bovis* strain 1173 P₂, Pasteur Institute) by the method of Carswell et al. (5). Fourteen days later, they received an intravenous injection of 25 μ g of *Salmonella enteritidis* LPS W (Difco Laboratories, Detroit, Mich.)

labeled with ^{51}Cr (7). Mice were bled 2 h later, and pooled serum was stored in small portions over liquid nitrogen. In some experiments TNS was absorbed with 10% heat-killed bacteria, the mixture was kept at 4°C for 30 min before centrifugation, and the whole procedure was repeated once. As controls for TNS, we used serum obtained either from normal mice 2 h after an injection of $25\text{ }\mu\text{g}$ of LPS (LPS serum) or from BCG-treated mice not given LPS (BCG serum). Sera were injected subcutaneously into newborn mice and intravenously into adult mice. Before treatment, newborns were randomized and redistributed in litters of usually seven or eight per mother (25).

Measurements of radioactivity (7) and evaluation of toxicity in adrenalectomized mice (7) were made by using ^{51}Cr -LPS in saline. Results showed that 2 h after injection of $25\text{ }\mu\text{g}$ of LPS to BCG-infected mice, endotoxin had been removed very rapidly since mouse serum contained only approximately $0.5\text{ }\mu\text{g}$ of toxic LPS per ml.

Infection studies. The *Klebsiella pneumoniae* strain (Pasteur Institute Collection no. 7823) capsular type 2 has been used in the past in similar experiments (6, 25). *Listeria monocytogenes* strain EDG was obtained from G. B. Mackaness (Trudeau Institute, Saranac Lake, N.J.). For serum absorption experiments, bacteria from a 16-h culture were killed at 60°C for 1 h. All challenges were performed with a single stock of bacterial culture in the logarithmic phase of growth stored over liquid nitrogen. Bacterial inoculation was by the intravenous route into adult mice (using groups of eight mice) and by the subcutaneous route into 8-day-old mice (25). The minimal lethal dose of *K. pneumoniae* was less than 10 organisms in adult C3H mice and about 10^3 organisms in 8-day-old B6AK mice. *Listeria* inoculations were, respectively, 6×10^4 and 2×10^3 organisms by the same routes of injection. Deaths were recorded for 15 days. The *P* values were calculated by the adjusted χ^2 method (30). Estimation of viable bacterial counts in vivo was performed according to reported methods (26).

RESULTS

Antibacterial activity of TNS in adult C3H/He mice. To rule out a possible direct bactericidal activity of TNS, *K. pneumoniae* and *L. monocytogenes* were incubated with undiluted serum at 37°C for 5 h. No differences in bacterial multiplication were found between TNS and the various control sera (from normal mice or from mice treated with BCG or with LPS). We previously reported that *K. pneumoniae* proliferated very rapidly in the C3H/He subline and that treatment with LPS was unable to protect them or to check this rapid rate of bacterial division (24), even after a small inoculum of about 10 organisms (Parant et al., unpublished data).

The course of infection was followed in both high- (eB) and low- (He) responder mice by viable counts performed in blood, liver, and

spleen. These counts were made 5 h after the intravenous injection of 2.5×10^3 *Klebsiella* or 48 and 72 h after injecting 6×10^4 *Listeria* by the same route. There was a rapid increase in the total number of *Klebsiella* recovered from the very susceptible He mice: 1.2×10^6 organisms (or approximately nine divisions) after 5 h versus 1.3×10^5 in eB mice (Table 1). Moreover, in contrast to what was observed after an injection of LPS in high responders, bacteria were not destroyed (total numbers: 1.2×10^6 versus 4.5×10^3); blood clearance was not stimulated, since considerably fewer organisms were found in the liver and spleen than in the blood. The two He control groups receiving serum from donors treated with BCG or LPS showed the same pattern of distribution and the same elevated total number of bacteria. In contrast, the effect of TNS on the number (100-fold less than in the controls) and distribution of viable *Klebsiella* was very marked after 5 h and similar to what was observed in LPS-stimulated high responders (Table 1).

Although the susceptibility to *L. monocytogenes* of untreated He controls was somewhat similar to the susceptibility of high responders, the response of He mice to LPS or to serum from LPS- and BCG-treated mice was insignificant. In contrast, after treatment by TNS, *L. monocytogenes* was very effectively destroyed, since the numbers recovered from spleen and liver were significantly reduced. At these times of infection, negligible amounts of *Listeria* were recovered in the blood compartment (Table 1). The survival experiments confirmed that TNS renders these susceptible mice capable of destroying virulent bacteria.

C3H/He mice started to die soon after the intravenous injection of a small number of *K. pneumoniae*. Administration of $1\text{ }\mu\text{g}$ or greater amounts of LPS at 1 day or 2 h before the challenge did not modify the mortality rate, whereas such treatment very effectively protected the closely related C3HeB/Fe subline. In contrast, an intravenous injection of 0.5 ml of TNS 2 h before the challenge protected 75% of the animals, whereas a slight delay in mortality was observed when mice received serum from donors treated with LPS only. The serum from mice treated with BCG only was totally inactive (Fig. 1). In additional experiments, recipient C3H/He mice could not be protected by serum from BCG-treated donors to which $0.5\text{ }\mu\text{g}$ of LPS (corresponding to the residual amount of endotoxin found in TNS) had been added.

The role of TNS in the resistance of C3H/He mice to *Listeria* infection was also measured. Preliminary experiments showed that this sub-

TABLE 1. Influence of TNS on distribution of living bacteria in adult C3H mice

Bacterial inoculum	Time (h)	Group ^a	Treatment	Recovered viable bacteria ^b			
				Total no.	Blood	Spleen	Liver
<i>Klebsiella</i> , 2.5×10^3	5	C3HeB/Fe	Controls	133×10^3	$96 \pm 19 \times 10^3$	$29 \pm 8 \times 10^3$	$8 \pm 2 \times 10^3$
			LPS (saline)	4×10^3	$2 \pm 0.6 \times 10^3$	$1 \pm 0.4 \times 10^3$	$1 \pm 0.3 \times 10^3$
		C3H/He	Controls	$1,230 \times 10^3$	$1,186 \pm 322 \times 10^3$	$44 \pm 4 \times 10^3$	0
			LPS (saline)	$1,162 \times 10^3$	$1,082 \pm 386 \times 10^3$	$80 \pm 6 \times 10^3$	0
			LPS serum	750×10^3	$722 \pm 195 \times 10^3$	$28 \pm 8 \times 10^3$	0
			BCG serum	$1,286 \times 10^3$	$1,240 \pm 542 \times 10^3$	$45 \pm 9 \times 10^3$	0
			TNS	18×10^3	$8 \pm 4 \times 10^3$	$5 \pm 2 \times 10^3$	$5 \pm 0.6 \times 10^3$
<i>Listeria</i> , 6×10^4	48	C3HeB/Fe	Controls	$8,220 \times 10^4$		$6,630 \pm 810 \times 10^4$	$1,590 \pm 320 \times 10^4$
			LPS (saline)	183×10^4		$105 \pm 30 \times 10^4$	$78 \pm 23 \times 10^4$
		C3H/He	Controls	$7,755 \times 10^4$		$5,325 \pm 627 \times 10^4$	$2,430 \pm 814 \times 10^4$
			LPS (saline)	$5,340 \times 10^4$		$3,225 \pm 465 \times 10^4$	$2,115 \pm 760 \times 10^4$
			LPS serum	$4,950 \times 10^4$		$3,045 \pm 670 \times 10^4$	$1,905 \pm 960 \times 10^4$
			BCG serum	$7,050 \times 10^4$		$3,600 \pm 280 \times 10^4$	$3,450 \pm 410 \times 10^4$
			TNS	860×10^4		$200 \pm 95 \times 10^4$	$660 \pm 160 \times 10^4$
	72	C3HeB/Fe	Controls	$1,740 \times 10^7$		$230 \pm 87 \times 10^7$	$1,510 \pm 544 \times 10^7$
			LPS (saline)	2×10^7		$1 \pm 0.3 \times 10^7$	$1 \pm 0.3 \times 10^7$
		C3H/He	Controls	$1,673 \times 10^7$		$63 \pm 33 \times 10^7$	$1,610 \pm 913 \times 10^7$
			LPS (saline)	$2,076 \times 10^7$		$35 \pm 11 \times 10^7$	$2,040 \pm 815 \times 10^7$
			LPS serum	$4,104 \times 10^7$		$104 \pm 57 \times 10^7$	$4,000 \pm 970 \times 10^7$
			BCG serum	$1,146 \times 10^7$		$29 \pm 11 \times 10^7$	$1,117 \pm 480 \times 10^7$
			TNS	62×10^7		$4 \pm 3 \times 10^7$	$58 \pm 28 \times 10^7$

^a Groups of five mice treated intravenously with 0.5 ml of serum at h 2 or with 1 μ g of LPS in saline as control.^b Given as mean $\pm$ standard deviation. FIG. 1. Protective activity of TNS in adult C3H/He mice infected intravenously with 2×10^3 *K. pneumoniae*. Cumulative results of two identical experiments with eight mice in each group are given; mice had been challenged 2 h after intravenous injection of 0.5 ml of serum. FIG. 2. Protective activity of TNS in adult C3H/He mice infected intravenously with 6×10^4 *L. monocytogenes*. Cumulative results of three identical experiments with eight mice in each group are given; mice had been challenged 2 h after intravenous injection of 0.5 ml of serum.

line appeared to be somewhat more resistant than other mouse strains, including the other C3HeB/Fe subline. Nevertheless, these mice were not protected against this organism by LPS (Parant et al., unpublished data). Serum from donors treated by LPS or BCG only was also devoid of significant activity (Fig. 2). In contrast, TNS had a very marked protective effect in mice infected with *Listeria*.

Antibacterial activity of TNS in 8-day-old mice. LPS can protect B6AK F₁ hybrids if they are at least 2 weeks old but not during the 1st week. In the following assays, 8-day-old F₁ hy-

brids were challenged subcutaneously with *Klebsiella* or with *Listeria* after having been treated with sera obtained from the various groups of donors used in the previous experiments. TNS absorbed by *Klebsiella* or by *Listeria* was also administered to additional groups of newborn mice. After either challenge, more than 90% of the controls died between days 5 and 7. Cumulative numbers of survivors at day 15 obtained in several similar experiments are reported in Table 2. The results show that only TNS-treated groups were significantly protected against both infections as compared with all

TABLE 2. Protection by TNS of 8-day-old mice infected subcutaneously with either *K. pneumoniae* or *L. monocytogenes*

Treatment at day -1 ^a	% Survival after 15 days ^b	
	<i>Klebsiella</i> infection	<i>Listeria</i> infection
Controls	2.2 (2/89)	2.6 (2/78)
LPS, 0.5 µg (saline)	13.3 (4/30)	0 (0/41)
BCG serum	0 (0/36)	20 (9/45)
LPS serum	10.3 (3/29)	15.1 (5/33)
Unabsorbed TNS	53.6 ^c (30/56)	71.4 ^c (55/77)
TNS absorbed with <i>Klebsiella</i>	62.2 ^c (23/37)	78.9 ^c (15/19)
TNS absorbed with <i>Listeria</i>	57 ^c (16/28)	71.4 ^c (15/21)

^a 0.1 ml of undiluted serum.

^b After subcutaneous infection with 1.5×10^3 *K. pneumoniae* or 2×10^3 *L. monocytogenes*. Numbers of survivors/total are given in parentheses.

^c $P < 0.01$.

other control groups. Moreover, the results demonstrate that the protective activity of TNS was not at all decreased by previous absorption with homologous or heterologous heat-killed bacteria.

DISCUSSION

This paper presents the effect of TNS on nonspecific resistance to bacterial infections. TNS was obtained as described by others from mice infected with BCG before an injection of LPS. Either BCG or LPS has been repeatedly shown to increase resistance to infectious challenges. However, serum from mice treated with only one of these bacterial agents displayed no or only a very slight activity. The protective action of TNS described here is particularly striking since hosts chosen in this study were very susceptible to certain infections. These hosts (8-day-old hybrids or C3H/He adults) were also selected because of their unresponsiveness to the protective effect of LPS. Therefore, the influence on nonspecific resistance of small amounts of residual LPS in TNS samples could be ruled out. In B6AK newborn mice and He adults, the activity of TNS was equally marked against infection by two different types of organisms, *K. pneumoniae* and *L. monocytogenes*. Clearance and distribution experiments showed that *Klebsiella* could be removed very rapidly from the bloodstream and that both organisms could be destroyed in mice after an injection of TNS. Enhanced resistance was not related to the presence of natural antibodies, since the full effectiveness of TNS was retained after TNS was repeatedly absorbed by heterologous or homologous organisms.

During early postnatal life, mice have been found to be immunologically deficient. The onset of resistance to *Salmonella typhimurium* (4) or to oral infection by *Escherichia coli* (23) is around 10 days of age and around 21 days of age in the case of *Klebsiella*. Yet mice challenged with *Klebsiella* could be protected by LPS from 14 days of age (Parant et al., unpublished observation). Before this age, death can only be delayed for a short time by endotoxin administration (25, 27, 31). We are currently examining responses of younger animals (fewer than 8 days old) to determine whether TNS can protect them against infection as is the case after administration of muramyl dipeptide, a synthetic immunostimulant (25). Inability to respond to antigenic stimulation during early postnatal life has been ascribed to lack of maturity or to strong suppressive activity of cells, or both (22). Immunological immaturity in newborn mice may also be due to the lack of competent macrophages, as was shown by cell transfer in immunization studies (1, 3, 13). Moreover, newborns given nonimmune adult macrophages were totally protected against the lethal effect of *Listeria* organisms to which they were highly susceptible (3).

The genetic defect of C3H/HeJ mice that limits responsiveness to many of the effects of LPS has been shown to involve different cell types (6, 8, 10, 11, 29, 32, 34). The failure of endotoxin to elicit an increased resistance to bacterial infections in C3H/He mice does not depend only on its inability to induce mitogenic responses of B cells, since a trichloroacetic acid extract was also inactive in this system although it produced lymphocyte proliferation (6). Unresponsiveness to LPS seemed to be related largely to macrophage defects, yet appeared to also require the participation of LPS-resistant bone marrow cells as was reported in experiments on LPS-enhanced resistance to *Klebsiella* (10). LPS-induced tumor necrosis studies also indicated that the effects on macrophages were mediated through lymphoreticular cells (20). In many respects, acquisition of tumor killing by activated macrophages seems to parallel bactericidal activity (14). In addition to inhibiting tumor growth in vivo and in vitro, TNS has immunological properties which have been compared with macrophage effects. Thus, TNS replaces a factor which is produced by LPS-responsive macrophages in the presence of LPS and which repairs the LPS macrophage defect of C3H/HeJ mice, making spleen cell cultures able to generate immune responses to TNS conjugated with autologous erythrocytes (15). Other immunological functions of TNS have been de-

scribed (substitution for helper T-cells [16], maturation of B cells [19]) which are consistent with the assumption that TNS is a macrophage product (5, 17, 21). So far, purification procedures have not been able to dissociate these immunological activities from the ability to necrotize tumors (12, 17). Whether increased resistance to infections and tumor necrosis can be ascribed to the same factor also remains an open question. In addition, further experiments are required to determine the nature of the cell or cells involved. Nevertheless, our findings suggest that LPS exerts its influence on nonspecific resistance by the production of humoral mediators. Our data also demonstrate that natural immunity of a genetically deficient or immature host can be restored and enhanced by soluble factors which have already been shown to be capable of activating defective cells (29).

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