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On the role of macrophages in anthrax

(*Bacillus anthracis*/lethal toxin/tumor necrosis factor/interleukin 1/silica)

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ABSTRACT **_Bacillus anthracis_, the causative agent of anthrax, produces systemic shock and death in susceptible animals, primarily through the action of its lethal toxin. This toxin, at high concentrations, induces lysis of macrophages _in vitro_ but shows little or no effect on other cells. We found that when mice were specifically depleted of macrophages by silica injections, they became resistant to the toxin. Sensitivity could be restored by coinjection of toxin-sensitive cultured macrophages (RAW 264.7 cells) but not by coinjection of other cell lines tested. These results implied that macrophages mediate the action of lethal toxin _in vivo_ and led us to investigate their role in death of the mammalian host. Sublytic concentrations of lethal toxin, orders of magnitude lower than those required to induce lysis of RAW 264.7 cells, were found to induce these cells to express interleukin 1 (IL-1) and tumor necrosis factor _in vitro_. Passive immunization against IL-1 or injection of an IL-1 receptor antagonist protected mice from toxin challenge, whereas anti-tumor necrosis factor provided little, if any, protection. These results imply that systemic shock and death from anthrax result primarily from the effects of high levels of cytokines, principally IL-1, produced by macrophages that have been stimulated by the anthrax lethal toxin.**

Death of a mammalian host from microbial infection involves a complex cascade of events, which are incompletely characterized, even in the most thoroughly studied diseases. In bacterial infections, mechanisms of pathogenesis are perhaps most clearly defined in diseases in which the major symptoms are caused by potent toxins. However, even where we understand the action of a toxin at the biochemical level [e.g., diphtheria (1)], there are major gaps in our knowledge of the cellular and physiological links between its primary biochemical action and overt symptoms.

Anthrax has been both a scourge and an historically important model in understanding infectious diseases (2, 3). The disease, which afflicts humans, other mammals, and several species of birds, reptiles, and amphibians, is initiated by introduction of spores of *Bacillus anthracis* into the body, usually via a minor abrasion, an insect bite, or inhalation (4, 5). After spore germination and local multiplication, the bacteria may spread to regional lymph nodes and from there to the blood stream, where they reach high concentrations ($>10^8$ per ml), generating the systemic form of anthrax (4). This form is nearly always fatal, the victim succumbing with nonspecific, shock-like symptoms. One defining characteristic of systemic anthrax is the suddenness of the fatal shock; indeed, the first overt sign of the disease in animals is often death itself (4).

Pathogenesis of *B. anthracis* depends upon three plasmid-encoded virulence factors: an antiphagocytic poly-D-glutamic acid capsule and two proteinaceous toxins, termed edema toxin and lethal toxin (LeTx) (6–8). The two toxins are believed to contribute to different forms of the disease. When injected intradermally, edema toxin acts as an adenylate cyclase that induces edema reminiscent of that seen in cutaneous anthrax (6–8). LeTx, on the other hand, appears to be the central effector of shock and death from systemic anthrax. Thus, the symptoms of systemic anthrax are mimicked by injection of LeTx alone (9, 10); immunity to LeTx protects animals from bacterial infection (11); and LeTx-deficient strains are attenuated (12, 13).

LeTx is formed by combining two proteins: lethal factor (LF, 83 kDa), which is believed to enter and act within sensitive cells, and protective antigen (PA, 90 kDa), which mediates the attachment and entry of LF into cells (4, 6, 8, 14). Studies in cell culture indicate that LeTx, in contrast to edema toxin, is selectively cytotoxic for macrophages (9). These cells lyse within 1–2 hr of exposure to high concentrations of LeTx (9, 10), whereas essentially all other types of cells tested appear unaffected, even though they, like macrophages, internalize the toxin (6). Here we explore potential roles of macrophages as mediators of symptoms and death induced by the LeTx during anthrax infections.

EXPERIMENTAL PROCEDURES

Macrophages were depleted from 6-month-old BALB/c mice (≈1:1 male/female) by treatment on days 1–5 with SiO_2 (15) (50 mg per day i.p. and 30 mg per day i.v.). On day 6, animals were challenged with LeTx. Toxin challenges, by i.v. injection via the tail vein, consisted of LeTx (100 μg PA plus 20 μg LF) or endotoxin (10 μg) from *Salmonella typhimurium* (Sigma). Some animals were supplemented i.v. with 10^8 cultured RAW 264.7 cells by simultaneous injection with toxin. RAW 264.7 cultures (ATCC TIB-71), originally derived from virally transformed, murine peritoneal macrophages, maintain most macrophage-specific markers and capabilities. Negative-control replacement cells included Vero cells (African green monkey kidney), CHO-K1 (Chinese hamster ovary), or IC-21 (a LeTx-resistant murine peritoneal macrophage line), all of which have been shown to be insensitive to the LeTx (6). Silica was obtained from Sigma and suspended in sterile phosphate-buffered saline at a working concentration of 500 mg per ml. Anthrax LeTx was prepared, purified, and assayed as described (10). In the indicated experiment, mice were passively immunized with 400 μl of neutralizing rabbit anti-mouse cytokine antiserum [anti-interleukin 1 (anti-IL-1) or anti-tumor necrosis factor (anti-TNF)] or with nonimmune normal rabbit serum (Genzyme) i.p. 24 hr before toxin challenge or with 12.5 μg of

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Abbreviations: IL-1, interleukin 1; LeTx, lethal toxin; LPS, lipopolysaccharide; PA, protective antigen; LF, lethal factor; TNF, tumor necrosis factor.

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Table 1. LeTx challenge of macrophage-depleted mice

Group	Silica	Cells injected	Toxin	Death/*n*	*P* value*
1			LeTx	11/12†‡	<0.006
2				0/3	<0.001
3	+		LeTx	0/10	<0.001
4	+			0/4	<0.001
5	+	RAW 264.7	LeTx	3/3§	<0.006
6	+	RAW 264.7		0/3¶	<0.006
7		RAW 264.7	LeTx	3/3§	<0.006
8		RAW 264.7		0/2¶	<0.012
9			LPS	2/2†	<0.012
10	+		LPS	0/2	<0.012
11	+	RAW 264.7	LPS	2/2§	<0.012
12	+	IC-21‖	LeTx	0/4	<0.001
13	+	Vero‖	LeTx	0/4	<0.001
14	+	CHO-K1‖	LeTx	0/4	<0.001

Macrophages were depleted from 6-month-old BALB/c mice by treatment on days 1–5 with SiO_2 (15). On day 6, animals were challenged i.v. with LeTx, LPS, or mock cells (see *Experimental Procedures*). Animal groups 5–8 and 11 were supplemented i.v. with 10^8 cultured RAW 264.7 cells by simultaneous injection with toxin.
*P values were determined by using Fisher's exact test (16).
†Deaths occurred 24–36 hr after toxin challenge.
‡The lone survivor looked sick for 4 days before recovering.
§Deaths occurred within 4 hr after toxin challenge. This more rapid time to death probably represents a greater and more rapid exposure of toxin to the exogenous macrophages, as compared with the normal battery of these phagocytes.
¶Cultured cells are transformed, and mice generated tumors after 3–4 weeks.
‖Vero, CHO-K1, and IC-21 cells have been shown to be insensitive to LeTx (6) and were used as negative-control replacement cells.

human IL-1 receptor antagonist (R & D Systems) i.v. 15 min before toxin challenge (as above). ELISA reagents were purchased from Genzyme and used as specified by the manufacturer. Macrophage growth and test media were assayed for endotoxin using the *Limulus* amebocyte lysate kit supplied by BioWhittaker (N283). All reagents contained minimal endotoxin contamination (<0.03 endotoxin units/ml).

RESULTS AND DISCUSSION

First we tested the hypothesis that macrophages mediate the lethal effects of LeTx on the whole organism. Fine silica particles (SiO_2, 1- to 5-μm diameter, derived from quartz) are selectively toxic for macrophages *in vivo* and *in vitro* and can cause >90% depletion of these cells in animals (15). When phagocytosed by macrophages, silica particles apparently disrupt lysosomal membranes, causing leakage of lysosomal contents and destruction of the cell by a "suicide-bag" mechanism (15). We treated mice with a 5-day regimen of silica injections, which had been shown to eliminate macrophages from the blood, peritoneum, liver, spleen, and certain other organs (but may not decrease the macrophage population in the lungs or brain) (15). Upon challenge on day 6, mice were found to have become resistant to a lethal dose of LeTx (100 μg PA plus 20 μg LF) (Table 1). Silica-treated animals could be resensitized to LeTx by injection of cultured RAW 264.7 murine macrophage-like cells but could not be resensitized by any of three other cell lines that do not respond to the toxin, including IC-21, a toxin-insensitive murine peritoneal macrophage line. These results strongly support the notion that macrophages serve as cellular mediators of the lethal action of LeTx *in vivo*.

The systemic shock caused by *B. anthracis* infections in humans or animals, or by LeTx in test animals, vaguely resembles that seen during Gram-negative bacterial sepsis. During Gram-negative infections, bacterial endotoxin, or lipopolysaccharide (LPS), stimulates macrophages and other cells to produce, among other factors, IL-1 and TNF (17–19). At low levels these cytokines act to stimulate the immune response of the host, but at higher levels they mediate damage to the host, even to the degree of fatal shock (17–19). Consistent with this model, silica injections rendered mice

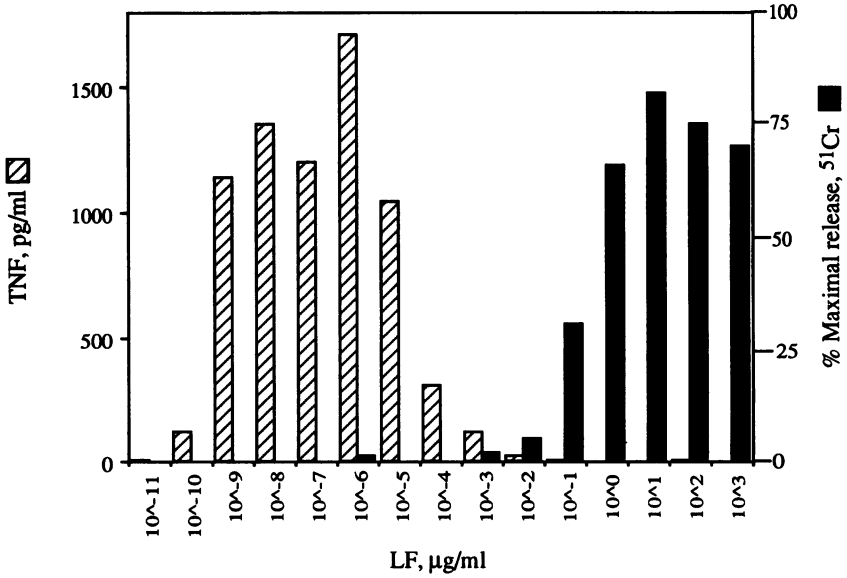

FIG. 1. Dose–response of LeTx on cultured macrophages. Production of TNF (hatched bars) and cytotoxicity (solid bars) were measured as a function of LeTx concentration. Raw 264.7 cells were grown nearly to confluence (≈1 × 10^6 cells per well) in 24-well plates, as described (10). Cells to be monitored for cytotoxicity were loaded with $Na_2\,^{51}CrO_4$ (1 μCi/ml; 1 Ci = 37 GBq) for 16 hr, washed twice (10), and challenged with LeTx (PA at 0.1 μg/ml + LF, as indicated) in a 1.5-ml vol per well. After 6 hr at 37°C, test medium was removed and analyzed for TNF content by ELISA or analyzed for cytotoxicity by counting released ^{51}Cr. ^{51}Cr release was normalized for spontaneous leakage (<1000 cpm per well) and plotted as the percentage of total radioactivity in the cell (≈11,500 cpm per well) (10). Experiments measuring TNF release and cytotoxicity were run in parallel (duplicates); three experimental series were run for both ^{51}Cr release and TNF release each. SEM values were <17% of the indicated value (cytokine measurement) and <6% of the indicated value (^{51}Cr assay) (n = 3). In the absence of PA, LF at concentrations of 1, 0.1, or 0.01 μg/ml gave TNF values of 75, 67, and 105 pg/ml, respectively.

Table 2. Protection of mice from LeTx action

Group	Inhibitor(s)	Toxin	Death/*n*	*P* value*
1	NRS	LeTx	6/7	<0.006
2	Anti-IL-1	LeTx	2/8	<0.04
3	Anti-TNF	LeTx	5/8	<0.57
4	Anti-IL-1 + anti-TNF	LeTx	0/6	<0.005
5	IL-1ra	LeTx	0/4	<0.006

Mice were passively immunized with 400 μl of neutralizing rabbit anti-mouse cytokine antiserum (anti-IL-1 or anti-TNF) or with nonimmune normal rabbit serum (NRS) i.p. 24 hr before challenge with LeTx. All deaths occurred 24–48 hr after toxin administration. Mice were injected with 12.5 μg of human IL-1 receptor antagonist (IL-1ra) i.v. 15 min before toxin challenge.
**P* values were determined by using Fisher's exact test (16).

resistant to LPS, and sensitivity to LPS could be restored by injecting RAW 264.7 cells (Table 1).

We next explored the possibility that LeTx, like LPS, might induce cytokine production by macrophages and that these cytokines might be responsible for death of the anthrax victim. *In vitro* LeTx caused induction of both IL-1 and TNF in RAW 264.7 cells, beginning 2 hr after challenge (Figs. 1 and 2). In the presence of 0.1 μg of PA per ml, production of TNF was detectable at concentrations of LF as low as 10^{-10} μg/ml and reached a broad maximum over LF concentrations of 10^{-9}–10^{-5} μg/ml. TNF production declined to undetectable levels at higher, lytic concentrations of LF. Neither PA nor LF alone induced significant levels of IL-1 or TNF expression. At low doses of LeTx, a single molecule of LF induced production maximally of $>10^5$ molecules of TNF and IL-1 per macrophage, representing a remarkable molecular amplification.

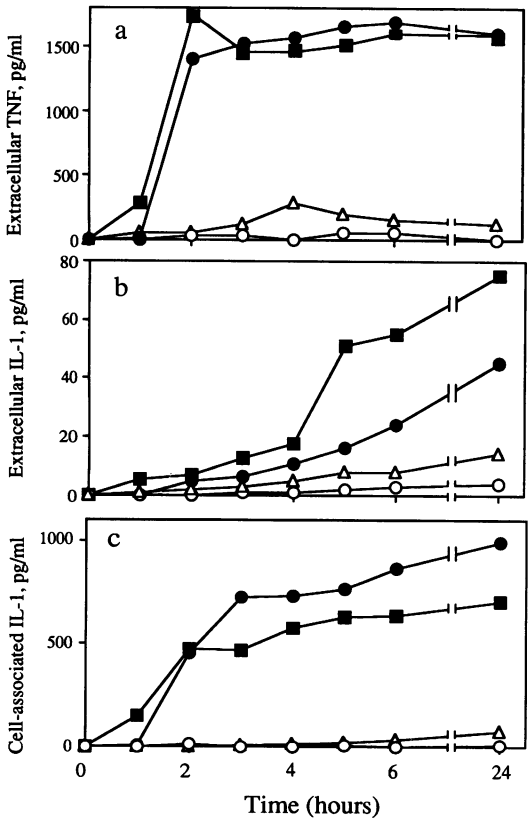

FIG. 2. Time course of cytokine expression. Cultured RAW 264.7 macrophage-like cells were prepared, as described for Fig. 1 and challenged with a sublytic amount of LeTx, with PA or LF alone, or with control medium (data not shown). At the indicated times after toxin addition, test medium was removed, and the levels of cytokine were determined by ELISA (as in Fig. 1). (*a*) TNF released into medium. (*b*) IL-1β released into medium. (*c*) Cell-associated IL-1β. Toxin samples were as follows: ●, LeTx (PA, 0.1 μg/ml, plus LF, 10^{-6} μg/ml); △, PA alone (0.1 μg/ml); ○, LF alone (10^{-6} μg/ml); ■, LPS (10 ng/ml). Virtually no cell-associated TNF was produced under any of the challenge conditions (data not shown). Control medium caused virtually no intra- or extracellular cytokine expression. Measurements of cell-associated cytokine concentrations were determined after lysis of cells with 0.05% Triton X-100. Data points represent the mean of three trials run in duplicate. SEM <14% of the indicated value (n = 3).

To test for induction of IL-1 and TNF production by LeTx *in vivo* and its possible physiological significance, we injected cytokine-neutralizing antisera into mice 24 hr before challenge with LeTx (Table 2). Antiserum against IL-1 partially protected against the toxin, whereas the combination anti-IL-1 and anti-TNF conferred complete resistance. Also, injection of human IL-1 receptor antagonist protected animals from LeTx challenge (20). These observations support the notion that death from systemic anthrax can be directly attributed to the effects of cytokines, especially IL-1, produced by LeTx-stimulated macrophages.

In vitro TNF was exported from RAW 264.7 cells rapidly after synthesis, whereas IL-1 remained almost entirely cell-associated (Fig. 2). Accumulation of IL-1 intracellularly has also been noted with other stimuli (21). This raises the possibility that IL-1 accumulates intracellularly under the influence of low levels of LeTx during anthrax infections and is released within a short period when a lytic threshold of toxin, produced by the exponentially growing bacteria, is reached (Fig. 1). Conceivably the rapid release of IL-1 may contribute to the dramatic "sudden death" seen during anthrax.

The results presented imply that macrophages play a double-edged role in anthrax, serving not only as protectors against bacterial infection but also serving as mediators of lethality. Our data represent evidence that macrophages and their products can mediate death of the host in a Gram-positive bacterial infection, as they do in Gram-negative bacterial sepsis, although by apparently different mechanisms. The mode of action of LeTx is not known, but it is likely to be enzymatic, by analogy with edema toxin and other toxic proteins that act intracellularly (6, 8). In a recent report, Klimpel *et al.* (22) have proposed that LF may be a zinc-dependent protease. In contrast, LPS (nonproteinacious) is believed to act from the cell surface, via membrane-receptor signaling. Also, although it is now clear that lethal shock can be induced by several Gram-positive "superantigen" toxins, in these cases host T-cell, not macrophage, production of cytokines is implicated (23–25).

Our results demonstrate that physiologically important effects of lytic toxins may occur at low concentrations of these proteins, without bursting target cells. Possible stimulatory effects of sublytic doses of other cytolytic/hemolytic protein toxins on cells of the immune system should be examined in light of these results with anthrax lethal toxin. The model also suggests that anticytokine (or other anti-shock) therapies may be useful supplements to antibiotics and more traditional treatments for anthrax. In addition, our results may help to explain the long-noted and paradoxical inverse correlation among animal species between susceptibility to LeTx and susceptibility to infection by *B. anthracis* (26). Thus, animals that are relatively resistant to infection are highly sensitive to the toxin and *vice versa* (26, 27). From our results, greater numbers (or potency) of macrophages, while enhancing defense against bacterial invasion, might also be expected to increase sensitivity to LeTx, LPS, and possibly other bacterial virulence factors (19, 28). Evolution of a more effective host immune response by expansion of the population of macrophages may therefore ultimately be lim-

ited by the ability of certain infectious agents to exploit the potential of the host's macrophages to cause self-damage.

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