Macrophages Release Tumor Necrosis Factor Alpha and Interleukin-12 in Response to Intracellular *Bacillus anthracis* Spores

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Herein we report that infection of a murine macrophage cell line with *Bacillus anthracis* results in the production of tumor necrosis factor alpha and interleukin-12 (IL-12). When infected with *B. anthracis* spores in combination with lipopolysaccharide, macrophages release increased amounts of IL-12. We found no evidence of inhibition of cytokine responses in macrophages infected with *B. anthracis* spores.

Anthrax is a zoonotic disease caused by infection of the mammalian host with *Bacillus anthracis*, a gram-positive, aerobic, spore-forming, rod-shaped bacterium (17; for reviews, see references 22 and 26). Anthrax is acquired when spores enter the host via the cutaneous, gastrointestinal, or inhalational routes. Inhalational anthrax, a rare event, rapidly leads to a highly lethal systemic infection (2, 7, 8, 16, 19, 27). *B. anthracis* carries two plasmids required for full virulence: pXO1, which encodes the components that combine to form the lethal and edema toxins (lethal factor, edema factor, and protective antigen), and pXO2, which carries the genes necessary for the expression of an antiphagocytic capsule (for reviews, see references 6 and 18).

The macrophage is thought to play a pivotal role in the early steps of anthrax pathogenesis. In inhalational anthrax, spores are phagocytosed by alveolar macrophages (3, 9, 11, 24, 25), which subsequently transport the spores to the regional lymph nodes. Spores germinate and multiply within the macrophages, eventually resulting in lysis of the macrophage (3, 9–11, 14). Vegetative cells multiply within the mediastinal lymph node and gain entry into the bloodstream (20, 24).

Hanna et al. demonstrated that the macrophage is essential to lethal toxin (LeTx) toxicity in vivo (12). When mice were depleted of macrophages by silica treatment, they became insensitive to LeTx. Hanna and colleagues showed at a cellular level that sublytic levels of LeTx trigger the production of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) by the macrophage cell line RAW 264.7 and that anti-IL-1 antibody or an IL-1 antagonist protected mice from toxin lethality. Subsequent publications have described studies that have contradicted this finding, reporting that purified LeTx inhibits cytokine responses to lipopolysaccharide (LPS) and postulating that this activity may play a role in the early stages of infection by dampening the immune response and permitting early replication (1, 4, 15, 21, 23).

In this study, we present the first report of the cytokine response to infection of a murine macrophage cell line with purified *B. anthracis* spores. We examined the responses of 10 cytokines (TNF-α, IL-6, IL-1β, granulocyte-macrophage colony-stimulating factor, IL-2, IL-4, IL-5, IL-10, IL-12, and gamma interferon [IFN-γ]) to infection of macrophages with two strains of *B. anthracis*. The *B. anthracis* 7702 strain carries the pXO1 plasmid but not the pXO2 plasmid, and therefore this strain produces LeTx but not a capsule. The *B. anthracis* 9131 strain carries neither the pXO1 nor the pXO2 plasmid and is therefore lacking both the LeTx and the capsule. We selected J774A.1 cells as an example of a LeTx-sensitive cell line (13). J774A.1 macrophages were maintained in high-glucose Dulbecco minimal essential medium containing 10% fetal calf serum, L-glutamine, and phenol red at 5% CO_2. Spores were prepared by the method described by Finlay et al. (5). Spore preparations were heat treated at 65°C for 30 min to kill any remaining vegetative cells. Spore material was purified by centrifugation through Renografin (Renocal-76; Bracco Diagnostics, Princeton, N.J.). The purified spore pellet was washed once with cold distilled water and stored at 4°C. A viability count was performed on the spore preparation, and the preparation was adjusted to 10^9 CFU/ml. Modified Ziehl-Neelsen staining revealed that the preparations contained 100% spores.

Macrophages were seeded at 10^6/ml and were allowed to attach to 24-well plates for 2 h prior to infection. Monolayers were infected at a multiplicity of infection of 1:1 and were incubated for 30 min at 37°C. Gentamicin (10 μg/ml) was added to remove extracellular *B. anthracis*. After an additional 30-min incubation, monolayers were washed, and fresh complete medium (without antibiotics) was added. For all experiments, this was considered to be the zero time point. For cytokine determinations, J774A.1 cells were cultured in phenol-red-free and high-glucose Dulbecco minimal essential medium containing 10% serum and L-glutamine. For cultures that were treated with LPS, *Escherichia coli* O111:B4 LPS (1 μg/ml; Sigma Chemical Co.) was added to the cell monolayers at the zero time point. Samples of supernatant were taken at 2.5, 5, and 7.5 h postinfection. Aliquots were stored at −70°C until analysis was performed. Cytokine concentrations in culture supernatants were measured with a mouse cytokine Ten-Plex antibody bead kit (Biosource International, Camarillo, Calif.)

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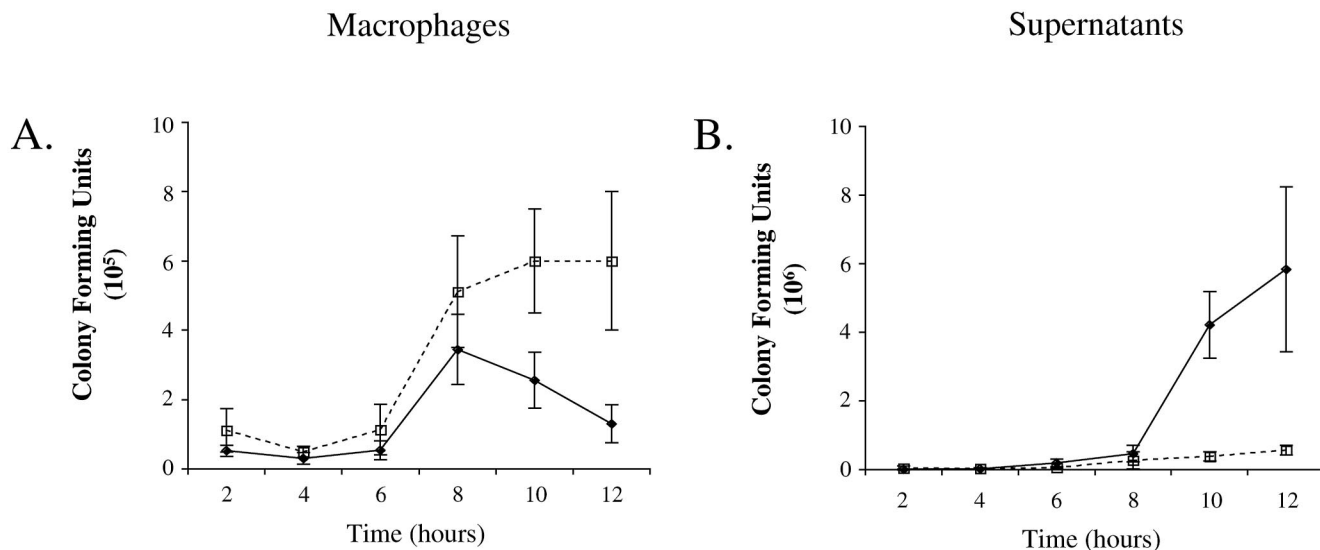
Macrophages

Supernatants

FIG. 1. Growth of *B. anthracis* strains 7702 and 9131 in J774 macrophages. Macrophages were infected with *B. anthracis* spores prepared from *B. anthracis* strain 7702 (filled diamonds) and *B. anthracis* strain 9131 (open squares), as described in the text. CFU were determined and are shown as a function of time postinfection. At each time point, the numbers of CFU present inside the macrophages (A) and in the supernatant (B) were determined. Four experiments were performed, and the results of a representative experiment are shown. Each value reported is the average for three samples ± the standard deviation.

and a Luminex model 100 analyzer (Luminex Corporation, Austin, Tex.).

Initial experiments were performed to study the growth of *B. anthracis* within J774A.1 macrophages. Measurements were made by counting viable *B. anthracis* organisms in macrophages and supernatant samples at 2, 4, 6, 8, 10, and 12 h postinfection (Fig. 1). After an initial lag phase of approximately 4 h, *B. anthracis* strain 7702 multiplies within J774A.1 macrophages until 8 to 10 h postinfection, at which time the cells lyse, releasing *B. anthracis* into the supernatant. In contrast, after the initial lag phase, *B. anthracis* strain 9131 multiplies within the macrophage but fails to cause cell lysis. The termination time of cytokine experiments at 7.5 h was therefore chosen to measure cytokine release prior to cell lysis. These experiments confirmed the sensitivity of the J774A.1 cell line to lysis after infection with a pXO1$^+$ strain of *B. anthracis*.

When macrophages were infected with purified *B. anthracis* spores alone, TNF-α was released as early as 5 h following infection, and its level continued to increase up to 7.5 h postinfection (Fig. 2A). The pXO1$^+$ strain caused an earlier release of higher levels of TNF-α than the pXO1$^-$ strain did. No other cytokines were induced upon infection of macrophages by *B. anthracis* spores alone (Table 1) with the exception of IL-12, which increased significantly between 5 and 7.5 h postinfection with spores from strain 7702 (Fig. 2A).

We observed the expected strong induction of TNF-α and IL-6 and the induction of IL-1β, IL-10, and IL-12 in response to the addition of LPS alone (Fig. 2B and Table 1). Our results indicate that the IL-12 response to LPS treatment was enhanced in *B. anthracis*-infected cells relative to uninfected cells (Fig. 2B and Table 1). Under all conditions studied, the cytokines IFN-γ, IL-2, IL-4, and IL-5 were produced at levels at or below the detection limit of the assay (data not shown). In contrast to previously reported results with purified LeTx (1, 4,

15, 21, 23), we found no evidence of an inhibitory effect on cytokine production by the macrophages in response to infection with spores from *B. anthracis* strain 7702 or 9131. LeTx-expressing strain 7702 caused a more rapid TNF-α response than did strain 9131. In addition, cells infected with spores from strain 7702 produced a small amount of IL-12, while cells infected with spores from the LeTx-deficient strain 9131 produced none. The absence of an IL-12 response to infection with strain 9131 spores may indicate that a factor encoded by plasmid pXO1 mediates IL-12 stimulation, either directly or through a regulatory pathway.

In this study, we present results indicating that a live infection of a mouse macrophage cell line with *B. anthracis* spores triggers TNF-α and IL-12 responses and enhances the LPS-induced production of IL-12. The stimulation of proinflammatory cytokines such as TNF-α is an important step in the activation of inflammatory pathways such as cellular recruitment, adhesion, and coagulation. The production of IL-12 may direct the immune system toward a cell-mediated helper T-cell response.

Previous reports indicated that purified LeTx inhibits cytokine production in macrophages (1, 4, 15, 21, 13). In those studies, the authors suggested that the observed LeTx inhibitory activity may be relevant during the early stages of infection. However, we observed no inhibition of cytokine induction upon infection of macrophages with spores capable of expressing LeTx. The infection of macrophages by *B. anthracis* spores results in the expression of multiple factors by the bacterium. The expression of a combination of regulatory pathways, activating cell surface protein factors, and toxic mediators, as well as the mechanical effects of a large intracellular population of expanding vegetative cells, may effect an influence on macrophage signaling, cytokine release, and the production of bactericidal oxygen and nitrogen radicals. Our results indicate
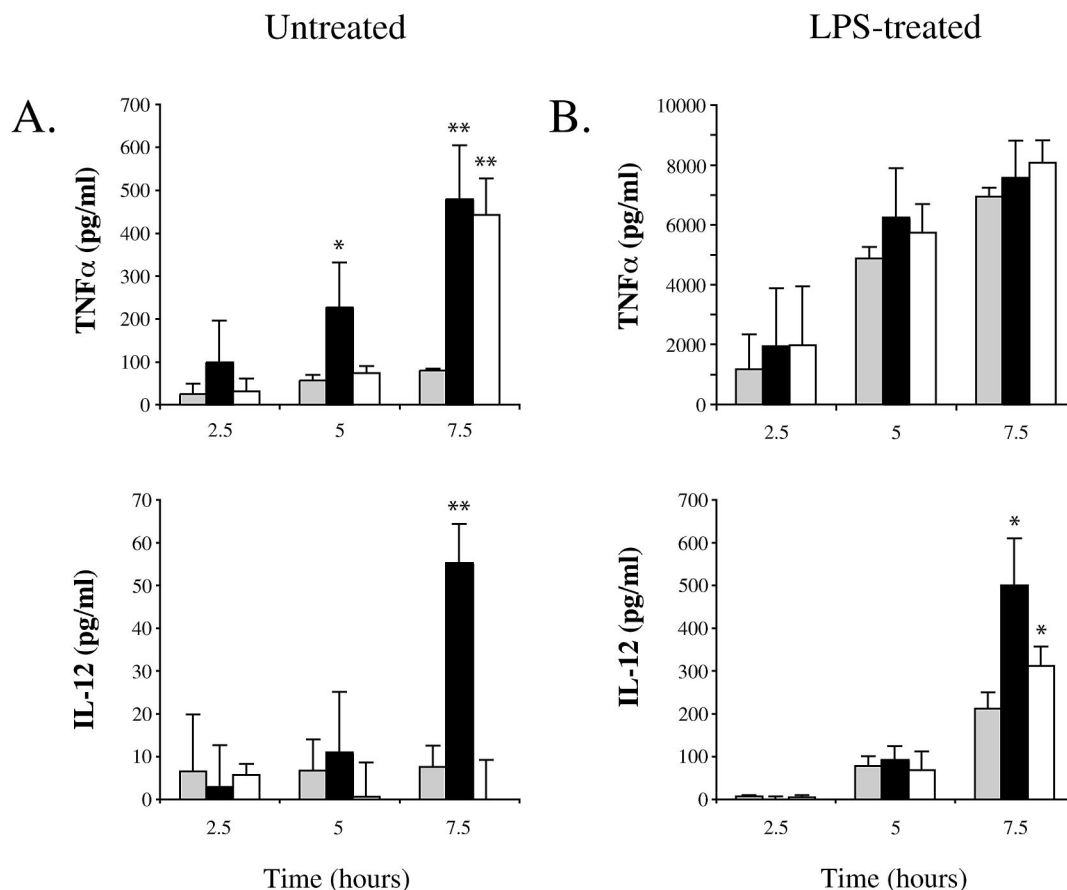
Untreated

LPS-treated

FIG. 2. Release of selected cytokines by macrophages in response to infection by *B. anthracis* strains 7702 and 9131. (A) Macrophages were left untreated (gray bars) or infected with *B. anthracis* spores prepared from *B. anthracis* strain 7702 (black bars) or *B. anthracis* strain 9131 (white bars), as described in the text. (B) Macrophages were treated with LPS alone (gray bars) or in combination with infection with *B. anthracis* spores prepared from *B. anthracis* strain 7702 (black bars) or *B. anthracis* strain 9131 (white bars), as described in the text. In all cases, cytokine concentrations were determined at the indicated time points. Four experiments were performed, and the results of a representative experiment are shown. Each value reported is the average for three samples ± the standard deviation. Statistical significance was determined using Student's *t* test analysis. In all cases, means were compared to those of the uninfected control groups. *, $P < 0.05$; **, $P < 0.01$.

either that LeTx is not produced at sufficient levels during the initial infection of macrophages to exert an inhibitory effect on cytokine production or that the inhibitory effect is not sufficient to overcome the stimulatory effects of a replicating intracellular pathogen.

The macrophage is thought to play a central role in the progression of anthrax from a local infection to a systemic disease. This model predicts that the macrophage carries anthrax spores from the site of infection to the regional lymph nodes, where the bacterial population expands and enters the bloodstream (9, 11, 24, 25). If this model is correct, the ability of *B. anthracis* to transit through the macrophage is critical to its ability to cause systemic disease. A detailed analysis of the interaction between the macrophage and the pathogen is es-

TABLE 1. Expression of cytokines by *B. anthracis*-infected macrophages

Cytokine[b]	Concn (pg/ml) after indicated treatment[a]					
	None	7702 infection	9131 infection	LPS	LPS + 7702 infection	LPS + 9131 infection
IL-6	49 ± 34	28 ± 55	6 ± 26	5,380 ± 750	7,152 ± 1,703	8,178 ± 1,828
IL-10	14 ± 7	1 ± 4	2 ± 1	82 ± 10	93 ± 17	84 ± 16
IL-1β	18 ± 15	4 ± 35	1 ± 30	119 ± 26	114 ± 16	128 ± 13
GM-CSF	72 ± 37	64 ± 34	60 ± 22	97 ± 32	110 ± 21	91 ± 13

[a] Macrophages were infected with *B. anthracis* spores with and without LPS-treatment, as described in the text. Cytokine concentrations are reported for each treatment group at 7.5 h postinfection. Four experiments with infections were performed, and the results of a representative experiment are presented. Each reported value represents the average for three samples ± 1 standard deviation.

[b] The limit of detection of the assay for IL-6, IL-1β, and granulocyte–macrophage colony-stimulating factor (GM-CSF) was 10 pg/ml, and that for IL-10 was 15 pg/ml.

sential to our understanding of the progression of *B. anthracis* infection. The results of this study provide insight into the effects of *B. anthracis* infection of the macrophage on the host's immune response. The results reported herein indicate that during the early stages of infection, the production of cytokines by macrophages is not inhibited by the intracellular *B. anthracis* spores. On the contrary, our results suggest that the host is stimulated to mount a proinflammatory response and is primed to mount a cell-mediated response towards what is, at this early stage of the infection, an intracellular pathogen.

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