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Effects of BCG Infection on the Susceptibility of Mouse Macrophages to Endotoxin

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Mice infected intravenously with *Mycobacterium bovis* (BCG) are 100 to 1,000 times more sensitive to the lethal effects of bacterial lipopolysaccharides (LPS). Since BCG infection results in macrophage activation and LPS may cause pathophysiological effects through interaction with this cell type, it was of interest to determine whether macrophages from BCG-infected animals were more susceptible to the toxic effects of LPS in vitro. When LPS-susceptible, C57BL/6 mice were infected with BCG, a significant reduction in the 50% lethal dose of LPS was first observed after 7 days and persisted for several weeks. Macrophages from these animals had greatly increased susceptibility to LPS in vitro, which correlated with the development of acquired cellular resistance as determined by their ability to inhibit the growth of *Listeria monocytogenes*. In contrast, BCG infection of C3H/HeJ mice, a strain resistant to LPS, did not alter the 50% lethal dose of LPS for these animals or increase the sensitivity of their peritoneal macrophages to LPS in vitro. These results indicate that susceptibility of BCG-infected mice to the lethal effects of LPS parallels the susceptibility of their macrophages in vitro; release of vasoactive substances from LPS-susceptible activated macrophages in vivo may be, in part, responsible for lethality.

Experimental infection of mice with facultative intracellular bacteria increases their susceptibility to the lethal effects of bacterial lipopolysaccharide (LPS) (1, 12, 32-34). The mechanism for this enhanced susceptibility has not been determined. Intracellular infections characteristically activate macrophages, causing increased accumulation of lysosomal enzymes (5, 24). LPS causes extensive vacuolation and death of macrophages in vitro; similar morphological alterations have been observed in vivo (18, 25). It is possible that macrophages that are activated by infection are killed more readily by LPS; release of toxic or vasoactive substances might then contribute to increased mortality.

The present series of experiments was designed to provide further insight into this phenomenon by studying the effect of BCG infection on LPS-induced cytotoxicity and lethality, using LPS-susceptible (C57BL/6) and LPS-resistant (C3H/HeJ) mice (9, 30).

MATERIALS AND METHODS

Animals. C57BL/6 and C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Mice were fed standard laboratory chow and acidified-chlorinated water ad libitum and were used when 8 to 10 weeks old.

Bacteria. *Mycobacterium bovis* BCG (Tice) was maintained by weekly transfers in Dubos liquid medium (Difco Laboratories, Detroit, Mich.) containing 5% albumin. A human isolate of *Listeria monocytogenes* was adapted to mice by serial passage and grown in brain heart infusion (Difco) at 37°C for 18 h.

Endotoxin. LPS, extracted by the phenol/water technique from a galactoseless mutant of *Salmonella typhimurium* (SL 684), was generously provided by J. W. Shands, Jr., University of Florida College of Medicine, Gainesville. The mitogenic and cytotoxic activities of LPS from this strain have been described previously (18-20).

Experimental procedure. Mice were infected intravenously with 10^8 BCG obtained from a 21- to 28-day-old culture. At regular intervals thereafter, groups of five mice were challenged intravenously with one of five serial fivefold dilutions of LPS. Uninfected mice received similar doses of LPS at each time interval. Deaths resulting from LPS were recorded, and the 50% lethal dose (LD_{50}) was determined by using the method of Reed and Muench (22). Additional groups of normal and BCG-infected mice received 5×10^5 viable *L. monocytogenes* intravenously to demonstrate acquired cellular resistance.

Quantitation of *L. monocytogenes*. Spleens and livers from listeria-infected mice were removed 48 h after challenge and homogenized in 5 ml of sterile distilled water. Serial 10-fold dilutions were prepared, and 0.01-ml aliquots were plated on nutrient agar;

colonies were counted after incubation at 37°C for 24 h.

Cell preparation and tissue culture conditions. Peritoneal leukocytes from BCG-infected and normal mice were harvested by injecting 4 ml of RPMI 1640 containing 1 U of heparin per ml into the peritoneal cavity and aspirating the fluid contents via a 19-gauge needle. Cells were washed, counted, and suspended at 2×10^6 cells per ml in a complete tissue culture medium, which was supplemented with 10% fetal calf serum or, in selected experiments, with adult, heat-inactivated human serum (6). Adherent cell monolayers were prepared by adding 0.3 ml of the peritoneal leukocyte suspension to sterile glass cover slips (22 by 22 mm) as described previously (18).

Peritoneal leukocytes (2×10^6 in 1 ml of complete tissue culture medium) were added to plastic tubes (12 by 75 mm, no. 2003; Falcon Plastics, Oxnard, Calif.); adherent cell monolayers were placed in 35-mm plastic petri dishes (Falcon, no. 3001) containing 1 ml of complete tissue culture medium. All cultures were incubated under Mishell-Dutton conditions for 48 h (6). Immediately thereafter, culture viability was determined by trypan blue exclusion. Results are reported as the mean percent viable cells ± standard error of the mean.

Cellular differentiation and histochemical staining for acid phosphatase. Peritoneal leukocytes from normal and BCG-infected mice were pelleted onto glass microscope slides by cytocentrifugation (Cytospin SCA-0030, Shandon Southern Products, Ltd., Cheshire, England), stained with May-Grünwald-Giemsa stain, and examined by light microscopy. Differential counts of at least 300 cells were performed according to morphological and functional criteria described previously (18, 25). Acid phosphatase activity of peritoneal leukocytes was determined by a modification of the method described by Li et al. (17).

Quantitation of LPS in serum. Venous blood from a human volunteer was aseptically collected, transferred to pyrogen-free (180°C for 4 h) glass tubes, and incubated at 37°C for 2 h. Serum was separated from cellular elements by centrifugation, treated with chloroform (16), and diluted 1:10 with nonpyrogenic water (Travenol, Deerfield, Ill). Serial twofold dilutions were prepared, and 0.1-ml samples were added to an equal volume of *Limulus* amebocyte lysate (Microbiological Associates, Bethesda, Md.) and incubated at 37°C for 1 h. This assay detected LPS at levels of ≥0.06 ng/ml. No LPS was detectable in human serum, but fetal calf serum used in these studies was positive.

Statistical analysis. Regression analysis of log transformed data and statistical comparisons using Student's *t* test were carried out by Daniel E. Kastner, Baylor College of Medicine.

RESULTS

Experiments using LPS-sensitive mice. (i) Effect of BCG infection on susceptibility to LPS in vivo. BCG infection increased the susceptibility of C57BL/6 mice to LPS. In a representative experiment (Table 1), the LD_{50} of LPS administered intravenously to uninfected mice was 130 μg; this decreased to 2 μg 7 days after BCG infection and to 0.8 μg and 0.7 μg, respectively, 14 and 21 days after BCG infection. Increased susceptibility to LPS was temporally associated with increased ability to inhibit the growth of *L. monocytogenes*.

(ii) Effect of BCG infection on peritoneal leukocyte populations. The numbers and kinds of leukocytes present in the peritoneal cavity were altered very little by BCG infection. The mean number of nucleated cells obtained from 10 normal mice was 3.74×10^6 (Table 2). This number decreased slightly 7 days after BCG infection and then increased to 3.99×10^6 and 4.90×10^6, respectively, 14 and 21 days after infection. These differences were not significant ($0.1 < P < 0.25$). A slight tendency toward greater numbers of macrophages was observed, but this also failed to achieve statistical significance. Peritoneal macrophages recovered from mice during periods of peak resistance to infec-

TABLE 1. *Effect of BCG infection on susceptibility of C57BL/6 mice to LPS and resistance to listeria*

Days after BCG infection	LD_{50} of LPS (μg)	Log_{10} listeria recovered per organ[a]	
		Liver	Spleen
0	130	7.74 (7.61–7.89)	7.18 (6.92–7.38)
7	2.2	5.78 (5.02–6.36)	4.85 (4.03–5.56)
14	0.8	5.27 (4.62–5.96)	4.34 (3.30–5.48)
21	0.7	6.74 (6.28–7.19)	5.83 (5.28–6.35)

[a] Arithmetic mean; range is noted in parentheses.

TABLE 2. *Differential counts of peritoneal leukocytes from BCG-infected and normal C57BL/6 mice*

Time after BCG (days)	Number of nucleated cells per mouse × 10^6 (mean ± SEM)[a]	Mononuclear leukocytes[b]			Polymorphonuclear leukocytes		
		Mφ	SL	LL + blasts	Neutrophils	Basophils	Eosinophils
0	3.74 ± 0.46	55	24	16	1	3	1
7	3.23 ± 0.25	55	24	14	0	6	1
14	3.99 ± 0.38	63	20	14	1	2	0
21	4.90 ± 0.89	63	19	14	1	3	0

[a] SEM, Standard error of the mean.
[b] Abbreviations used: Mφ, macrophages; SL, small lymphocytes; LL, large lymphocytes.

tion appeared to be activated, based on their increased size and increased numbers of cytoplasmic granules that stained positively for acid phosphatase.

(iii) Effect of BCG infection on susceptibility of leukocytes to LPS in vitro. Experiments were then conducted to determine the susceptibility of peritoneal leukocytes from normal and BCG-infected mice to LPS in vitro. Unseparated peritoneal leukocytes were incubated for 48 h at 37°C with varying concentrations of LPS from 0.5 to 50 μg/ml. In the absence of LPS, more than 90% of cells from uninfected mice remained viable (Fig. 1). A lower percentage of these cells survived when incubated with LPS; this decreased survival was dose related between 0.5 and 50 μg/ml. Survival of leukocytes from BCG-infected mice was decreased in the absence of LPS, with fewer than 50% of cells from 10- and 17-day infected mice remaining viable. Addition of LPS (0.5 to 50 μg/ml) caused further dose-related decreases in the viability of peritoneal leukocytes from these mice. The observed differences in viability were not due to extensive loss of cells. When cells adhering to the monolayer were counted by inverting the cover slip on a hemacytometer, there was no apparent decline in the number of cells during the 48-h incubation.

(iv) Effect of BCG infection on susceptibility of macrophages to LPS in vitro. Varying concentrations of LPS were then added to the adherent cell monolayer (macrophages) from normal and BCG-infected mice. The percentage of cells remaining viable at each concentration of LPS after 48 h of incubation at 37°C was used to determine regression equations, and the slope of each experimental line was compared to the slope of the control using Student's *t* test. There was no difference in the survival of macrophages from normal mice or from mice infected 7 days previously with BCG; in both cases, LPS caused a dose-dependent decrease in survival of peritoneal macrophages (Fig. 2). In contrast, macrophages from mice infected 14 and 21 days earlier had decreased viability in vitro, and addition of LPS caused greater rates of killing of these cells than of uninfected or 7-day-infected cells (slopes of curves for 14- and 21-day-infected mice were significantly greater

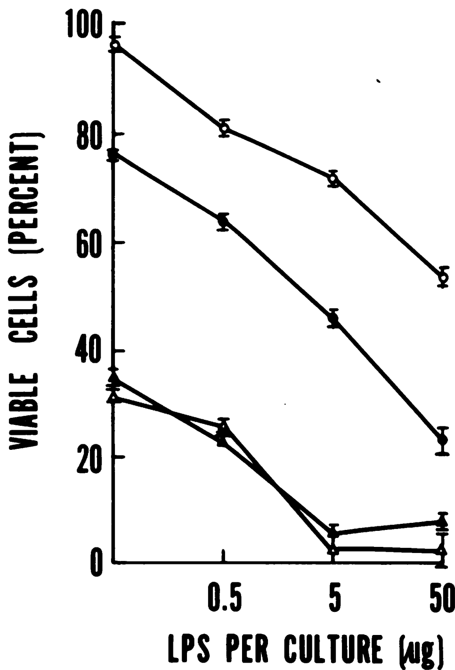

FIG. 1. *Effects of BCG infection on the susceptibility to LPS of peritoneal leukocytes. Peritoneal leukocytes (2 × 10⁶) from uninfected controls, C57BL/6 (○), or from mice infected 5 (●), 10 (△), or 17 days (▲) previously with BCG, were incubated in culture tubes (12 by 75 mm) with the indicated concentrations of LPS. Cell viabilities, determined after 48 h by trypan blue exclusion, are expressed as the mean of duplicate cultures ± standard error of the mean.*

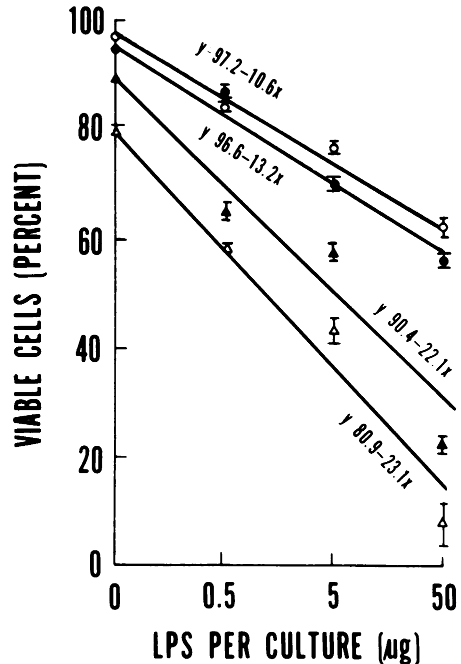

FIG. 2. *Cytotoxicity of LPS for macrophages from BCG-infected mice. Adherent cell monolayers were prepared using peritoneal leukocytes from normal (○) C57BL/6 mice and from mice 7 (●), 14 (△), and 21 (▲) days after BCG infection. Results are expressed as the mean of triplicate cultures ± standard error of the mean.*

than of uninfected mice, $P < 0.005$).

The possibility that LPS contamination of fetal calf serum (27) was responsible for decreased viability of BCG-infected leukocytes in the absence of added LPS was studied by repeating these experiments using aseptically collected adult human serum instead of fetal calf serum. Incubation of macrophages in complete tissue culture medium supplemented with this human serum was associated with significantly improved viability (Table 3), although the decreased survival of BCG-infected macrophages indicated that factors inherent to the cells themselves are also partly responsible.

Experiments using LPS-resistant mice. (i) Effect of BCG infection on susceptibility to LPS in vivo. Entirely different results were obtained when similar studies were carried out in C3H/HeJ mice. In four separate experiments, BCG infection did not alter the susceptibility of C3H/HeJ mice to LPS, although acquired cellular resistance could be demonstrated by suppressed growth of *L. monocytogenes*. Data from a representative experiment (Table 4) show that the LD_{50} of LPS for C3H/HeJ mice, with or without BCG infection, ranged from 3,200 to 3,600 μg. BCG infection produced no significant change in peritoneal cell populations of C3H/HeJ mice. Total cell counts and differentials before or after infection did not differ significantly from those of C57BL/6 mice.

TABLE 3. *Viability of peritoneal leukocytes from C57BL/6 mice in media supplemented with heterologous sera*

Cell source	Serum	Culture viability[a] (%)
Normal leukocytes	FCS[b]	98 ± 2
	Human	99 ± 4
BCG leukocytes	FCS	55 ± 3
	Human	81 ± 0
Normal adherent cells	FCS	97 ± 1
	Human	95 ± 2
BCG adherent cells	FCS	76 ± 2
	Human	94 ± 1

[a] Mean ± standard error of the mean.
[b] FCS, Fetal calf serum.

TABLE 4. *Effect of BCG infection on susceptibility of C3H/HeJ mice to LPS and resistance to listeria*

Days after BCG infection	LD_{50} of LPS (μg)	Log_{10} listeria recovered per organ[a]	
		Liver	Spleen
0	3,300	7.97 (7.62–8.33)	7.57 (7.20–8.16)
10	3,600	5.61 (5.22–6.16)	4.86 (4.57–5.17)
15	3,200	5.22 (4.75–5.76)	4.69 (4.47–5.39)
21	3,400	4.72 (4.02–5.34)	4.84 (4.11–6.00)

[a] Arithmetic mean; range is noted in parentheses.

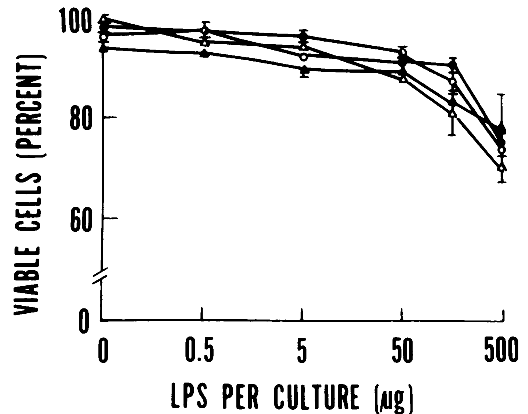

FIG. 3. *Effects of BCG infection on the toxicity of LPS for C3H/HeJ macrophages. LPS was added to cultures containing peritoneal adherent cells from C3H/HeJ mice. Experimental details and organization of this figure are as described in Fig. 2.*

(ii) Effect of BCG infection on susceptibility of macrophages to LPS in vitro. Monolayers of peritoneal adherent cells from C3H/HeJ mice were prepared at varying intervals after BCG infection and incubated for 48 h with 0.50 to 500 μg of LPS. No reduction in macrophage viability was seen at LPS concentrations of 0.5 to 50 μg/ml (Fig. 3). At higher concentrations, LPS was toxic for both normal and BCG-infected macrophages, but differences among these groups were not discernible.

DISCUSSION

Results of these studies show that BCG infection increases the sensitivity of macrophages from LPS-susceptible mice to the lethal effects of LPS in vitro. This increased macrophage sensitivity developed concomitantly with acquired cellular resistance to heterologous bacteria, correlating closely with a reduction in the LD_{50} of LPS. Very different results were obtained with C3H/HeJ mice, a strain resistant to many of the biological activities of LPS (9, 13, 14, 28, 30, 31, 35). The LD_{50} of LPS for BCG-infected C3H/HeJ mice and the survival of their macrophages in vitro during incubation with LPS were unchanged when compared to uninfected controls. Although LPS-refractory mice are usually resistant to only a 25-fold increase in LPS dose, a 5,000-fold difference in lethality was demonstrated after BCG infection. Comparison of LPS-induced responses in BCG-infected mice of these strains may provide a means for elucidating the mechanisms of endotoxicity.

We (18, 25) and others (8) have previously shown that the lethality of LPS for mice parallels the in vitro susceptibility of their macro-

phages to LPS. The present study extends these findings by demonstrating that BCG infection renders macrophages from LPS-susceptible mice more sensitive to LPS but does not alter the sensitivity of macrophages from LPS-resistant mice. These results provide further evidence that a major target for lethal effects of LPS is the macrophage.

The mechanism of LPS-mediated cytotoxicity in BCG infection can probably be divided into two separate stages. First, phagocytosis and/or interaction with antigen-stimulated lymphocytes during experimental infection causes macrophage activation with increased accumulation of lysosomal enzymes and/or biological active mediators. Second, exposure to LPS, possibly promoted by the increased capacity of activated macrophages for particle uptake, leads to a release of these substances, perhaps as a result of cell autolysis. The way in which these substances enhance lethality is obscure, although altered vasoactivity may be, in part, responsible.

The failure to increase the sensitivity of C3H/HeJ mice to the lethal effects of LPS appears to result not from an inability of BCG to activate macrophages but from the inability of LPS to elicit the appropriate response(s) from the macrophage. This supports the earlier suggestion (35) that cells from LPS-resistant mice lack receptors that interact with LPS.

One of the most striking features of BCG macrophages is their capacity to kill tumor cells in vitro (4, 7, 21). Toxicity for tumor cells does not result from a phagocytic event but requires cell-to-cell contact and appears to be mediated by exocytosis of lysosomes into the tumor cell cytoplasm (10) or by release of soluble toxins into the microenvironment (15). Several recent observations provide evidence that LPS may play an integral role in the killing of tumor cells by BCG-infected macrophages. Hibbs et al. demonstrated that BCG macrophages were not cytotoxic in vitro unless incubated in sera contaminated with LPS (11). Furthermore, the cytotoxic activity of these cells could be augmented by the addition of purified LPS preparations. Interestingly, Ruco and Meltzer reported that peritoneal macrophages from BCG-infected C3H/HeJ mice were only minimally cytotoxic in vitro (23). These studies indicate that tumor cell killing by BCG macrophages can be modified by LPS under culture conditions similar to those used in this study and suggest that the tumoricidal mechanism and the autolytic process may be similar.

The possibility that LPS augments the tumoricidal capacity of macrophages from BCG-infected animals suggests a mechanism by which lethality of LPS is increased in BCG infection. Macrophages with the greatest killing activity appear to be localized to the liver and spleen, the organs which are also responsible for clearing LPS after intravenous injection. Interestingly, previous reports have demonstrated severe hypoglycemia and hepatic damage following LPS administration in BCG-infected mice (3, 26). To date, evidence that BCG-infected macrophages can kill normal cells has not been provided conclusively. It is possible, however, that LPS-treated BCG macrophages could be considerably more active in this respect.

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