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Correlation of Increased Metabolic Activity, Resistance to Infection, Enhanced Phagocytosis, and Inhibition of Bacterial Growth by Macrophages from Listeria- and BCG-Infected Mice

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Macrophages from mice infected with facultative intracellular organisms such as *Listeria monocytogenes* and BCG have been shown to resist infection by antigenically unrelated intracellular bacterial parasites. This study compares phagocytosis, bacterial growth inhibition, and oxidation of glucose by macrophages from normal mice, mice infected with listeria or BCG, or mice immunized with killed listeria in incomplete Freund's adjuvant. Macrophages from listeria- and BCG-infected mice ingested more listeria; 67 and 57%, respectively, had three or more cell-associated bacteria versus 22% of controls ($P < 0.001$). Peritoneal macrophages from listeria- and BCG-infected animals significantly ($P < 0.001$ covariance analysis) inhibited growth of listeria in suspension, whereas control macrophages had no such inhibitory effect. The rate of oxidation of glucose-*1*-^{14}C was higher in macrophages from listeria- and BCG-infected mice than from either uninfected animals or those immunized with killed listeria. During phagocytosis of killed or live bacteria, or latex particles, the rate of glucose oxidation was increased ($P < 0.01$). These data suggest that the cellular immunity after infection by an intracellular organism is associated with an increase in metabolic activity of macrophages, namely, an increase in the rate of glucose oxidation resulting in enhancement of phagocytosis and killing.

Mice infected with *Listeria monocytogenes*, BCG, and *Besnoitia jellisoni* or treated with compounds such as polyinosinic:polycytidilic acid resist infection by intracellular bacteria (8, 10, 16). Such protection appears to be independent of serum factors and has been attributed to enhanced function of macrophages. The purpose of the present study was to compare one aspect of metabolism of resting and phagocytizing macrophages obtained from normal mice, from animals infected with *L. monocytogenes* or BCG, and from mice immunized with killed listeria suspended in incomplete Freund's adjuvant. In addition, we assessed the phagocytic activity and inhibition of growth of listeria by macrophages from untreated mice and from animals injected with viable BCG, or living or killed *L. monocytogenes*.

MATERIALS AND METHODS

Mice. Conventionally raised female mice (Charles River Laboratory strain), 35 to 40 days old (22 to 25 g), were used in all the experiments.

Bacteria. A strain of *L. monocytogenes* isolated from a patient with meningitis was passed through mice several times. The median lethal dose (LD_{50}) was approximately 10^7 organisms by the subcutaneous and 10^6 by the intraperitoneal route.

A single colony of listeria was inoculated into Todd-Hewitt broth and incubated at 37 C for 18 hr. The number of colony-forming units at this time was 2×10^9 to 3×10^9 per ml. For each experiment, a sample was centrifuged at 7,000 $\times$ *g* (5 C), washed twice in iced Hanks balanced salt solution (HBSS), and resuspended to its original volume in HBSS. Listeria were killed by exposure to ultraviolet light for 30 min; the inactivated organisms failed to grow in Todd-Hewitt broth at 37 C for 48 hr.

BCG (Lilly) was reconstituted in distilled water to yield 4×10^5 to 9×10^5 viable organisms per 0.1 ml.

Inoculation of mice. Listeria-infected mice were inoculated intraperitoneally with 2.0×10^5 to 2.5×10^5 viable bacteria; BCG-infected animals received 4×10^5 to 9×10^5 viable organisms intravenously 3 weeks and again 3 days before either challenge with live listeria or collection of their macrophages; and listeria-immune mice were given a subcutaneous in-

jection of 10^9 killed organisms in incomplete Freund's adjuvant.

Macrophages. The peritoneal cavities of mice were injected with 4.0 ml of iced HBSS containing 10 units of heparin per ml, the abdomens were massaged, and the peritoneal cells were harvested by aspiration. The yield from normal, listeria- or BCG-infected animals averaged 3×10^6 leukocytes per mouse; 65 to 70% of these were macrophages; 25 to 30%, lymphocytes; 3%, neutrophils; and 1%, mast cells. Peritoneal leukocytes from groups of treated or control mice were pooled, washed in HBSS with heparin, resuspended to give equal concentrations of macrophages, and held at 5 C until use.

Enumeration of bacteria in spleen. Animals were killed in groups of five at 25, 48, 72, 96, 120, and 144 hr after intraperitoneal inoculation with 2×10^5 listeria. The spleens were removed, suspended in 5 ml of sterile distilled water, and homogenized in no. B size grinding flasks (A. H. Thomas) by power-driven Teflon rods. Serial dilutions were made, and 0.1-ml fractions were spread onto the surface of Eugon agar (Difco). Colonies were counted after incubation at 37 C for 24 hr.

Phagocytosis and growth inhibition studies. Equal numbers of washed macrophages (10^6 to 2×10^6) from mice infected with BCG or listeria and from uninfected controls were suspended in a total volume of 1 ml containing 20% calf serum, 5% complement, and 75% HBSS. Live listeria (10^7 to 2×10^7) were added to yield a bacteria to macrophage ratio of 10:1. These suspensions were then distributed in duplicate into 3.0-ml polyethylene capped tubes (Falcon Plastics) and tumbled at 37 C. For studies of phagocytosis, the shaking was stopped after 30 min and duplicate smears of each reaction mixture were made on cover slips and stained with Wright stain. Two hundred macrophages were examined, and cells were considered to be actively phagocytic when three or more bacteria appeared to be within the cytoplasm. The ability of macrophages to inhibit growth of listeria was studied in the following manner. Suspensions containing cells and organisms, as described above, were incubated at 37 C with tumbling for various periods of time. At the end of 30, 60, 120, and 180 min, 0.5 ml was removed, added to 4.5 ml of sterile distilled water and sonically treated (Biosonik I, intensity 50) for 30 sec. This procedure disrupted macrophages without affecting the viability of the bacteria. Samples (0.1 ml) of 10-fold serial dilutions were spread on Eugon agar for counting.

Metabolic studies. Equal numbers of macrophages (in 0.5 ml of HBSS), prepared as described above from control and experimental animals, were placed in a series of 25-ml Erlenmeyer flasks (center well). To each of these was added 0.4 ml of HBSS containing 40% calf serum and 10% complement (GIBCO). Latex particles or killed or live bacteria, suspended in 0.1 ml of HBSS, were then added. Experimental mixtures contained either bacteria or latex particles in a ratio of 100 particles to one macrophage; 0.1 ml of HBSS was added to control flasks. Two types of controls were included in the study: (i) listeria without macrophages and (ii) macrophages alone. One-fourth microcurie of glucose-*1*-^{14}C (New England Nuclear Corp.) in 0.2 ml was added sequentially to each flask which was then sealed immediately with a rubber cap and placed in a shaking water bath at 37 C. After 30 min, 0.4 ml of hydroxide of hyamine was injected into a glass cup placed in the center well, and 1 ml of 2 N HCl was added to the macrophage suspension to release intracellular $^{14}CO_2$. The glass cups were removed and placed into vials containing 10 ml of scintillation fluid [0.4% diphenyloxazole, 0.1% 1,4-bis-2-(5-phenyloxazolyl) in toluene]. Radioactivity was measured in a scintillation counter (Nuclear-Chicago). The quantity of $^{14}CO_2$ produced by macrophages phagocytizing live listeria was calculated by subtracting the production of $^{14}CO_2$ by listeria alone. The contribution of the other cell types in the peritoneal suspension (lymphocytes and the small number of polymorphonuclear leukocytes) to total glucose oxidation appeared to be negligible.

RESULTS

In vivo resistance to Listeria monocytogenes infection in listeria- or BCG-infected mice. Uninfected mice and those which had been given a subcutaneous injection of 0.1 LD_{50} listeria 7 days previously were injected intraperitoneally with 2×10^5 listeria. The spleens of the immunized animals consistently contained fewer

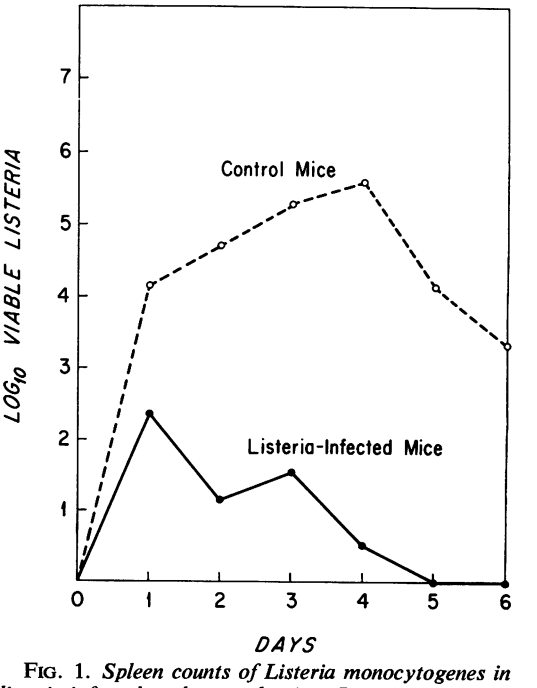

FIG. 1. *Spleen counts of Listeria monocytogenes in listeria-infected and control mice. L. monocytogenes (2.5×10^5) were inoculated ip into control mice and animals infected subcutaneously with 0.1 LD_{50} listeria 7 days before.*

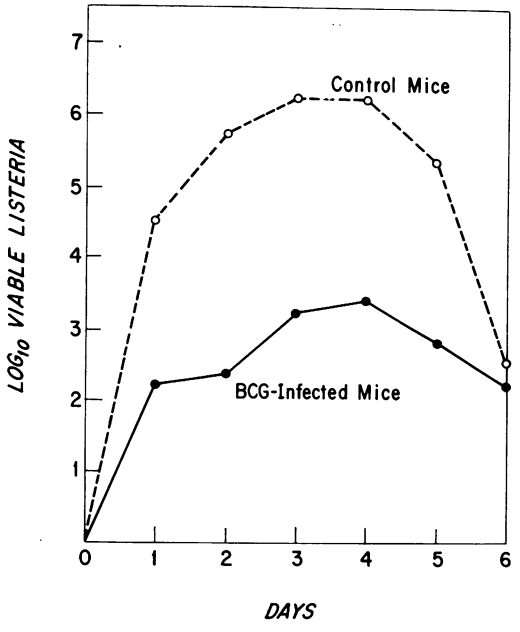

FIG. 2. *Spleen counts of Listeria monocytogenes in BCG-infected and control mice. L. monocytogenes (2.5×10^5) were inoculated ip into control mice and animals infected iv with 4×10^5 to 9×10^5 BCG, 3 weeks and 3 days before listeria challenge.*

TABLE 1. *Phagocytosis of listeria by macrophages from uninfected mice and mice infected with listeria or BCG*

Group tested	Macrophages with 3 or more cell-associated listeria/total no. counted	Difference vs. control[a]
Control	223/1,000 (22%)	
Listeria-infected	532/800 (67%)	$P < 0.001$
BCG-infected	453/800 (57%)	$P < 0.001$

[a] χ^2 test.

organisms (Fig. 1); after 4 days, listeria had been virtually eliminated. The spleens of controls continued to harbor live bacteria for at least 6 days. Mice inoculated with BCG at 3.5 weeks and at 3 days prior to infection with listeria also had many fewer organisms in their spleens than did the control animals (Fig. 2).

Direct observation of phagocytosis. Listeria were ingested more avidly by macrophages from animals infected with either BCG or *L. monocytogenes* than by cells obtained from uninfected animals (Table 1).

Growth inhibition. The growth of listeria was inhibited to a significantly greater degree by macrophages from infected animals (*L. monocytogenes* or BCG) than by those obtained from control mice (Fig. 3 and 4; $P < 0.001$ covariance analysis). In the presence of normal macrophages, multiplication of listeria paralleled the growth of this organism in the absence of cells. Serum obtained from animals which had survived two sublethal infections with listeria did not enhance growth inhibition (Fig. 5).

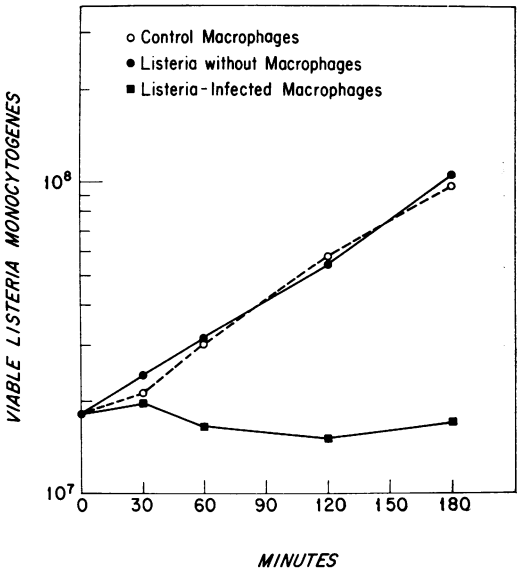

FIG. 3. *Growth inhibition of listeria by macrophages from listeria-infected and control mice ($P < 0.001$ covariance analysis). Listeria-infected macrophages were obtained from peritoneal cavities of animals inoculated ip with 2.2×10^5 listeria 7 days before.*

Metabolic studies. Macrophages from mice infected with listeria consistently oxidized glucose-*1*-^{14}C at a higher rate than those obtained from control animals (435 counts per min per million cells versus 262 counts per min per million cells; $P < 0.05$; Table 2). Phagocytosis of live or killed listeria or latex particles by macrophages from infected mice (*L. monocytogenes* or BCG) was accompanied by significantly greater increases in oxidation of glucose than phagocytosis by control cells. These data indicate that macrophages from infected animals demonstrate both a higher baseline activity and greater increment in metabolism during phagocytosis than macrophages from uninfected animals. On the other hand, glucose metabolism of macrophages obtained from mice immunized 10 days previously with killed listeria did not differ significantly from controls (Table 2).

DISCUSSION

Animals infected with BCG, *L. monocytogenes*, *Brucella*, or *Toxoplasma gondii*, all of which

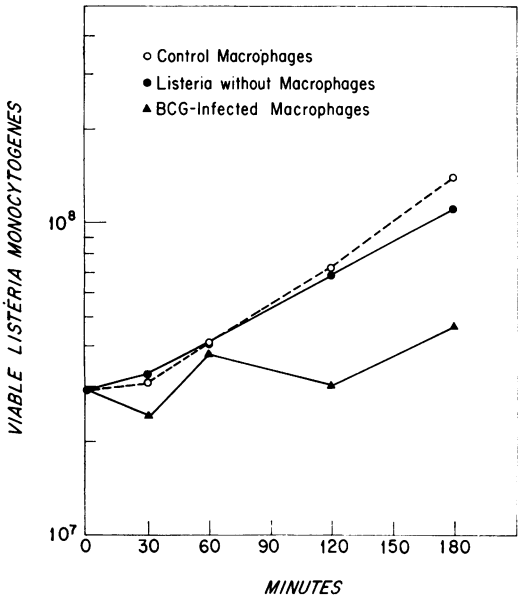

FIG. 4. *Growth inhibition of Listeria monocytogenes by macrophages from BCG-infected and control mice ($P < 0.001$ covariance analysis). BCG-infected macrophages were obtained from peritoneal cavities of animals inoculated iv with 4×10^5 to 9×10^5 BCG 3 weeks and again 3 days before.*

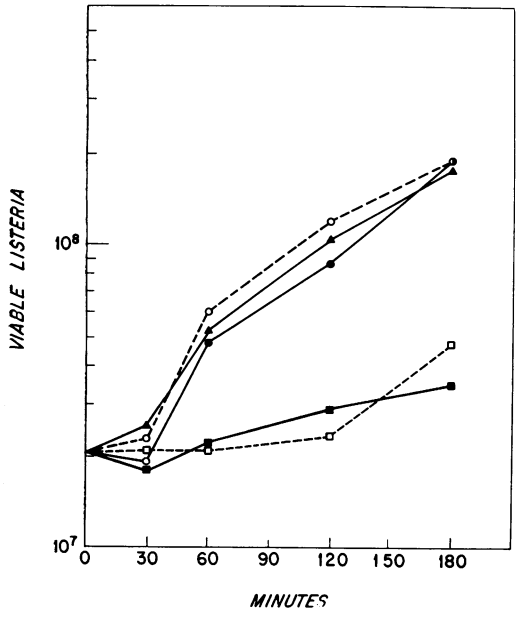

FIG. 5. *Growth inhibition of Listeria monocytogenes by peritoneal macrophages. Symbols:* (○) *control macrophages and normal mouse serum,* (●) *control macrophages and anti-listeria serum,* (▲) *Listeria monocytogenes alone,* (□) *listeria-infected macrophages and normal mouse serum, and* (■) *listeria-infected macrophages and anti-listeria serum.*

TABLE 2. *Oxidation of glucose by nonphagocytizing and phagocytizing macrophages*

Macrophage	State of phagocytosis (counts/min/10^6 macrophages)			
	No particles	Latex	Listeria	
			Killed	Live
Control	262	390	346	664
Listeria-infected	435[a]	1,735[a]	1,650[a]	1,595[a]
BCG-infected	416[a]	1,517[a]	1,143[a]	1,243[a]
Listeria-immunized	353[b]	505[b]	505[b]	858[b]

[a] Student t test; $P < 0.01$ compared to control macrophages.

[b] No significant difference compared to controls.

survive and multiply within macrophages, have been shown to be resistant to challenge with the same or other facultative intracellular organisms (8, 10, 16). This has been attributed to three properties of such macrophages: (i) resistance to the cytotoxic effects of ingested bacteria, (ii) killing of organisms, and (iii) inhibition of bacterial multiplication (3, 6, 9). With listeria, only live organisms induce this type of resistance (4).

The results of the present studies with respect to the protection of mice previously infected with listeria or BCG against subsequent challenge with *L. monocytogenes* confirm those of Mackaness (10). In addition to this phenomenon, other properties of BCG- or listeria-stimulated macrophages were demonstrated. These included increased phagocytic capacity, enhanced metabolic activity, and a greater degree of inhibition of bacterial growth. Our studies indicating that anti-listeria serum does not enhance the killing of listeria by macrophages confirm the observation that resistance to infection with listeria is directly related to cellular and not to humoral immunity (13). In addition, the similarity between the rate of glucose oxidation by macrophages obtained from animals immunized with killed listeria and by those obtained from normal animals is consistent with Coppel and Youmans' finding that live, not killed, listeria confer resistance to subsequent listeria challenge (4). Nonetheless, whereas our data are consistent with activation of macrophages in the absence of serum antibody, they do not exclude the possibility that cytophilic antibody may be responsible for the heightened activity of the "immune macrophages." It should be noted that, although BCG infection conferred both in vivo resistance to listeria challenge and in vitro enhancement of metabolic, phagocytic, and growth-inhibiting capacities of peritoneal macrophages when exposed to live listeria, the magnitude of these

activities was distinctly greater after infection with *L. monocytogenes*. Thus, although BCG infection conferred nonspecific macrophage activation, listeria infection produced an even greater macrophage response which suggests that there was some specificity in the immune reaction induced by viable listeria. The specificity of this reaction resides with the lymphocyte (11) which secretes factors known to enhance macrophage function (14, 18).

Although objection may be raised to our use of a relatively avirulent strain of *L. monocytogenes*, the fact that there was a convincing difference between bacterial counts in spleens of infected and control mice suggests that virulence of the organism was not a critical factor. Despite the fact that the listeria were not opsonized prior to their exposure to macrophages, there was a highly significant difference in growth inhibition between macrophages from control and infected animals. Furthermore, the addition of anti-listeria serum to control and immune macrophages, which served to opsonize the listeria, did not enhance growth inhibition.

Our method of studying growth inhibition of listeria measured a composite of extracellular multiplication and intracellular killing of the organisms. It did not delineate the fate of the bacteria once ingested by macrophages. Nonetheless, a significant difference was demonstrated between infected (BCG and listeria) and control macrophages. In addition, our techniques were designed to study in vitro functions of macrophages; they cannot be compared with those designed to study in vivo bacterial killing such as experiments by Blanden et al. (3) who studied the ability of peritoneal leukocytes to ingest and inactivate salmonella within the peritoneal cavities of mice.

It has been found that "activated" macrophages spread more rapidly and fully on glass, contain more mitochondria and intracytoplasmic vesicles, have a more extensive Golgi apparatus and many more electron-opaque particles, presumably representing lysosomes, and exhibit a higher degree of hydrolytic enzyme activity (1, 2, 7, 17, 19).

It is surprising that in the course of extensive studies of "activated" macrophages few observations have been made of their metabolic activity. Evans and Myrvik (5) demonstrated that BCG vaccination stimulated the rate of oxygen uptake and hexose monophosphate shunt activity of rabbit alveolar macrophages. Mackaness (12) has cited unpublished data which claim to show an increased respiratory rate in "activated" peritoneal macrophages. Nathan et al. (11) reported that, when these cells are exposed to mediators from lymphocytes, they exhibit increased adherence to glass, enhanced phagocytosis, and increased hexose monophosphate shunt activity. However, Hard (7) has suggested that "resting" (nonphagocytizing) macrophages utilize more oxygen, have more adenosine triphosphate, and incorporate ^{14}C-glycine into protein at a faster rate than "immune" cells. Although there is some disagreement regarding the contributions of glycolysis and hexose monophosphate shunt activity during phagocytosis, it has been generally accepted that oxygen uptake is increased (15, 20; D. Musher, G. T. Keusch, and L. Weinstein, *submitted for publication*). Our observation that macrophages from mice infected with listeria or BCG oxidize glucose-*1*-^{14}C at a faster rate than cells from normal or immunized animals indicates clearly that exposure of mice to live organisms significantly increases one aspect of the metabolic activity of their macrophages. Moreover, this important determinant appears to correlate well with (i) resistance to listeria in vivo, (ii) enhanced phagocytosis, and (iii) significant inhibition of bacterial growth in vitro. Our data suggest that the cellular immunity that follows infection by an intracellular organism is due to an increase in metabolic activity of macrophages, as reflected by increased glucose oxidation, which results in enhancement of phagocytosis and killing. The specific biochemical events responsible for the increased phagocytic and bactericidal activities of resistant cells after infection with live intracellular organisms remain to be clarified.

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