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Time Course of Antilisterial Activity by Immunologically Activated Murine Peritoneal Macrophages

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Murine peritoneal macrophages were rapidly rendered listericidal after exposure to lymphokine-rich supernatants (LRSs) derived from antigen-pulsed *Listeria monocytogenes*-immune spleen cells. A 6-h incubation period with LRSs was sufficient to induce microbicidal activity in resident macrophages. In vitro induction of macrophage listericidal activity by constant exposure to LRSs persisted for 18 h, after which time spleen cell factors were no longer capable of modifying intracellular inactivation of *Listeria*. Results obtained by utilizing a short assay indicated that the killing kinetics is extremely rapid, with large numbers of bacteria destroyed during the first 15 min of infection. Intracellular killing at this time appeared to be greatly dependent upon the stage of growth from which the microorganisms were harvested. Induction of bactericidal macrophages by infection of mice with a sublethal dose of virulent *Listeria* cells and subsequent intraperitoneal elicitation with heat-killed homologous bacteria was similarly a transient event. Macrophages harvested 18 h after antigenic challenge displayed dramatic antibacterial activity during the first 22 h in culture. After 22 h, activity was lost, and stasis was observed during the ensuing 23 h. At 68 h, macrophages were devoid of antilisterial action. Activity, however, could be recalled after incubation with LRSs.

Peritoneal macrophages from experimental animals can be activated immunologically in vivo by sublethal infection with facultative intracellular parasites (13, 14) or in vitro to become antimicrobial by supernatants derived from antigenic stimulation of specifically sensitized lymphocytes (4, 5, 8, 11, 25). Macrophages activated via immunological modalities develop a plethora of biochemical changes which may or may not be associated with their enhanced capacity to restrict the intracellular growth of microorganisms (9). Time course studies on the activation phenomena have produced various findings, depending upon the procedure employed to obtain activation, the species of animal, and the assessed parameter of activation. Guinea pig macrophages preincubated with lymphocyte mediators for a period of 72 h exhibit a significant increase in glucose oxidation (21, 22) and bacteriostasis (5, 11). In the murine system (12), lymphocyte supernatants rich in migration inhibitory factor increased $^{14}CO_2$ production from [1-^{14}C]glucose by 24 h. When sensitized lymphocytes and antigen are incubated directly with macrophages, antibacterial activity against *Listeria monocytogenes* is observed after 24 h (26). A 4-h incubation of macrophages with a 45,000-molecular-weight lymphokine fraction was sufficient to induce the cells to become refractory to rickettsial infection. Continued incubation of these cultures for 24 h resulted in increased intracellular rickettsiacidal activity (19). The development and loss of tumoricidal activity during lymphokine treatment in vitro follows a reproducible time course in which resident macrophages develop optimal cytotoxic activity after 6 to 14 h. Cytotoxic activity decreases thereafter and returns to control levels between 24 and 36 h (25).

Little information, however, is available regarding time course studies on the acquisition and loss of antibacterial activity by macrophages activated immunologically in vivo. We recently described bactericidal macrophage populations derived from mice which had been immunized intraperitoneally with a sublethal dose of *L. monocytogenes* and subsequently boosted with heat-killed homologous organisms (6, 7). Macrophages harvested 18 h after the injection of eliciting antigen exhibited potent listericidal activity in comparison with resident or elicited cells. The present investigation presents a temporal analysis of macrophage activation for killing of *Listeria* in cells treated with lymphokine-rich supernatants of antigen-stimulated spleen cells and describes the retention and loss of

bactericidal activity by macrophage cultures from *Listeria* immune-boosted animals.

MATERIALS AND METHODS

Bacteria. Smooth *L. monocytogenes* A4413 (serotype 4b), a virulent facultative intracellular parasite, was utilized as the target microorganism in all experiments.

Collection and cultivation of macrophages. Peritoneal macrophages from CD-1 and inbred C57BL/6 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were harvested, processed, and cultivated according to Harrington-Fowler et al. (6). Macrophages were allowed to attach to 16-mm plastic dishes (Costar, Cambridge, Mass.) for 2 h in a 10% CO_2 atmosphere at 37°C. Nonadherent cells were withdrawn, and the monolayers were reincubated for an additional 15 h to ensure removal of any residual adherent polymorphonuclear leukocytes and contaminating lymphocytes. Histological examination of cover slips stained with a Diff-Quik system revealed a rapid development of confluent monolayers consisting of morphologically distinct macrophages. Viability of the adherent population was routinely confirmed by dye exclusion.

Infection and enumeration of cultures. Procedures for ascertaining the postphagocytic fate of *L. monocytogenes* have been detailed elsewhere (28). Briefly, 10^6 macrophages were infected with 10^5 bacteria harvested in different phases of the growth cycle and opsonized with pooled homologous serum. After 40 min of phagocytosis, the monolayer was washed and incubated for 30 min with an antibiotic mixture consisting of 100 μg of streptomycin and 100 U of penicillin per ml to sterilize residual extracellular bacteria. The cultures were washed with fresh Dulbecco modified Eagle medium to remove the pulsed drug, and zero time determinations of viable intracellular bacteria were assessed by plate counts of lysates of macrophages fractured by being scraped with a plastic 1.0-ml pipette. In replicate cultures, the medium was changed at 0.5-h intervals for 6 h, after which time a final intracellular count was performed. This assay described the intracellular fate of *Listeria* between the period immediately after termination of phagocytosis (90 min) and the ensuing 6 h. The technique is limited by the absence of information regarding bacterial interactions with macrophages during the period of time allotted for phagocytosis and extracellular sterilization. Accordingly, the dynamics of the host-parasite relationship during the initial hour of the experiment were determined as follows. One million adherent macrophages in Costar chambers were infected in triplicate with a multiplicity of infection of 10^7 to 10^8 opsonized bacteria per macrophage culture for 10 min after which the monolayer was washed 10 times to remove excess extracellular microorganisms. Macrophages were then fractured as described above for a zero time viable count, and phagocytosis was simultaneously enumerated with cover slip cultures. Replicate monolayers were incubated for an additional 15 and 45 min, after which cultures were again enumerated for viable bacteria and phagocytosis to estimate the short-term intracellular fate of the microorganisms. Cover slips stained with Diff-Quik did not reveal substantial amounts of extracellular bacteria. Similarly, infected monolayers visualized by electron microscopy indicated that extensive washing without antibiotic treatment removed most of the external particles.

Preparation of immunologically activated macrophages. *Listeria*-immune antigen-elicited (LIAE) macrophages were obtained from mice after intraperitoneal immunization and after elicitation with viable and heat-killed *Listeria*, respectively, as described previously (6).

For activation in vitro, macrophage monolayers were incubated for various intervals with a 20% preparation of the supernatants derived from antigenically stimulated splenic lymphocyte cultures. Lymphokine-containing supernatants were obtained by a modification of the procedure of Adler et al. (1) as adapted for mouse cells (2). Spleens from normal (uninfected) and *Listeria* immune-boosted mice were dissociated by gentle teasing followed by pressing minced tissue through a 100-mesh sterile sieve apparatus into RPMI tissue culture medium with antibiotics. Single-cell suspensions obtained by serial aspirations through no. 19 and no. 23 gauge needles were centrifuged at 250 × *g* for 10 min at 4°C and resuspended to a concentration of 5×10^6 viable cells per ml in antibiotic-free RPMI medium. For cell counting, a sample was withdrawn and erythrocytes were lysed with 2% acetic acid. For the preparation of lymphocyte culture supernatants from mice sensitized to *L. monocytogenes*, lymphocytes were exposed to 10^7 washed, heat-killed whole cells (*Listeria* antigen) for 3 days at 37°C. Supernatant fluids from replicate cultures were pooled, centrifuged at 450 × *g* for 15 min at 4°C, divided into aliquots, and stored at 4°C until used. Lymphokine preparations prepared in this manner did not display any intrinsic antibacterial activity against *Listeria*.

RESULTS

It has been determined in our laboratory that highly activated macrophage populations can be produced by a two-step immunization schedule consisting of an intraperitoneal injection of a sublethal dose of viable *L. monocytogenes* followed by elicitation into the peritoneum with heat-killed homologous bacteria. Because polymorphonuclear leukocytes contaminate the culture during the first 14 h of incubation, experiments were generally performed after overnight culture and at later intervals, when the percentage of neutrophils in the monolayer did not exceed 1%. Employing the standard assay, which measures antimicrobial activity at 90 min and at 6 h postinfection, it was noted that LIAE macrophages expressed high levels of listericidal activity during the initial 17 h of cultivation (Table 1). Antibacterial activity, however, was not detected at 22 h, and stasis was observed during the ensuing 23 h. In separate experiments, macrophages were shown to be devoid of all antilisterial activity at 48 h when intracellular growth of the microorganism was recorded. Extension of the incubation to 68 h yielded monolayers which supported maximal growth of bac-

TABLE 1. Kinetics of decline of bactericidal activity by *Listeria*-immune antigen-elicited macrophages[a]

Time in culture[b] (h)	Viable intracellular bacteria[c] at:	
	Zero time	6 h
2	6,300 (805)	1,845 (40)
12	5,698 (732)	1,912 (220)
15	7,580 (442)	1,035 (733)
16	9,230 (394)	2,330 (191)
17	6,830 (286)	2,712 (163)
22	12,230 (110)	12,960 (120)
45	7,840 (300)	7,460 (10)
48	733 (28)	1,408 (73)
48	2,917 (633)	3,867 (1,272)
68	13,760 (914)	32,487 (2,442)

[a] Macrophages were collected 18 h after elicitation of 7-day-old immune animals with an intraperitoneal injection of 10^7 heat-killed *Listeria monocytogenes* cells.

[b] Hours that macrophages remained in culture before infection. Macrophages were adherent and viable at all time intervals.

[c] Data in each experiment represent the average and standard deviation (parentheses) of duplicate determinations performed on four macrophage cultures at the indicated time intervals.

teria. Results obtained from studies measuring the intracellular bacterial fate during the first hour of the experiment suggested that the killing kinetics was extremely rapid (Table 2). At cultivation intervals of 16 and 22 h, large numbers of bacteria were killed within 15 min, but not after 90 min, as noted above (Table 1). The rapidity of killing might indicate that bacterial viability is terminated immediately upon contact with the cell membrane, precluding the necessity for phagolysosome formation and subsequent fusion. The loss of viability during the ensuing 45 min progressed at a fairly constant rate in both the 16- and 22-h macrophage cultures, with an average of 50% of the *Listeria* killed during the first hour of the assay. After 24 h in culture, the LIAE monolayer expressed bacteriostasis between zero time and 15 min, and the capacity to kill the microorganisms abated after 68 h.

Other experiments showed that resident macrophages could rapidly be rendered listericidal after incubation in lymphokine-rich supernatants (LRSs) derived from antigenically stimulated *Listeria* immune-boosted spleen cells (Fig. 1). The results are presented as the ratio of intracellular bacteria at 6 h to those at zero time. Ratios of less than one denote bactericidal activity, whereas those approximating unity or greater represent stasis and intracellular growth, respectively. In contrast to previous reports regarding the time course of macrophage activation to antilisterial activity by lymphokine preparations (4, 5, 8, 11), an extraordinarily rapid response was observed in the current experiments. Resident macrophages expressed high capacities for bacterial killing after a 6-h preincubation with LRSs, and this activity persisted for nearly 20 h, after which the bactericidal action waned with time. In monolayers pretreated with control supernatants, the ratio varied from 1.2 to 2.2 during the first 18 h and was never less than one (data not shown). It was also observed that in comparison with untreated macrophages, cultures incubated with lymphokine preparations in excess of 40 h appeared less able to cope with intracellular *Listeria*.

The kinetics of listericidal activity by LRS-treated macrophages as depicted during the first hour of assessment of intracellular bacterial fate is shown in Table 3. From these experiments, it appeared that a 6-h preincubation period of resident macrophages with LRSs was sufficient to render the cells bactericidal. At least 50% of the bacteria were killed within 15 min in either CD-1 or C57BL/6 murine macrophages. In all experiments, there was essentially no killing of *Listeria* within control macrophages during this interval. It is important to note that some antibacterial activity was apparent in resident macrophages after incubation with control supernatant. From experiment 1 (Table 3), it appeared that the total number of microorganisms destroyed by LRSs and control-treated macrophages was of approximate magnitude after 1 h. However, concurrent assessment of phagocytosis with stained cover slips presented compelling evidence that antibacterial activity was virtually more rapid and extensive in lymphokine-treated cells than indicated by viable intracellular counts. With one exception (experiment 3), enumeration of Diff-Quik-stained cover slips revealed a two- to threefold increase in phagocytic index in the LRS-treated macrophages. Because zero time viable bacterial counts of treated cells were generally equal to or less than those derived from fractured control monolayers, it can be assumed that stained preparations represented a significant percentage of bacteria which

TABLE 2. Kinetics of decline of bactericidal activity by *Listeria*-immune antigen-elicited macrophages[a]

Time in culture[b] (h)	Viable intracellular bacteria[c] at:		
	Zero time	15 min	1 h
16	99,100 (3,432)	67,233 (4,981)	40,200 (2,599)
22	88,100 (2,900)	67,800 (1,000)	52,975 (1,175)
24	92,867 (660)	95,000 (5,374)	68,400 (1,431)
45	47,100 (2,500)	50,500 (3,700)	48,700 (700)
63	63,200 (3,600)	67,650 (8,150)	38,750 (1,950)
68	137,067 (8,290)	148,667 (2,853)	166,167 (1,926)

[a–c] See footnotes to Table 1.

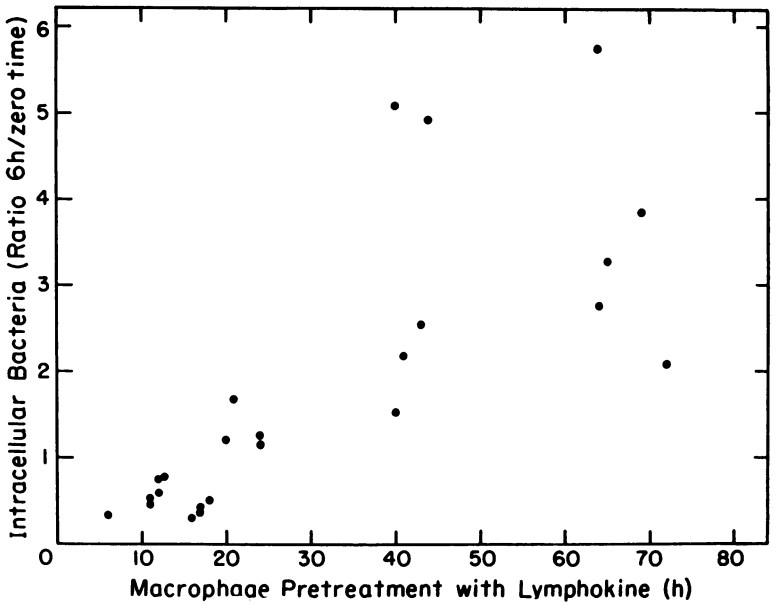

FIG. 1. Kinetics of lymphokine activation of macrophages to antilisterial activity. The ratio of the number of intracellular bacteria at 6 h to that at zero time is shown on the ordinate. Each point represents a separate experiment performed after treatment of macrophages with lymphokine-rich supernatants at the indicated intervals.

were killed shortly after interaction with lymphokine-activated macrophages.

LRSs were additionally shown to be capable of restoring listericidal activity to immune-boosted macrophages whose antibacterial activity had declined after lengthy cultivation (Table 4). Immune macrophages maintained in culture in excess of 24 h failed to display bactericidal activity when assessed after 15 min. In contrast, macrophages cultivated at intervals between 29 and 63 h displayed rapid antibacterial activity when pretreated for 6 h with lymphokine preparations. The restoration of antilisterial activity was similarly evident when the assay was conducted over a 6-h period in which LIAE cells cultivated for 48 h supported intracellular microbial growth, whereas those treated briefly with spleen cell supernatant displayed potent bactericidal action (data not shown).

These studies were expanded to include experiments which determined the fate of *Listeria* in macrophages as a function of their growth cycle. Cultures of *L. monocytogenes* display a typical bacterial growth curve as determined by concurrent measurements of colony-forming units with optical density readings at 660 nm. Table 5 shows the effect of growth phase on bacterial susceptibility to killing by resident macrophages. The data indicate that during the first 15 min of the assay, unstimulated macrophages were unable to kill *Listeria* cells harvested at the peak of their late logarithmic growth. At all other phases tested, however, the microorganisms appeared to be susceptible to short-term killing by resident cells. A comparison of the fate of bacteria taken at peak logarithmic growth in resident and LIAE macrophages is shown in Table 6. Bactericidal activity is expressed within the initial 15 min by the immune cells but not by resident macrophages, a finding which is consistent with previous observations presented in this investigation concerning the heightened capacity of immune cells to rapidly inactivate the microorganism. The phase of bacterial growth does not appear to influence the intracellular fate of *Listeria* when assessed after 6 h. Bacteria harvested at mid-logarithmic to early stationary phase exhibit intracellular growth in resident macrophages at this time (data not shown).

DISCUSSION

We previously reported that immunologically activated macrophages display bactericidal activity towards *L. monocytogenes* only when the experimental animals receive an injection of heat-killed bacteria 18 h before harvest of macrophages (6). In this study, we show that macrophage bactericidal activity in vitro is transitory and progressively decreases after 18 h. Bacteriostasis is evident after 22 h of cultivation, and at 46 h, macrophages support intracellular microbial growth. The observation that macrophage activation is lost under in vitro conditions sug-

TABLE 3. Activation of macrophages for antilisterial activity after incubation in splenic lymphocyte culture supernatants

Macrophages[a]	Incubation[b] time (h)	MOI[c]	Viable intracellular bacteria[d] at:			Phagocytic index[e]
			Zero time	15 min	1 h	
Expt 1						
Lymphokine	12	26	41,450 (1,250)	27,775 (425)	28,375 (1,425)	0.460
Control	12	26	50,800 (500)	47,100 (1,300)	41,600 (1,350)	0.195
Expt 2						
Lymphokine	6	30	88,900 (500)	45,450 (2,450)	29,725 (925)	0.650
Control	6	30	93,550 (5,000)	89,900 (2,100)	51,900 (1,100)	0.192
Expt 3						
Lymphokine	4	18	37,550 (1,650)	34,850 (3,650)	15,550 (750)	0.219
Control	4	18	32,150 (750)	36,150 (1,050)	27,700 (500)	0.221
Expt 4[f]						
Lymphokine	6	33	103,400 (600)	57,200 (3,600)	61,800 (3,200)	0.691
Control	6	33	106,000 (1,040)	108,600 (2,000)	96,000 (1,600)	0.271

[a] Macrophages were incubated with supernatants derived from *Listeria*-immune spleen cells sensitized with heat-killed homologous bacteria for 72 h. Controls were incubated with supernatants obtained from antigenically stimulated normal spleen cells.
[b] Time of exposure of lymphocyte supernatants with macrophages before infection.
[c] Multiplicity of infection denotes infectivity ratio (number of bacteria per macrophage).
[d] See Table 1, footnote [c].
[e] Mean number of organisms ingested per cell.
[f] Experiment performed with C57BL/6 mice.

gests that persistence of cell-mediated immunity requires continual modulation of macrophage functional capabilities by immunologically stimulated lymphocytes. In this regard, LIAE macrophages could be restored to full listericidal activity after brief treatment of the 48-h culture with LRSs. Nogueira and Cohn (24) similarly observed a rapid loss of microbicidal activity against *Trypanosoma cruzi* by in vivo-activated macrophages explanted into culture. Restoration of microbicidal activity was obtained with supernatants of antigen-pulsed immune spleen cells, indicating that persistent stimulation of lymphoid cells may be of importance in organ clearance of intracellular pathogens in the intact host. The idea of continual modulation is consistent with the in vivo requirement for elicitation of immune cells in the production of bactericidal macrophage populations. Introduction of antigen into the peritonea of immune animals would initiate local activation at the site(s) where the microorganisms and their products

TABLE 4. Restoration of bactericidal activity in immune-boosted macrophages[a] after incubation with splenic lymphocyte culture supernatants

Time in culture[b] (h)	Treatment	Viable intracellular bacteria[c] at:		
		Zero time	15 min	1 h
15	None	48,800 (3,800)	37,700 (1,100)	16,700 (141)
29	None	205,567 (5,839)	208,467 (7,487)	131,133 (9,794)
29	Lymphokine[d]	173,967 (17,467)	148,067 (6,214)	86,870 (6,014)
39	None	61,500 (5,515)	57,600 (7,920)	23,100 (3,677)
39	Lymphokine	53,200 (3,959)	39,600 (4,243)	15,600 (2,404)
39	None	122,000 (20,000)	149,500 (7,500)	111,000 (3,600)
39	Lymphokine	78,250 (550)	62,400 (6,240)	37,300 (1,300)
63	None	63,200 (3,600)	67,650 (8,150)	38,750 (1,950)
63	Lymphokine	46,300 (900)	34,100 (1,100)	16,450 (2,130)

[a–c] See footnotes to Table 1.
[d] Macrophages were incubated for 6 h with a 20% lymphokine preparation. See Table 3, footnote *a*.

TABLE 5. Effect of bacterial growth phase on antilisterial activity of resident CD-1 macrophages

Phase of growth[a]	No. of expts	Intracellular bacteria[b] (ratio 15 min/zero time)
Logarithmic[c]	4	0.64 ± 0.35
Late logarithmic/peak[d]	4	1.05 ± 0.03
Early stationary[e]	4	0.89 ± 0.14
Stationary[f]	2	0.67 ± 0.38
Decline[g]	1	0.64

[a] Cultures of *L. monocytogenes* A4413 cultured without shaking in tryptose broth at 37°C.

[b] Ratio or intracellular bacterial counts determined at zero time and at 15 min. Ratios greater than one represent bacterial growth. Bactericidal activity is represented by ratios of less than one. Results are expressed as mean and standard deviation ($P < 0.05$) of the indicated number of experiments.

[c] Represents the interval when cultures show minimal turbidity and spectrophotometric readings increase with time.

[d] Interval of time approximately 30 min before and 20 min after optical density readings level off.

[e] Sixty-minute interval after that described in footnote *d*.

[f] Time period after early stationary phase and before spectrophotometric readings begin to diminish.

[g] Phase when spectrophotometric readings decline with time.

stimulate macrophages, probably via lymphokines produced by the influx of committed lymphocytes into inflammatory sites (10, 15).

Time course studies of macrophage activation in vitro indicate that incubation of monolayers with either antigen-stimulated lymphocytes or supernatants of antigen-sensitized spleen cells results in heightened antimicrobial capabilities after variable periods of latency. In the guinea pig model, Simon and Sheagren (26) observed that macrophages exhibited antilisterial activity after 24 h of incubation with tuberculin or bovine gamma globulin-sensitized lymphocytes with specific antigen. Their system, however, presented technical difficulties, and it was not clear whether activity was bacteriostatic or bactericidal. Supernatant fluid from *Toxoplasma gondii*-stimulated spleen cells derived from chronically infected guinea pigs caused diminution in the growth rate of intracellular *Listeria* after preincubation for 72 h (11). The intracellular growth of *L. monocytogenes* can also be inhibited by lymphocyte culture products obtained from *Listeria*-immune lymphocytes. In mice, supernatant fluids derived from immune lymphocyte cultures stimulated in vitro with *Listeria* antigen are markedly inhibitory to the multiplication of intracellular bacteria (8). In these studies, supernatants were added to cultures of infected macrophages, and growth of *Listeria* was measured 24 h after infection. In some experiments, inhibition of growth was observed 12 h postinfection but was not as marked as it was after 24 h. Using the homologous system, Cole (4) pretreated glass-adherent mouse peritoneal cells for 18 h before infection with live *L. monocytogenes* cells. Time course studies on the fate of the bacteria indicated 35% killing after 7 h of infection, with minimal bactericidal activity evident at 48 h postinfection.

In marked contrast to the data obtained from these studies, we observed rapid killing of intracellular *Listeria* after relatively short-term incubation of resident macrophages with experimental supernatants derived from immune-boosted lymphocytes stimulated with homologous antigen. Resident macrophages expressed listericidal activity when assessed at 15 min after a 6-h preincubation in LRS fluids (Table 3). Antibacterial activity, however, was short-lived and was not detectable after 18 h. Our results are consistent with the observation that resident peritoneal macrophages rapidly develop tumoricidal activity in vitro after treatment with supernatants of purified protein derivative-stimulated *Mycobac-*

TABLE 6. Fate of *Listeria* cells grown to peak logarithmic phase in resident and immune CD-1 macrophages

Expt no.	Macrophages[a]	Viable intracellular bacteria[b] at:		
		Zero time	15 min	1 h
1	Resident	22,750 (919)	24,150 (2,758)	19,375 (35)
	LIAE	30,000 (283)	23,050 (3,606)	15,675 (672)
2	Resident	54,650 (350)	63,100 (900)	52,350 (4,150)
	LIAE	114,400 (11,200)	89,100 (1,300)	87,400 (3,000)
3	Resident	36,450 (750)	34,700 (200)	22,100 (1,000)
	LIAE	33,500 (1,500)	23,750 (2,450)	22,900 (1,900)

[a] Macrophages were cultured for 15 h before assay for intracellular bacterial fate. *Listeria*-immune antigen-elicited (LIAE) macrophages were prepared as described in Materials and Methods.

[b] Data in each experiment represent the average and standard deviation (parentheses) of duplicate determinations performed on four macrophage cultures, utilizing infecting inocula prepared from bacteria grown to late logarithmic phase (see footnote *d* to Table 4).

terium bovis BCG-immune lymphocyte cultures (25). The time course for lymphokine-induced macrophage activation indicated that cytotoxic activity was evident after 4 h of incubation. Tumor cytotoxicity became maximal by 8 to 12 h and then progressively decreased to control levels by 36 h. In both systems, loss of lymphokine responsiveness was not due to cell death or to depletion of active supernatants; it appeared to reflect a diminished sensitivity with time to lymphokine signals. The observation that resident macrophages became less capable at restricting intracellular growth of *Listeria* after prolonged incubations with LRSs suggests depletion or exhaustion of antimicrobial metabolites.

Lymphokines obtained from immune-boosted spleen cells were considerably more active in stimulating macrophages than were culture supernatants of antigen-stimulated cells derived from unelicited *Listeria*-immune animals. In this regard, experimental supernatants used for this investigation may have been of unusually high potency in the promotion of macrophage activation for intracellular killing of bacteria. This could explain why earlier workers did not report lymphokine induction of macrophage antibacterial activity until after extended periods of latency. Alternatively, previous investigations may have overlooked the possibility of rapid lymphokine responsiveness by macrophages in culture.

At this juncture, there is insufficient information available to speculate on which antibacterial systems are used either by in vivo- or in vitro-activated macrophages. However, relevant reports indicate that both immune-boosted and lymphokine-stimulated macrophages acquire the capacity to release substantial amounts of hydrogen peroxide in response to membrane perturbation (18, 20, 23), and studies are in progress to determine whether antibacterial activities of activated macrophages correlate closely with their production of oxygen intermediates.

Although many studies have been performed on the listericidal activity of immune macrophages, few have critically evaluated the bacterial fate in resident cells. Early in vivo studies (16) revealed appreciable killing of *L. monocytogenes* cells 3 h after infection, after which bacterial growth was observed in the spleens and livers of infected mice. Corresponding killing kinetics were recently reported in vitro by using resident macrophages (27). Data presented in this report describe significant bactericidal action by unstimulated macrophages during the first hour postinfection. Although information is available regarding the ability of resident mononuclear phagocytes to inactivate a wide variety of microorganisms (3, 17), the antimicrobial systems have not been explored. Killing systems inherent to resident macrophages appear to be only partially effective against the facultative intracellular pathogens and may become rapidly exhausted during active infection. Further, density-separated subpopulations of resident macrophages differ in their ability to control intracellular growth of *Listeria* (7). The early destruction of bacteria by these cells could be the result of specific bactericidal subpopulations whose microbicidal activity is ultimately eclipsed by unrestricted intracellular replication within nonfunctional subsets. In this manuscript, we present data indicating that the killing of *L. monocytogenes* cells by resident macrophages may be due in part to bacterial factors associated with their growth characteristics. Bacteria in various stages of growth exhibited variable resistance and susceptibility to intracellular killing (Tables 5 and 6). In contrast to cells harvested during stationary and mid-logarithmic growth, microorganisms harvested from the late logarithmic/peak phase expressed maximal refractoriness to intracellular inactivation by resident macrophages when assayed during the initial 15 min of the experiment. Accordingly, short-term assays were consistently performed by using bacteria grown to late logarithmic phase to maximize differences observable in the intracellular bacterial fate when comparing antimicrobial activity in resident and immune antigen-elicited cells. This finding emphasizes the importance of precisely defining the stage of microbial growth before assessing the postphagocytic fate of the bacteria.

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