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In Vitro Antilisterial Activity of Soluble Product(s) Released from *Listeria*-Immune Murine Peritoneal Macrophages

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The importance of macrophages as effector cells in cellular reactions of immunity and hypersensitivity is well established (8). Macrophages activated as a result of specific immunological events exhibit increased microbicidal activity not only for the primary organism used for immunization purposes but also against unrelated intracellular pathogens (7). It is believed that the acquisition of this bactericidal or bacteriostatic activity is conferred on macrophages by materials released during the interaction of specifically immune lymphocytes with the antigen (Fowles et al., 1973; Godal et al., 1971; Jones and Youmans, 1973; Krahenbuhl and Remington, 1971; Mackaness, 1971; Patterson and Youmans, 1970). The antimicrobial activity in these studies refers to the intracellular inhibition and/or killing of the organisms; no attempts were made to assess the effect of activated macrophages on the extracellularly residing organisms. In an earlier study with the pathogenic yeastlike fungus *Cryptococcus neoformans*, we observed that freshly collected peritoneal macrophages from mice pretreated with a variety of nonspecific agents or from specifically immune mice possessed the capacity to exert anti-cryptococcal effect both on intracellular yeasts as well as those present in the extracellular milieu (Sethi et al., 1971; Sethi and Pelster, in press). Our results suggest that soluble product(s) released from mouse macrophages immune to *Listeria monocytogenes* can exert antilisterial activity under in vitro conditions.

White male mice were immunized intraperitoneally with approximately 3×10^6 *Listeria monocytogenes* organisms belonging to an attenuated strain (type 1). Conventional procedures were used for harvesting and in vitro maintenance of macrophages from the peritoneal cavities of immunized or unimmunized control mice. Briefly, washings from the peritoneal cavities of individual mice were pooled together and suspended in Eagle medium containing 10% fetal calf serum (GM) and the cells were allowed to settle onto glass cover slips placed in Leighton tubes. Nonadherent cells were completely washed away after 4 h and adherent cells (approximately 10^6 per cover slip) were fed with either fresh GM or GM containing killed (100 C for 30 min) *Listeria* (type 1; 10^6/ml). After 5 h of incubation the supernatants were centrifuged and passed through a membrane filter (Millipore Corp.) to remove suspended *Listeria* or detached cells. The high buffering capacity of the medium prevented significant changes in its pH during 5 h of incubation with immune or nonimmune macrophages. Samples (1 ml) of the supernatants were then inoculated with 0.1 ml of washed suspension (7.9×10^4) of either virulent *L. monocytogenes* organisms (strain LB 20; type 4b) or attenuated organisms (type 1) to make a total volume of 1.2 ml. After 5 h of incubation at room temperature, during which the cultures were subjected to gentle agitation, viable bacterial counts were carried out on brain heart infusion agar by the pour plate method using appropriate dilutions. The results of such experiments are summarized in Table 1.

It follows from these results that specifically immune peritoneal macrophages are capable of releasing a soluble product(s) into the extracellular milieu capable of inhibiting and/or killing *Listeria*. That the observed antilisterial activity of active macrophage supernatants was not due to clumping effect was confirmed by phase-contrast microscope examination of the test samples. The antilisterial activity was maximum in supernatants from immune macrophages cultured in presence of killed *Listeria*, although antilisterial activity was also detected to some extent in supernatants from immune macrophages not exposed to the antigen. It is possible

TABLE 1. *Antilisterial activity of supernatants from immune macrophages*[a]

Culture supernatant from:	Treatment	Bacterial counts[b] in reaction mixture after 5 h of incubation
Nonimmune macrophages	No	5.17 ± 0.15
Nonimmune macrophages plus killed *Listeria*	No	5.20 ± 0.17
Immune macrophages	No	4.98 ± 0.13
Immune macrophages plus killed *Listeria*	No	3.34 ± 0.17
	Diluted 1:3	4.25 ± 0.16
	Heated (50 C, 30 min)	3.20 ± 0.14
	Heated (100 C, 5 min)	5.22 ± 0.18
Medium without macrophages		5.34 ± 0.13

[a] Original inoculum contained 7.9×10^4 *Listeria*/1.1 ml (strain EB 20; type 4b).

[b] Bacterial counts were determined from triplicate runs. The tabulated values represent log of the geometric mean for six experiments (calculated from log-transformed data) ± the standard error of the mean.

that immune macrophages released antilisterial product(s) before the washing procedure to which macrophages were subjected which might have removed the released factor(s). Evidence against the possibility of the nutritional deterioration of the culture medium as the cause of observed antilisterial effect is provided by the fact that active supernatants could exert marked antilisterial activity even diluted to one-third of its original strength with fresh culture medium. The active supernatant fluids stored at −20 C for a period of 2 months have been found to retain antilisterial activity. The antilisterial activity of the supernatants was not abolished by heating them at 56 C for 30 min but was destroyed when the active supernatants were heated at 100 C for 5 min. Viability counts carried out on supernatants from nonimmune macrophages incubated alone or with killed *Listeria* were not significantly different from those attained in GM alone. The nature of antilisterial substance(s) present in active supernatant fluids has not been established. However, it appears unlikely that the observed antilisterial effect was due merely to the exocytosis of lysosomal hydrolases in the culture medium since the detectable levels of lysosomal hydrolases in culture fluid from normal and immune macrophages were found to be not significantly different whether cultured with or without the antigen. It may be mentioned that freshly harvested immune macrophages demonstrated the usual traits of macrophage "activation" and possessed increased intracellular listeriacidal capacity when compared to macrophages from nonimmune animals.

On the basis of above results it is logical to consider the possible role of the soluble product(s) elaborated by host macrophages in limiting the extracellular population of organisms under in vivo conditions. It is interesting to note that whereas the supernatants from *Listeria*-immune lymphocyte cultures stimulated in vitro with antigen had no direct antilisterial effect but they could inhibit the growth of organisms inside normal mouse macrophages (4). On the other hand, it has been reported that mouse lymphocytes stimulated by phytohemagglutinin-P release lymphokines which possess antifungal activity (10). However, whereas the mouse lymphokine preparations exerted antifungal activity after 20 to 48 h of incubation, the soluble immune macrophage product(s) seems to exert a comparatively rapid antilisterial effect. Immune macrophage supernatants which possessed antilisterial activity were also found to have cytotoxic property for mammalian L cells. However, this observation does not necessarily imply that the factor(s) responsible for antilisterial activity is identical with the known macrophage cytotoxins (3, 11). Furthermore, the data provided also does not prove that the macrophage product(s) mediating antilisterial activity is identical to that responsible for inhibiting the extracellular growth and/or killing of cryptococci reported earlier (K. K. Sethi et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 131, 1973; K. K. Sethi and B. Pelster, Proc. Symp. "Macrophage activation," in press).

We are now investigating the exact nature, mode of action, and the specificity of the antilisterial principle released from listeria-immune macrophages.

APPENDIX

After completion and submission of this manuscript, a paper describing similar results appeared (R. C. Bast, R. P. Cleveland, B. H. Littman, B. Zbar, and H. J. Rapp. 1974. Acquired cellular immunity: extracellular killing of *Listeria monocytogenes* by a product of immunologically activated macrophages. Cell. Immunol. **10**:248–258).

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