THE ENHANCEMENT OF MACROPHAGE BACTERIOSTASIS BY PRODUCTS OF ACTIVATED LYMPHOCYTES*

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Resistance to certain infections appears to be partly dependent upon a population of macrophages which exhibits altered functions, including an enhanced capacity to deal with a number of microorganisms (1-4). Such macrophages are said to be “activated,” and their in vivo activation has been shown to require the interaction of specifically sensitized T lymphocytes with the appropriate antigenic agent (5, 6). Once activated, macrophages exhibit nonspecific antimicrobial activity (7).

In vitro studies have confirmed this phenomenon. If peritoneal exudate cells (PEC, predominantly composed of macrophages and lymphocytes) are obtained from animals sensitized to purified protein derivative of tuberculin (PPD) or bovine gamma globulin (BGG) and incubated in vitro with the appropriate sensitizing antigen, the resulting macrophage monolayers exhibit enhanced bactericidal activity against an unrelated organism, Listeria monocytogenes (8). Further, macrophage monolayers produced from PEC of normal animals can be so activated by the addition of sensitive lymph node (9) or peritoneal (10) lymphocytes plus the antigen. The exact mechanism of the lymphocyte enhancement of macrophage bacteriostasis is unknown. One possibility is that the enhanced macrophage function is mediated by products of activated lymphocytes, such as migration inhibitory factor (MIF) (11, 12). Indeed, macrophages incubated in such lymphocyte mediators showed altered function (13, 14) and the activating substance is physicochemically indistinguishable from MIF (15).

It has been reported that normal macrophages incubated with supernatants from stimulated lymphoid cell cultures exhibit enhanced resistance to M. tuberculosis (16), M. Leprae murium (17), and L. monocytogenes (18). In one study, the organism used to sensitize lymphocytes was the same as that used to assess bacteriostasis; thus, nonspecific aspects could not be evaluated (16). In another, the supernatant came from mixed blood leukocyte cultures and the results were complicated by the proliferation of blood-derived macrophages in this medium (17). In a third, the supernatants were obtained from a mixture of antigen-stimulated toxoplasma sensitized spleen cells and peritoneal macrophages (18). On the other hand, two studies, including our early studies, showed that normal macrophages incubated with supernatants from stimulated lymphoid cell cultures exhibit enhanced resistance to M. tuberculosis, M. Leprae murium, and L. monocytogenes. The mechanism of this enhancement is unknown, but it is likely that the activating substance is a lymphocyte-derived factor, such as MIF.

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† Abbreviations used in this paper: BGG, bovine gamma globulin; Con A, concanavalin A; KRP, Krebs-Ringer phosphate buffer; MEM, minimal essential medium; MEM-S, 15% heat inactivated guinea pig serum; MIF, migration inhibitory factor; OCB-BGG, orthochlorobenzoyl bovine gamma globulin; PEC, peritoneal exudate cells; PPD, purified protein derivative.

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experiments, failed to document enhanced bacteriostasis when macrophages were incubated in supernatants from sensitized lymphocytes stimulated by antigen unrelated to the target bacteria (19, 20). In attempts to analyze this problem, we found that passage of tissue culture media through a Millipore filter (Millipore Corp., Bedford, Mass.) seriously affected the results, so, in subsequent experiments this procedure was discontinued.

The studies presented here indicate that partially purified MIF containing supernatants from cultures of stimulated lymphocytes enhance the bacteriostatic activity of normal macrophages infected with *Listeria monocytogenes*.

**Materials and Methods**

**Macrophage Monolayers.**—Casein-induced normal guinea pig PEC were harvested and plated at 4 × 10^6 cells/ml in tissue culture as previously described (14). After 2 h, nonadherent cells were removed, and the monolayers treated as discussed below.

**Preparation of Lymphocytes for Addition to Macrophage Monolayers.**—Lymph nodes from guinea pigs immunized with ortho-chlorobenzoyl bovine gamma globulin (OCB-BGG) were obtained as previously described (21). They were teased in Eagle’s minimal essential medium (MEM) containing penicillin 100 U/ml and streptomycin 100 μg/ml in order to minimize contamination during this process. The antibiotics were then removed by washing the cells two times in 100 volumes of MEM free of antibiotics (the last wash did not prevent the growth of *Listeria*).

**Preparation of Lymphocyte Mediators.**—Lymph node lymphocytes from Hartley guinea pigs were obtained and prepared for cultures as in prior experiments (21). The lymphocyte suspensions were incubated for 24 h in the presence or absence of concanavalin A (Con A) 10 μg/ml, Con A being added to control supernatants after the 24 h incubation period (22).

**Fractionation of Supernatants.**—The above supernatants were concentrated and chromatographed on Sephadex G-100 columns. The material eluting after the albumin marker (the partition coefficient = 0.19-0.45, 25-55,000 daltons) was collected (22) and tested for MIF activity (23). This fraction of the eluate from supernatants of Con A-stimulated lymphocyte cultures normally produced at least 40% inhibition of migration when compared to that produced by the same fraction of matched control supernatants. These fractions are herein identified as “MIF-rich fractions,” and those prepared from control supernatants as “control fractions.”

**Bacteria.**—*Listeria monocytogenes* were originally obtained from Dr. George Mackaness. Bacteria from the stock lyophilized culture were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). The broth was diluted in Krebs-Ringer phosphate buffer (KRP) and plated in warm agar, and the single colonies were used as a source of inocula for experiments. Log-phase broth cultures of *Listeria* were pulsed with 10 μCi/ml of [3H]thymidine (New England Nuclear, Boston, Mass.) for 1 h; aliquots of the broth were diluted 20-fold in chilled KRP. The bacteria thus suspended were then washed on a Millipore filter (0.45 μm) suction apparatus with normal saline. The filter was placed in KRP in a screw cap tube and the bacteria released from the filter by vigorous shaking on a Vortex apparatus. The concentration of organisms was estimated by nephelometry with a 550 nm Coleman Junior spectrophotometer (Coleman Instruments Div. Perkin Elmer Corp., Maywood, Ill.). The bacterial suspension for inoculating experimental monolayers was composed usually of approximately 2 × 10^7 bacteria/ml in KRP in MEM containing 15% heat inactivated normal guinea pig serum, although occasionally as many as 10^8 were used.

**Filtration of Media.**—The whole culture media containing supernatant fractions were initially sterilized by passage through 0.45 μm Millipore filters. Due to the consequences of this procedure, presented in the Results, the supernatant fractions were handled as follows: 100×
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Concentrated fractions were passed through filters which had been washed with 50 ml of medium or saline. The supernatant fractions were then diluted to their original concentration in unfiltered MEM (supplemented with 1 ml each of 100× concentrated amino acids [Microbiological Associates Inc., Bethesda, Md.] 40% L-glucose, and 200 mM L-glutamine, per 100 ml) and made to contain 15% heat inactivated guinea pig serum (MEM-S) but not containing antibiotics.

**Incubation of Macrophages with Either Lymphocytes or Their Products.**—Lymphocytes, $1.25 \times 10^6$ in one ml of MEM-S, were added, with or without 100 μg of OCB-BGG, to the macrophage monolayers, which were then incubated overnight at 37°C in a humidified 5% CO₂ and air environment. In experiments with supernatants the Sephadex G-100 fractions were made up to their original concentration in MEM-S. 1 ml of medium was used when cultures were incubated overnight and 2 ml when they were incubated 72 h. In the latter, 0.1 ml of a supplementary solution (14) was added daily.

**Injection of Monolayers.**—After the monolayers had been cultured with lymphocytes or with supernatant fractions for the appropriate time, the culture media were aspirated. 1 ml of bacterial suspension was added and the monolayer incubated at 37°C in 5% CO₂ for 10 min. The monolayers were then washed six times with 1 ml of sterile KRP, which removed the excess of Listeria not associated with the macrophages (less than 5% of cell-associated radioactivity was due to bacteria stuck to the culture vessel itself, as assessed by inoculating Petri dishes without macrophages). One set of dishes was reincubated in MEM-S, another was immediately processed as described below.

**Enumeration of Viable Bacteria.**—1 ml KRP was added to the washed monolayers and they were quickly frozen on dry ice. The monolayers were then thawed on a warm plate at 37°C and vibrated for 1–2 min on a Vortex apparatus to free monolayer material from the dish. Viability of Listeria was not affected by this procedure, as determined by separate experiments. Macrophages were disrupted, and 90% of adherent cells as well as radioactivity associated with them were released from the dish and accessible for sampling. In several experiments, monolayers were allowed to stand in distilled water for 20 min to facilitate freeze-thaw lysis; this itself did not alter Listeria viability. A 0.1 ml sample from the contents of frozen-thawed dishes was serially diluted in KRP and plated in warm agar (Brucella agar, Difco), so as to obtain between 50 and 250 colonies per plate using a different pipette for each dilution. Replicates of the appropriate dilution were averaged.

**Evaluation of the Number of Adherent Macrophages.**—The quantity of adherent cells remaining on each dish after six washes was assessed as cell protein and measured on a 0.2 ml sample by the method of Lowry et al. (24) and expressed as micrograms of protein per monolayer. Amounts of protein contributed by Listeria were found to be less than 5% of the total.

**Quantitation of the Uptake of Labeled Bacteria.**—Another 0.2 ml aliquot was dissolved in NCS (Amersham/Searle Corp., Arlington Heights, Ill.), placed in scintillation fluid (10 ml per vial, 6% PPO-POPOP in toluene) and cpm counted for 10–20 min in a Packard Tricarb scintillation counter (Packard Instruments Co., Downers Grove, Ill.) using external standards.

**Sampling at Subsequent Intervals after Injection.**—Dishes which had been inoculated with bacteria for 10 min, washed, and reincubated in MEM-S, were, at subsequent intervals, washed again six times and processed as above; the number of viable bacteria associated with the remaining cells and the amount of adherent protein were measured.

**Statistical Analysis.**—Arithmetic means of viable bacteria counts were calculated and compared using a two-tailed Student's t test for independent means. When compared to geometric means for the same sets of data, arithmetic means did not represent a significant distortion of the numbers of viable bacteria.

**RESULTS**

**Macrophage Bacteriostasis Induced by Sensitized Lymphocytes and Antigen.**—When sensitized lymphocytes plus antigen were incubated overnight on mono-
layers of normal macrophages, the macrophages showed marked enhancement of bacteriostasis. There were seven times fewer viable bacteria recovered in these monolayers as compared to macrophage monolayers which had received lymphocytes without antigen. These results confirmed previous reports (9, 10) and served to establish the ability of our assay system to demonstrate bacterial resistance in macrophages.

In earlier work, supernatants containing lymphocyte mediators had failed to confer bacterial resistance upon macrophages (19, 20). Experiments were carried out to determine whether supernatants or their fractions might at least have a supplementary effect on macrophage bacteriostasis if applied to monolayers in concert with lymphocytes and antigen. It was surprising to find that both MIF-rich and control supernatant fractions, rather than enhancing, abrogated the protective effect of the lymphocyte-antigen stimulus (Fig. 1). In analyzing these results it was noted that the culture media containing the Sephadex fractions had required sterilization by passage through Millipore filters, whereas the culture media without fractions had not. To determine whether the sterilization through a Millipore filter might account for these observations, sensitized lymphocytes with and without antigen were incubated with macrophages in filtered or unfiltered culture medium (no fractions were present in these media). Indeed, the filtered culture medium prevented the macrophage bacteriostasis induced by lymphocytes and antigen which was observed in the same experi-

![Graph](image)

**Fig. 1.** The effect of adding supernatant fractions to macrophage monolayers also containing lymphocytes and antigen. All cultures were incubated overnight, and then infected with *Listeria monocytogenes*. 22 h later, the amount of viable bacteria per microgram macrophage protein was assessed and is shown as means of triplicates ± SE. Note that the macrophages incubated with the lymphocytes and antigen, second bar, exhibit bacteriostasis relative to cultures lacking antigen, first bar, and that this effect is abrogated by the presence of adding control or MIF-rich fractions, last two bars. The macrophage monolayers with lymphocytes and antigen (without fractions) showed significantly lower bacteria per microgram macrophage protein than the rest \( P < 0.01 \).
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mment with unfiltered medium (Fig. 2). However, if the filter was washed with 50 ml of medium before use, the culture medium passed through this filter supported enhanced bacteriostasis better than that passed through unwashed filters. These results suggest that an inhibitory material, possibly a detergent, was eluting off the filter. To minimize the effect of filtration in subsequent ex-

Fig. 2. The effect of filtering the culture medium on macrophage bacteriostasis. Macrophages were incubated with antigen overnight in the presence of culture medium which had been passed through a Millipore filter. Some cultures received unfiltered medium. The resulting macrophage monolayers were infected with Listeria monocytogenes and viability assessed 10 min and 22 h later. Each point represents the mean of triplicate culture ± SE. The enhanced bacteriostasis seen in cultures of macrophages preincubated with lymphocytes and antigen was abrogated when the culture medium used had been filtered. The difference between bacteriostasis in filtered and unfiltered medium was significant P < 0.02.

periments, all filters were washed before use; furthermore, concentrated frac-
tions were filtered and then diluted 1:100 in unfiltered medium to their original concentration. The effect of Millipore filtration was not always present, varying from one lot of filters to another.

Macrophage Bacteriostasis Induced by Incubation with MIF-Rich Fractions for 72 h.—Experiments were now carried out to determine the effect of MIF-rich fractions on bacteriostasis by normal macrophages. Macrophage monolayers were incubated in MIF-rich or control fractions for 72 h, since this length of
time had been previously required for the enhancement of macrophage adherence and glucose oxidation (14, 15). In three experiments, macrophages incubated in MIF-rich fractions exhibited a 2- to 10-fold enhanced bacteriostasis compared to those incubated in control fractions (see Fig. 3).

It was important to determine whether the enhanced macrophage bacteriostasis in the MIF-treated monolayers was a consequence of the greater number of macrophages in these cultures than in controls. To compensate for the enhanced adherence of macrophages in MIF-rich cultures, 40% fewer PEC were initially plated in dishes which were to receive MIF-rich fractions. The results of this experiment are seen in Fig. 4. The control monolayers contained an average of 53 μg of protein before infection and contained 3,499 viable Listeria per microgram as measured 10 min after infection. 22 h later, the monolayers had lost 60% of their protein, and there was a 47-fold increase in viable Listeria per microgram macrophage protein. The MIF-treated monolayers had less macrophages to start with, 29 μg, and contained 5,281 bacteria per microgram macrophage protein. But, in contrast to controls, the monolayers had maintained their cell number after 22 h of infection, having lost only 9% of their protein, and growth of Listeria was limited to fourfold. Thus, despite there being less macrophages in MIF-treated monolayers and more Listeria per macrophage at the beginning of the experiment, bacteriostasis in these cultures was enhanced ten times over controls.

That the bacteriostatic effect was exerted by the activated macrophages themselves, and not by the different media in which they were cultured, is evidenced by the fact that in separate experiments, Listeria cultured without
Fig. 4, Enhanced macrophage bacteriostasis induced by 72 h preincubation with MIF-rich fraction even under conditions when there are less macrophages present at the time of infection in this group than in the controls. Peritoneal exudate cells were initially plated so that at the time of infection, there were about half as many macrophages in the MIF-treated monolayers than in the controls; despite this, MIF-treated monolayers were still markedly bacteriostatic compared to controls. Details are described in the section on Results. The points represent mean and SE of triplicate cultures (P < 0.03).

monolayers grew equally well in MIF-rich or control fractions. Thus, the MIF-rich fraction itself has no more bacteriostatic power than the control fraction but influences its target, the normal macrophage monolayer, to exhibit enhanced antibacterial properties.

Macrophage Bacteriostasis Induced by Incubation with MIF-Rich Fractions Overnight.—Because antigen-stimulated lymphocytes enhance macrophage bacteriostasis after an overnight incubation, macrophages were allowed to remain with fractions for this shorter period, and then assessed for bacteriostatic activity. Some of these experiments demonstrated a significant bacteriostatic effect in MIF-treated monolayers (see Fig. 5), but such results were not obtained consistently as is discussed below. In order to determine whether antigen-induced MIF-rich medium enhances macrophage bacteriostasis, an experiment was performed in which monolayers were incubated overnight with unfractionated supernatants. The cell-free supernatants were obtained from suspensions of sensitized lymphocytes cultured with or without 100 μg/ml of OCB-
Fig. 5. Enhanced macrophage bacteriostasis induced by preincubation overnight in MIF-rich fractions. Points represent mean and SE of triplicate cultures (P < 0.01).

BGG, and one of the control supernatants was reconstituted with antigen after removal of lymphocytes (14). As can be seen in Fig. 6, this MIF-rich supernatant did enhance bacteriostasis after an overnight incubation with normal macrophages. These results indicate that an overnight exposure to MIF-rich media suffices in some experiments to cause the normal macrophages to exhibit enhanced bacteriostasis.

Comparison of Macrophage Bacteriostasis Induced by Lymphocytes or Their Products.—The various modes of inducing macrophage bacteriostasis in these studies are compared in Fig. 7. The number viable Listeria/microgram macrophage protein in control groups divided by the number in the MIF-treated groups yields a ratio which is plotted for each experiment. A high ratio represents an experiment in which bacteria grew more readily in the control macrophages than in the treated macrophages; a ratio of one implies that growth was similar in both.

Incubation with antigen-stimulated lymphocytes appeared to be most effective in enhancing bacteriostasis. Control macrophages, incubated with lymphocytes without antigen, contained an average of 7.26 times more bacteria than those treated with lymphocytes plus antigen; a statistically significant (P < 0.05) difference in viable Listeria/microgram protein between the two groups
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Fig. 6. Enhanced macrophage bacteriostasis induced by preincubation overnight in supernatants from OCB-BGG-stimulated sensitized lymphocytes. Points represent mean and SE of triplicates (P < 0.01).

occurred in 8 of 11 experiments. Moreover, bactericidal activity was observed in previous studies (8, 9) as well as in one experiment from this series; this has not been observed when macrophages are incubated with MIF-rich fractions.

In experiments where macrophages were incubated for 72 h in supernatant fractions, Listeria grew an average of 5.53 times more in the control than in the MIF-rich treated macrophages; a statistical significant difference (P < 0.03) was seen in four of four experiments.

When macrophages were incubated overnight with MIF-rich fractions, the control/MIF-rich ratio was only 1.98; there was a significant difference (P < 0.05) in viable Listeria/microgram protein in only 3 of 12 experiments.

Thus, antigen-stimulated lymphocytes incubated in vitro directly with normal macrophages seem to be more potent than their cell-free mediators in conferring enhanced bacteriostasis; nevertheless the mediators themselves have a definite effect.

DISCUSSION

The results presented here support the concept that macrophage bacteriostasis can be enhanced by lymphocyte mediators. Normal macrophage mono-
Fig. 7. Comparison of macrophage bacteriostasis induced by lymphocytes or their products. This summarizes the data of bacteriostasis by macrophages which were pretreated either for 20 h with sensitive lymphocytes and antigen (controls not receiving antigen) or for 20 h or 72 h with MIF-rich fractions (as compared to control fractions) and then infected with Listeria monocytogenes. Viability of bacteria was assessed in all cases after 22 h of infection. Each point represents the ratio obtained by dividing the viable Listeria/microgram protein in control monolayers by that of treated monolayers for one experiment. Horizontal lines indicate the mean ratio for the experiments in each group. A ratio of 1 (dashed line) indicates growth in both control and experimental was the same.

layers, after incubation for 72 h in MIF-rich fractions of supernatants from stimulated lymphocytes, exhibited antilisterial activity up to 10 times greater than monolayers incubated in control fractions. Even when the plating density of macrophages was adjusted so that at the time of infection there were fewer macrophages in the MIF-treated than in control monolayers, the MIF-treated monolayers survived better and accomplished significantly enhanced bacteriostasis compared to the control monolayers.

Patterson and Youmans (16) sensitized mice with M. tuberculosis H37Ra and then cultured the splenic lymphocytes with H37Rv. Normal mouse macrophages, when cultured in the resultant supernatants, inhibited the intracellular growth of viable H37Rv. Although it is likely that the observed results were due to a lymphocyte mediator, the use of the same organism as sensitizer and target as well as the use of unfractionated supernatants makes it difficult to evaluate the extent of nonspecific enhancement of macrophage resistance and separate it from specific factors, including antibody. Although we have been especially interested in the nonspecific enhancement of cellular immunity by lymphocytes, there may well be specific components operative in these systems, as has been reemphasized recently by Frenkel (25).

In a different system Godal et al. (17) found that normal rabbit blood monocytes, when incubated in supernatants from mixed leukocyte cultures, showed nonspecific resistance to M. Leprae murium. An interesting finding in their study is that such supernatants were able to stimulate proliferation of blood-derived
macrophages. At 7 days of culture, when the bacilli began to multiply, there were many more macrophages in experimental than in control cultures, although each had been inoculated with an equivalent number of bacilli. It is thus difficult to determine whether the observed bacteriostasis was due to increased number of macrophages per se or an enhancement of individual macrophage capability.

Krahenbuhl and Remington (18) obtained spleen cells from guinea pigs chronically infected with *Toxoplasma gondii*. These were incubated for 72 h with monolayers of normal macrophages and soluble *T. gondii* antigen and the culture supernatants collected. When these supernatants were added to fresh normal macrophage monolayers and incubated another 72 h, the resultant monolayer exhibited enhanced listeriostasis. It would be of interest to know if the sensitized spleen cells would produce active supernatants if cultured for 3 days without the macrophages, thus separating any contribution from the macrophages. Kinetic experiments of Nathan et al. (15) showed no evidence that lymphocyte mediators caused macrophages to produce a substance which activated other macrophages in terms of increased adherence or glucose oxidation. However, there is no evidence at present that these parameters of altered cell function are directly connected with bacteriostasis and that in this case, macrophage products could be involved. Indeed, it is of note that passage of medium through a Millipore filter does not prevent inhibition of macrophage migration, or enhanced cell adherence and glucose oxidation, although it may prevent enhanced bacteriostasis by lymphocytes or their mediators. This latter effect is perhaps due to a material such as detergent eluting from the filter. It would be interesting to know how the eluted material might affect the macrophage's function vis-à-vis bacteriostasis; however, it does not appear to affect the macrophage's ability to respond to MIF. Further, whether the mediator responsible for enhancing macrophage bacteriostasis is MIF or another mediator remains to be determined.

In our experiments, MIF-rich fractions enhanced macrophage bacteriostasis at 72 h. The effect at 20 h was variable and consistent with the experiments reported by Simon and Sheagren (19).

The most marked enhancement of macrophage bacteriostasis was found when lymphocytes and antigen were incubated directly with macrophages; such cultures occasionally demonstrated bactericidal activity, i.e., less bacteria were present after culture than before. Further, this enhancement of macrophage bacteriostasis was induced in 20 h. It is not known why lymphocytes themselves have a stronger influence upon macrophages vis-à-vis bacteriostasis than their mediators. They may produce essential components which are more labile than mediators such as MIF. Alternatively, direct contact between the lymphocyte and macrophage may be required for effective bactericidal activity. Anatomic interaction between lymphocytes and macrophages has been documented (26–29) and further work is required to determine whether such a mechanism is operative in enhancement of macrophage bacteriostatic or bactericidal activity.
SUMMARY

It was reported previously that the incubation of normal guinea pig macrophages with partially purified products of activated lymphocytes resulted in altered macrophage function including increased cell adherence to culture vessels, spreading, phagocytosis, and glucose carbon-1 oxidation. Studies reported here demonstrate that such macrophages also exhibit enhanced bacteriostasis. Lymphocytes were stimulated with concanavalin A, the culture supernatant was chromatographed over Sephadex G-100 and the fraction of mol wt 25,000–55,000, rich in lymphocyte mediators, was cultured with normal guinea pig macrophages for 1–3 days. Macrophages incubated with fractions from unstimulated lymphocyte cultures served as controls. The resulting macrophage monolayers were infected with Listeria monocytogenes. Macrophages incubated with mediator-rich fractions exhibited 2- to 10-fold enhanced bacteriostasis compared to controls. Further studies indicate that this enhancement was attributable to intrinsic changes in the macrophages and not simply a consequence of the number of macrophages on the monolayers. The studies support the concept that macrophage bacteriostasis can be enhanced by lymphocyte mediators. However, macrophages, which have been preincubated directly with sensitive lymphocytes and antigen exhibit even greater bacteriostasis and sometimes bactericidal capacity, suggesting that either a labile lymphocyte factor or direct lymphocyte macrophage interaction may also be involved in bactericidal activity.

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