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Interactions Between Salmonellae and Macrophages of Guinea Pigs

IV. Relationship Between Migration Inhibition and Antibacterial Action of Macrophages

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The in vitro macrophage migration inhibition test was used to detect the development of delayed-type hypersensitivity in guinea pigs infected with *Salmonella typhimurium*. Four different preparations from supernatants of *S. typhimurium* cultures were used as the antigens in this test. They included the concentrated bacterial antigens, the high-molecular-weight (>50,000) antigens, the ammonium sulfate-precipitated antigens, and the ribonuclease-treated antigens. All four antigen preparations were shown to inhibit the migration of peritoneal macrophages of salmonella-infected (immune) guinea pigs from capillary tubes, in comparison with cells of normal control animals. By use of the high-molecular-weight antigens and the ammonium sulfate-precipitated antigens, the production of the migration inhibition factor(s) was elicited from cultures of lymphocytes obtained from the peripheral blood of immune guinea pigs. The activity of the migration inhibition factor(s) was demonstrated by its ability to inhibit the migration of peritoneal macrophages of normal guinea pigs from capillary tubes. In contrast, normal peritoneal macrophages exposed to products of antigen-stimulated immune lymphocytes did not exhibit an enhanced phagocytic or bactericidal action against virulent *S. typhimurium* as compared with those of the normal control. The present study indicated that the bacterial antigens responsible for the elicitation of the production of the migration inhibition factor from lymphocytes of immune guinea pigs are inactivated by proteolytic enzymes, but not by ribonuclease, and have molecular weights of >50,000.

Experimental evidence in this laboratory has consistently shown that virulent salmonellae are effectively destroyed by macrophages of guinea pigs and mice (11, 16, 22). Macrophages from immune animals were not endowed with an enhanced capacity to accelerate the intracellular killing of virulent *Salmonella typhimurium* (11, 16), as found by other investigators (2, 6, 17, 24, 30). In contrast, the opsonizing effect of immune serum appears to play an important role in the acquired resistance of the host to salmonellosis (12, 14, 16, 19, 23). On the other hand, it is generally agreed that delayed-type hypersensitivity to bacterial antigens is a concomitant manifestation of the host in salmonellosis (4, 7, 27).

Current knowledge in immunobiology favors the concept that the antibacterial cellular immunity expressed by macrophages is mediated by products elicited from T-lymphocytes either specifically with antigens (5, 15, 21, 25) or nonspecifically with mitogens (8). In this context, the production of lymphokines from sensitized lymphocytes requires the elicitation with specific antigens, whereas the expression of enhanced antibacterial action in macrophages mediated by lymphokines is directed nonspecifically against different bacteria.

The present paper is intended to demonstrate the manifestation of delayed-type hypersensitivity in salmonella-infected guinea pigs by use of the in vitro macrophage migration inhibition test and to determine whether the antibacterial activities of macrophages can be altered by products of immune lymphocytes stimulated with salmonella antigens, using a cell culture procedure.

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MATERIALS AND METHODS

S. typhimurium. The stock virulent strain SR-11 (13) was used in this study. The mean lethal dose of

this strain was approximately 10 bacteria upon intraperitoneal injection into mice. The preparation of an optically standardized bacterial suspension in saline (containing approximately 2.5×10^9 bacteria/ml) was described previously (11, 13).

Preparation of bacterial antigens. Cultures of *S. typhimurium* were grown in tubes of 5 ml of tryptic soy broth (Difco) placed in a rotating drum at 37°C for 48 h. They were heated in a 60°C water bath for 30 min and then centrifuged at 2,000 × *g* for 30 min. The supernatant fluid was passed through a membrane filter (0.45 μm; Millipore Corp.), checked for sterility, and then concentrated to approximately 1/10 of its original volume with Lyphogel (Gelman Instrument Co.). This preparation is referred to as the concentrated bacterial antigens. When this preparation was ultrafiltered at 550 × *g* for 10 min through a Centriflo ultrafiltration membrane (Amicon Corp.) that eliminated substances of molecular weights <50,000, it is then referred to as the high-molecular-weight antigens.

Ammonium sulfate-precipitated antigens, hereafter referred to as the precipitated antigens, were prepared by treating 40 ml of heated *S. typhimurium* culture supernatant with an equal amount of a saturated solution of ammonium sulfate. After overnight refrigeration at 5°C, the precipitates were sedimented by centrifugation at 2,000 × *g* for 20 min at 5°C. The sediment was dissolved in 5 ml of phosphate-buffered saline (PBS; pH 7.3) and ultrafiltered at 550 × *g* for 10 min through a Centriflo ultrafiltration membrane to remove the ammonium sulfate. The antigenic preparation retained by the membrane was adjusted to a total volume of 2 ml with PBS.

Alternatively, 20 ml of the heated culture supernatant was treated with insoluble ribonuclease (RNase-agarose beads, Sigma Chemical Co.) prior to the precipitation with ammonium sulfate. The insoluble RNase was washed twice in distilled water before being added to the culture supernatant and agitated continuously for 30 min at room temperature. After removal of the insoluble RNase by centrifugation at 550 × *g* for 10 min, the ammonium sulfate extraction procedure was then followed as described above. This preparation is referred to as the RNase-treated antigens.

Tryptic soy broth was treated similarly as in the preparation of concentrated bacterial antigens and used as a control. All final preparations were filtered through a membrane filter (0.22 μm; Millipore Corp.) and checked for sterility before use.

Treatment of bacterial antigens with proteolytic enzymes. The insoluble trypsin (trypsin-polyacrylamide, Sigma Chemical Co.) or insoluble protease (protease-carboxymethyl cellulose, Sigma Chemical Co.) was suspended by adding 5 mg of each compound to 0.5 ml of PBS, pH 7.3. The suspensions were left at room temperature for 2 h with occasional shaking and then washed twice with PBS. Seven-tenths of 1 ml of the precipitated antigenic preparation was mixed with 0.35 ml of the soaked and washed insoluble enzyme preparations for 30 min, each in succession at room temperature. At each time, the insoluble enzyme was removed by centrifugation at 1,000 × *g* for 10 min. The final preparation was passed through membrane filters (0.22 μm; Millipore Corp.).

Guinea pigs. Male albino guinea pigs were purchased from commercial sources and fed with guinea pig pellets, supplemented with vitamins (Poly-Vi-Sol, Mead Johnson) in their drinking water. Each animal weighed between 500 and 800 g.

Infection of guinea pigs with *S. typhimurium*. Guinea pigs were injected intradermally in two sites with 10^5 virulent *S. typhimurium*. After 2 to 4 weeks, they were given an intraperitoneal injection of 2×10^4 bacteria of the same strain. These animals were referred to as immune guinea pigs and used 1 to 3 weeks later as donors of peritoneal exudate cells or circulating lymphocytes.

Procedures for the isolation and cultivation of lymphocytes. Lymphocytes were separated from the peripheral blood of guinea pigs. Blood was drawn by cardiac puncture with a 10-ml syringe containing heparin (The Upjohn Co.). A sterile Hanks solution containing 6% dextran (molecular weight, approximately 184,000; Pharmchem Corp.) was added to the heparinized blood in a volume ratio of one part of dextran to three parts of blood. This mixture was drawn up in a syringe that was held in an inverted position in a ring stand for approximately 20 min. After the sedimentation of the erythrocytes, the upper layer of turbid plasma containing leukocytes was pushed out from the syringe through a bent, 18-gauge needle. The leukocytes were sedimented by centrifugation at 150 × *g* for 10 min. This cell pellet was resuspended in 0.4 ml of the original supernatant fluid. Equal portions of this suspension were then layered onto two tubes, each containing 2.5 ml of 20% sodium diatrizoate (Oral Hypaque Sodium Powder, Winthrop Laboratories) and centrifuged at 220 × *g* for 10 min. The buffy coat was transferred into 5 ml of medium 199 (Microbiological Associates, Inc.) containing 2% homologous serum. The leukocytes were then collected by centrifugation at 320 × *g* for 10 min. The sedimented cells were resuspended in culture medium, composed of 20% normal homologous serum, 70% medium 199, and 10% isotonic 1.4% sodium bicarbonate (CO_2 saturated). The leukocytic population in the suspension was quantitated in a hemocytometer. Peripheral blood so treated yielded between 90 to 95% lymphocytes and approximately 1% erythrocytes.

The lymphocyte suspension was adjusted to 1.5×10^6 cells/ml and dispensed in 1-ml volumes into Vacutainer tubes, to which 0.02 ml of the antigenic preparation or the control broth and 20 μg of kanamycin per ml were added. The tubes were flushed with 5% CO_2 in air before incubation in an Eberbach water bath at 37°C with a horizontal shaking movement at approximately 72 cycles/min. After 48 h of incubation, the lymphocytes were removed by centrifugation at 1,100 × *g* for 15 min. The supernatant fluid was used as the culture medium in the macrophage migration test or for the peritoneal macrophages.

Technique for macrophage migration inhibition test. The procedure for macrophage migration was adapted from the in vitro test for delayed hypersensitivity described by Bloom and Glade (3). Peritoneal exudate cells, containing primarily monocytes, were harvested 3 to 5 days after an intraperitoneal injection of 10 ml of sterile mineral oil into guinea

pigs, as described in detail previously (13). Suspensions of peritoneal macrophages were drawn up in heparinized, microhematocrit capillary tubes (Fisher Scientific Co.) that were then sealed at one end with Plasticine. The cells were packed into one end of the capillary tubes by centrifugation at 150 × *g* for 5 min. The portion of the capillary tube containing the cell pellet was cut off at the cell-medium interface under a dissecting microscope and placed in Lexy culture chambers (Mini-Lab Co., Laval, Quebec, Canada). The chambers consisted of round wells with a diameter of 20 mm that held a volume of 0.5 ml of culture medium containing 2% by volume of the test antigens or of the control broth when applicable. Two capillary tubes of packed macrophages (6 to 8 mm long) were anchored to the side of each well with silicone grease. The wells were covered on the top with a prefitted round cover slip and sealed with a paraffin-petrolatum mixture. There were two openings at one side of the wells, through one of which the appropriate cell culture medium containing the bacterial antigens and kanamycin or the supernatant fluid from the antigen-stimulated lymphocyte culture was injected with a syringe and needle, while air was expelled through the other opening. The openings were then sealed with paraffin-petrolatum. After 24 h of incubation at 37°C, the distance of macrophage migration was observed under a dissecting microscope with ×30 magnification. The distance of migration was measured from the center of the capillary tube to the outer margin of migrating cells with a calibrated ocular micrometer. The percent inhibition of migration was determined by use of the following formula: % inhibition = 1 − (mean distance of migration in the presence of antigen/mean distance of migration in the absence of antigen) × 100.

Procedure for the determination of the fate of *S. typhimurium* within macrophages cultured in medium containing stimulated lymphocyte products. Peripheral lymphocytes of normal and immune guinea pigs were isolated and cultured in the presence of the 10^{-3} dilution of the precipitated antigens for 2 days after the procedure described above. The culture supernatant fluid was recovered by removing cells and debris at 1,100 × *g* for 15 min. Two volumes of freshly collected peritoneal macrophages from one normal guinea pig containing approximately 4.5×10^7 cells each were centrifuged at 150 × *g* for 10 min at 5°C. The cell sediment was resuspended in 7 ml of medium composed of 65% freshly prepared cell culture medium, 35% antigen-stimulated lymphocyte culture supernatant fluid prepared from either a normal or immune guinea pig, and 20 μg of kanamycin per ml of medium. The cell suspension was placed in a 25-ml silicone-coated Erlenmeyer flask and incubated in an Eberbach shaking water bath at 37°C for 16 to 18 h. The cells were then recovered by centrifugation at 150 × *g* for 10 min at 5°C and resuspended in Hanks solution containing 6 U of heparin per ml and 2% homologous serum. The supernatant medium was centrifuged at 1,400 × *g* for 15 min to remove culture debris and then used again in the cultivation of infected macrophages. The macrophage population was determined with eosin staining in a hemocytometer. Only macrophage suspensions containing over 90% unstained cells were infected with virulent *S. typhimurium*.

The procedures for the infection and cultivation of macrophages and for the determination of the fate of intracellular salmonellae by quantitative recovery of bacteria by sonic treatment were all described in detail previously (11). Briefly, after an overnight incubation in culture supernatant fluid derived from antigen-stimulated lymphocytes of either normal or immune guinea pigs, 3×10^7 macrophages of a normal guinea pig were resuspended in 4 ml of Hanks solution containing 20% normal homologous serum and mixed with *S. typhimurium* in a test tube at a ratio of 10 bacteria/cell. After 15 min of rotation at 37°C, the contents of each tube of infected cells were transferred separately into two 40-ml silicone-coated centrifuge tubes, each containing 32 ml of chilled Hanks solution with 40 μg of kanamycin per ml. The cells were collected by centrifugation at 150 × *g* for 10 min at 5°C and resuspended in 4 ml of corresponding culture supernatant in which they were previously maintained overnight and to which 0.4 ml of normal homologous serum was added. The kanamycin concentration in the culture medium was adjusted to approximately 40 μg/ml. The infected cell cultures were left in 25-ml Erlenmeyer flasks in an Eberbach shaking water bath at 37°C. Samples of infected cells were removed at 0-, 2-, 4-, and 6-h intervals. The ratio of viable bacteria per cell was determined for each interval.

RESULTS

Effect of bacterial antigens on the migration of normal and immune peritoneal exudate cells. Peritoneal exudate cells derived from normal or immune guinea pigs were packed in capillary tubes, placed in duplicates in the Lexy chamber, and cultured in medium containing either the concentrated bacterial antigens or the high-molecular-weight antigens. In preliminary experiments, the original preparations of the antigens and their 10-fold serial dilutions in PBS were tested in the cell culture medium. A substantial amount of inhibition of macrophage migration was seen in both the normal and immune cells in the presence of the undiluted antigen preparations, as compared with those in the medium containing the control broth. When 10^{-1} and 10^{-2} dilutions were used, there was a noticeable difference in the migration inhibition of the normal versus the immune cells, despite an appreciable migration inhibition in the normal cells. When the antigens were diluted to 10^{-3}, an apparent migration inhibition was seen in the immune cells, whereas the extent of migration in the normal cells did not appear to be significantly affected by the presence of the antigens.

Table 1 shows the average results from three experiments in which a 10^{-3} dilution of either the concentrated bacterial antigens or the high-molecular-weight antigens was used. The average percentage of migration inhibition in normal cells was 4 ± 3 (standard deviation [SD]) for the concentrated bacterial antigens and 1 ± 2

TABLE 1. *Migration inhibition in peritoneal macrophages derived from normal and immune guinea pigs and cultured in medium containing bacterial antigens*

Antigen	Migration inhibition (%)[a]	
	Normal cells	Immune cells
Control	0	0
Concentrated bacterial[b]	4 ± 3	28 ± 10
High molecular weight[b]	1 ± 2	37 ± 3
Precipitated[c]	10 ± 4	48 ± 6
RNase treated[c]	13 ± 3	47 ± 4

[a] Data are expressed as mean ± SD.

[b] A 10^{-3} dilution of the antigenic preparation was used.

[c] A 10^{-1} dilution of the antigenic preparation was used.

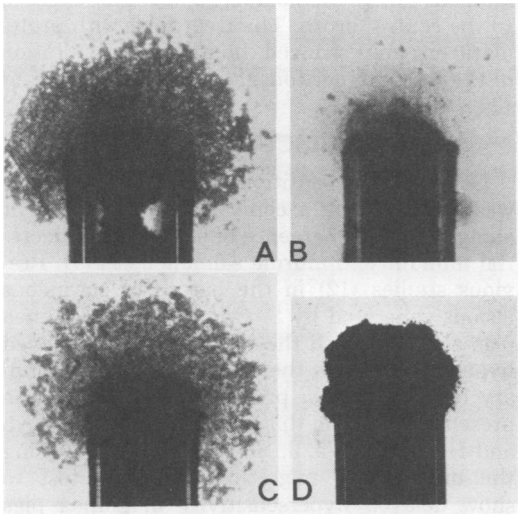

FIG. 1. *(A) Migration of peritoneal exudate cells of a normal guinea pig cultured in the presence of the concentrated bacterial antigens; (B) migration inhibition of peritoneal exudate cells of a salmonella-infected guinea pig cultured in the presence of the concentrated bacterial antigens; (C) migration of peritoneal exudate cells of a normal guinea pig maintained in the supernatant fluid of a culture of antigen-stimulated lymphocytes derived from a normal guinea pig; (D) migration inhibition of peritoneal exudate cells of a normal guinea pig maintained in the supernatant fluid of a culture of antigen-stimulated lymphocytes derived from a salmonella-infected guinea pig (original magnifications 8 · 5×).*

for the high-molecular-weight antigens, whereas the average migration inhibition in immune cells was 28 ± 10 and 37 ± 3%, respectively. Photographs of the comparative migration of normal and immune peritoneal macrophages from capillary tubes challenged with the concentrated bacterial antigens are shown in Fig. 1A and B.

In an initial attempt to characterize the chemical nature of the antigens that elicited the inhibition of macrophage migration, the bacterial culture filtrate was treated with ammonium sulfate. Also, a portion of the culture filtrate was pretreated with RNase before the precipitation with ammonium sulfate. Both preparations were tested in preliminary experiments, using 10-fold serial dilutions. In both cases, 10^{-2} and 10^{-3} dilutions failed to inhibit the migration of immune cells. Table 1 also shows the average results of three experiments in which a 10^{-1} dilution of either the precipitated antigens or the RNase-treated antigens was used. The average percentage of migration inhibition was 48 ± 6 for immune cells as compared with 10 ± 4 for normal cells when cultured in the presence of the precipitated antigens. When RNase-treated antigens were used, the comparative data were 47 ± 4% for immune cells and 13 ± 3% for normal cells.

Effect of lymphocyte products on the migration of normal peritoneal exudate cells. To detect the production of the migration inhibitory factor(s) (MIF) from antigen-stimulated immune lymphocytes, lymphocytes were isolated from peripheral blood of normal and immune guinea pigs and cultured in the presence of antigenic preparations or control broth for 48 h. The supernatant fluid was used as the test medium for the migration inhibition of peritoneal exudate cells harvested from normal animals.

Table 2 shows data from three experiments in which the 10^{-3} dilution of the high-molecular-weight antigens was used in the lymphocyte cultures. An average migration inhibition of 31 ± 16% was imposed on the peritoneal cells by the culture fluid of antigen-stimulated immune lymphocytes as compared with an average of 8 ± 5% by that of the normal lymphocytes.

When serial dilutions of the precipitated antigens were tested in lymphocyte cultures, the 10^{-3} dilution was the optimum concentration

TABLE 2. *Migration inhibition in peritoneal macrophages derived from normal guinea pigs and cultured in medium containing lymphocyte products elicited by bacterial antigens*

Antigen	Migration inhibition (%)[a]	
	Normal lymphocyte supernatant	Immune lymphocyte supernatant
Control	0	0
High molecular weight[b]	8 ± 5	31 ± 16
Precipitated[b]	9 ± 2	26 ± 5

[a] Data are expressed as mean ± SD.

[b] A 10^{-3} dilution of the antigenic preparation was used.

that elicited the production of specific MIF from immune lymphocytes. Table 2 also shows the results of three experiments in which the supernatant from antigen-stimulated immune lymphocytes inhibited the migration of normal macrophages by an average of 26 ± 5% as compared with that of the normal lymphocytes by an average of 9 ± 2%. Figures 1C and D are photographs of the comparative migration of normal macrophages from capillary tubes cultured in the supernatant fluid of normal and immune lymphocytes stimulated with the precipitated antigens.

Effect of lymphocyte products on the antibacterial action of normal peritoneal macrophages. Peritoneal macrophages from normal guinea pigs were cultured in medium containing antigen-stimulated lymphocyte products overnight. They were removed from their medium, infected with virulent *S. typhimurium*, and then returned to their original culture supernatant fluid for maintenance according to the procedure described above. The fate of salmonellae within macrophages cultured in the lymphocyte supernatant fluid derived from either normal or immune animals was compared over a 6-h period. Figure 2 shows the rate of intracellular destruction of these organisms plotted from the average of three sets of experiments. The phagocytic indexes, as defined previously (31), were 0.4 ± 0.19 (SD) for the infected macrophages exposed to the antigen-stimulated lymphocyte products from normal animals and 0.4 ± 0.17 for those exposed to antigen-stimulated lymphocyte products from immune animals. Under the condition of cell culture described here, exposure to products of antigen-stimulated immune lymphocytes did not enhance the bactericidal capacity of the normal macrophages against *S. typhimurium*, nor did it alter the phagocytic efficiency of these macrophages.

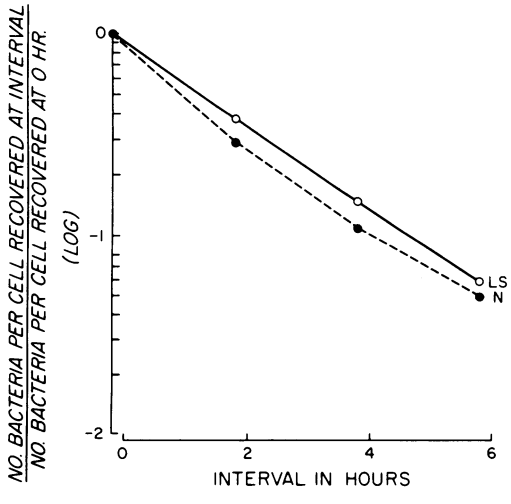

FIG. 2. *Comparative rate of destruction of S. typhimurium within normal macrophages maintained in the supernatant fluid of a culture of antigen-stimulated lymphocytes derived from a normal guinea pig (N) or from a salmonella-infected guinea pig (LS).*

Chemical nature of bacterial antigens. Table 1 shows that the migration inhibitory action of the precipitated antigens against immune peritoneal cells was not diminished by pretreatment with RNase. The precipitated antigens were also digested with trypsin and protease as described above and tested in cell culture against peritoneal exudate cells of immune guinea pigs. In two separate experiments, the average migration inhibition by the precipitated antigens was 46% as compared with cells exposed to the control broth. The treatment with proteolytic enzymes reduced the migration inhibition of the immune peritoneal cells to an average of 17%.

DISCUSSION

Delayed hypersensitivity to bacterial antigens was shown to be a concomitant development along with the emergence of acquired antibacterial immunity in murine salmonellosis (7). Previous studies (12) in the nature of cutaneous lesions generated by *S. typhimurium* in guinea pigs also suggested the involvement of delayed hypersensitivity in the development of secondary lesions. After a preliminary report on the present study from this laboratory (D. R. Mayo and H. S. Hsu, Va. J. Sci., **25**:105, 1974), using the macrophage migration inhibition test to show delayed hypersensitivity in guinea pigs infected with *S. typhimurium*, Cameron and van Rensburg (4) related host resistance to salmonellosis in guinea pigs by using a similar technique, except that their protein antigens were extracted from bacterial cells. The first objective of the present investigation was to demonstrate the presence of delayed hypersensitivity to salmonella antigens in salmonella-infected guinea pigs by use of the macrophage migration inhibition test and to determine the nature of the antigens responsible for this reaction.

Experimental data presented here showed that antigens prepared from culture supernatants of *S. typhimurium* could elicit the specific migration inhibition of peritoneal cells derived from previously infected guinea pigs. However, when peritoneal exudates from normal or salmonella-infected guinea pigs were initially tested with low dilutions of the concentrated bacterial antigens or of the high-molecular-

weight antigens, there was a consistently greater percentage of migration inhibition on the immune cells than on the normal cells, although the migration of the normal cells was also significantly inhibited. It would therefore be reasonable to presume that the migration inhibition of the normal cells was a result of their reaction with a variety of nonspecific antigens in the preparations, such as the common antigens among the gram-negative bacteria, rather than the result of the toxicity of the antigenic preparations. A subsequent dilution of the antigenic preparations to 10^{-3} removed the effect of the nonspecific antigens and showed a specific migration inhibition of the immune cells (Table 1).

The elimination of molecules with molecular weights of <50,000 from the bacterial culture filtrate by ultrafiltration did not diminish the migration inhibition activity of the antigenic preparations (Table 1). Protein was separated from the bacterial culture filtrate by ammonium-sulfate precipitation and tested in the culture medium for macrophage migration. A 10-fold dilution of the precipitated antigen preparation was also shown to elicit specific inhibition of cellular migration (Table 1). The bacterial culture filtrate was also treated with RNase before being subjected to the procedure of salt precipitation. No change in the antigenic activity of the treated antigenic preparation was detected (Table 1). On the other hand, when the same antigenic preparation was digested with trypsin and protease, its ability to induce migration inhibition of immune peritoneal cells was essentially eliminated.

To demonstrate the production of MIF by lymphocytes derived from guinea pigs infected with the virulent *S. typhimurium*, normal and immune lymphocytes were incubated with appropriate dilutions of the high-molecular-weight or the salt-precipitated antigens. The cell-free medium from immune lymphocyte cultures was shown to contain MIF, as evidenced by its ability to inhibit the migration of peritoneal macrophages of normal guinea pigs (Table 2).

The in vitro observations presented here are in agreement with those of Smith and Bigley (26), who reported that protein-rich fractions of *S. typhimurium* were responsible for the elicitation of MIF production by immune lymphocytes of mice in vitro. They also support the findings of Collins and Mackaness (7), who demonstrated a delayed hypersensitive reaction in footpads of salmonella-infected mice when injected with a protein-containing fraction obtained from cultures of several species of *Salmonella*.

The second objective of this investigation was to determine whether specific antigen-stimulated lymphocytes could produce lymphokines that might alter the antibacterial activities of macrophages against salmonellae. When a cell culture procedure similar in principle to those used successfully by other investigators in this type of experiments (8, 15, 18, 21, 25) was employed, the result shows that the supernatant fluid of antigen-stimulated lymphocytes of immune guinea pigs, despite its ability to inhibit the migration of macrophages, did not alter their bactericidal activity against *S. typhimurium* (Fig. 2), nor did it enhance their phagocytic capacity against these organisms as determined by the phagocytic indexes.

The failure to demonstrate enhanced antibacterial activities of macrophages against salmonellae under the influence of antigen-stimulated lymphocyte products does not necessarily negate the prevalent thesis regarding the role of lymphokines in the antibacterial action of macrophages (5, 8, 18, 25), nor does it dispute the functional capacity of lymphokines in acquired immunity against certain facultative intracellular bacteria such as *Mycobacterium tuberculosis* (21) and *Listeria monocytogenes* (15). Rather, the innate ability of macrophages to destroy *S. typhimurium* may indicate a maximum capacity for the bactericidal action of the macrophages against this pathogen and is therefore no longer subject to further activation by lymphokines. This is in agreement with previous observations (11, 16) that macrophages derived from immunized animals were not endowed with an enhanced capacity to destroy *S. typhimurium*.

Unlike *M. tuberculosis* (9, 10), authenticated experimental evidence to establish that *S. typhimurium* multiples within macrophages of the host is still lacking. Other investigators have also provided evidence of killing of *S. typhimurium* within mouse peritoneal macrophages (29) and by fractions of rabbit granulocytes (1). Examinations of histopathological specimens (H. S. Hsu and I. Nakoneczna, Proc. Natl. Meet. Reticuloendothel. Soc., 12th, Abstr. no. 81, 1975) revealed that the primary lesions in the reticuloendothelial system in murine salmonellosis consisted essentially of polymorphonuclear leukocytes at the initial stage of the infection, whereas the peripheral infiltration of mononuclear cells did not appear until 6 or 7 days after the infection. The latter coincided with the emergence of delayed hypersensitivity to bacterial antigens (7) and also with the terminal stage of a fatal primary infection. In contrast, the characteristics of a secondary lesion were marked by an early appearance of mononuclear cells and the formation of a granuloma. In the

final analysis, it is questionable whether the macrophages are in fact the crucial site of the initial bacterial proliferation in vivo. Hence, pathogenic salmonellae should not be classified as facultative intracellular parasites (6, 28). Rather, the antiphagocytic nature of the virulent *S. typhimurium* (31) would promote a rapid extracellular propagation of the pathogen in the tissue before the onset of acquired humoral immunity by the active production of specific opsonins (31) and cytophilic antibodies (11, 16). The beneficial role of delayed hypersensitivity is most likely manifested by an accelerated influx of the cellular and humoral elements into the site of infection. The view of an extracellular propagation rather than an intracellular invasion of salmonellae has largely been ignored by many investigators as the basic mechanism of pathogenesis in murine salmonellosis.

There is a general tendency to equate delayed hypersensitivity with acquired resistance in certain infectious diseases and to refer to their basic mechanisms as cell mediated or cellular immunity. It has been recognized, however, that these two phenomena are not synonymous, although they frequently emerge simultaneously after an infection. The dissociation of delayed hypersensitivity and acquired immunity in tuberculosis was discussed in detail recently by Youmans (32). In listeriosis, Osebold et al. (20) presented experimental evidence that separated acquired immunity from delayed hypersensitivity. Thus, there is now an increasing awareness in the literature to define clearly cellular hypersensitivity as the basis for the delayed hypersensitivity to antigens and cellular immunity as the basis for an enhanced antibacterial action of immune macrophages (18, 25). They are apparently two independent and distinguishable expressions of the altered functions of macrophages of the host in infectious diseases, although they may both be dependent on mediators produced by antigen-stimulated lymphocytes.

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