THE INFLUENCE OF IMMUNOLOGICALLY COMMITTED LYMPHOID CELLS ON MACROPHAGE ACTIVITY IN VIVO*

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Activated macrophages with highly microbicidal properties appear in the course of several important infections (1) and during a graft-versus-host reaction.[1] They contribute largely (2) and sometimes exclusively (3) to the mechanism of host resistance. The process of their activation involves immunological events which do not depend upon circulating antibody (4). Cells must therefore be considered for a role not only in the ultimate expression of this type of immunity but also in its mediation. Frenkel has shown convincingly that mice can be adoptively protected against an intracellular parasite, Besnoitia jellisoni, with immune lymphoid cells (5). The present work complements these findings by demonstrating that a high level of specific immunity and a proportional level of delayed-type hypersensitivity are conferred concurrently when living lymphoid cells from the spleens of Listeria-infected mice are injected intravenously into normal recipients.

Materials and Methods

Animals.—The mice used throughout these studies were specific pathogen-free females aged 5–6 wk. The colony, obtained from Charles River Breeding Laboratories, North Wilmington, Mass., has been maintained in an infection-free environment. Experimental animals were kept under Isocaps® (Lab Cages Inc., Hackensack, N. J.) on sterile litter and were fed on sterile, vitamin-enriched rations.

Organisms.—Listeria monocytogenes (strain EGD) has been maintained by constant passage in mice for the past 12 months. Suspensions for immunization or challenge were prepared from a 16 hr trypticase soy broth culture of infected spleen. The virulence of the organism has increased during passage from an initial intravenous LD_{50} of 1×10^5 to its current level of 5×10^3.

The strain of BCG (bacillus Calmette-Guérin) used was Tice (Trudeau Culture Collection). It was maintained on ATS[2] egg medium but was used as a 12 day primary subculture in Tween-albumin medium. This was dispersed by ultrasound (20 kcs for 20 sec) immediately prior to use. After enumeration in a Petroff-Hausser chamber, it was diluted to an appropriate concentration in Hanks' BSS (basic saline solution).

[1] Blanden, R. V. 1969. Increased antibacterial resistance in the presence of immunodepression from a GHV reaction. Submitted for publication.

[2] American Thoracic Society.

Immunization.—Mice were immunized against *Listeria monocytogenes* by the intravenous injection of living organisms in a dose which varied between 8×10^2 and 4×10^3. Some mice failed to survive the immunizing infection at the higher dose levels.

Preparation of Spleen Cell Suspensions.—Dissociated spleen cells were prepared aseptically from normal or immunized mice. Batches of five spleens were diced and extruded through a 60-mesh grid of stainless steel. After gentle pipetting, the crude suspensions were filtered through a 4 cm column of acid-cleaned cotton wool. They were then washed three times at 4°C in a large volume of Hanks' BSS containing 1% fetal calf serum (FCS). After a second passage through cotton wool, they were counted and brought to the desired concentration by appropriate dilution in 1% FCS in Hanks' BSS. The final cell suspensions were highly heterogeneous but consisted predominantly of lymphoid cells of varying sizes. They were almost devoid of recognizable macrophages which had been retained by the filter. Immediately before injection, the viability of the cells was assessed by dye exclusion using 0.05% trypan blue in BSS.

Test for Delayed Hypersensitivity.—Delayed hypersensitivity was measured in the footpad as previously described (6). The test antigens were prepared from the filtrate of a 96 hr trypticase soy broth culture of *L. monocytogenes*. The supernatant of a centrifuged culture was brought to 75% saturation with ammonium sulfate. The flocculent deposit was dissolved in 0.01 M phosphate buffer at pH 7.4, and separated into two protein-containing fractions by exclusion chromatography on Sephadex G-50. The material used for sensitivity testing was contained in the first fraction. It was lyophilized and redissolved in phosphate-buffered saline. The antigen gave good footpad reactions in *Listeria*-infected mice with a dose containing 2 μg protein. Routine tests were performed, however, with a dose of 20 μg protein in 0.02 ml. The injections were made with a 30 gauge needle introduced subcutaneously into the center of the plantar surface of the right hind foot. Reactions were measured at 24 hr with dial gauge calipers (Schnelltaster, H. C. Kröplin GmbH, Schlüchtern, Hessen, Germany). The difference in size between the right and left footpad (measured to 0.05 mm) was taken as the measure of hypersensitivity. The test antigen produced thickness increases which did not exceed 0.1 mm in unsensitized animals.

Measurement of Protective Immunity.—Protection against a challenge infection was measured in terms of the numbers of viable bacteria present in spleens and livers at intervals after the intravenous injection of approximately 4 LD_{50}'s of *L. monocytogenes*. The initial implantation of the challenge organism was obtained by plating homogenates of spleens and livers taken from five mice, 15 min after injection. Its subsequent behavior was determined in groups of five mice killed 24, 48, and sometimes 72 hr after challenge.

Microbicidal Activity of Peritoneal Macrophages.—Groups of mice were injected intraperitoneally with 0.1 ml of a suspension containing approximately 10^5 *L. monocytogenes* in 10% immune mouse serum obtained from animals which had survived a *Listeria* infection. The antiserum was used to promote maximum phagocytosis of bacteria within 10 min of residence in the peritoneal cavity, at which time, the peritoneal cavity of each mouse was washed out with 2.0 ml of cold BSS containing 10 IU heparin/ml. The washings were stood at 2°C until all samples had been recovered. Three aliquots of 0.3 ml were then diluted in 12 × 75 mm tubes containing 0.5 ml of 1% FCS. One was retained at 2°C, the others were incubated at 37°C for 30 min or 60 min. At these times one tube from each group was chilled to 2°C to halt further bacterial inactivation.[3] All tubes were then centrifuged for 8 min at 200 g. After plating 0.1 ml samples of the supernatant, which contained few, if any, extracellular organisms, the tubes were drained, and the deposited cells resuspended in 1.0 ml of 1% FCS in BSS. The cells were then disrupted by ultrasound (Biosonik) at 20 kcs for 10 sec. Volumes of 0.1 ml of disrupted cells were plated in duplicate to determine the initial number of intracellular bacteria and the percentage survival after 30 and 60 min.

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[3] Mackaness, G. B., and R. V. Blanden. 1969. Unpublished.

RESULTS

The Development of Immunologically Active Lymphoid Cells in the Spleens of Listeria-infected Mice.—It was learned from preliminary studies that an intravenous injection of 10^8 immune spleen cells, taken on the 7th day of a primary *Listeria* infection, would protect normal recipients against a lethal challenge with *L. monocytogenes*. Their rate of development and the persistence of the population of cells which could immunize recipient mice was studied in an experiment which could be performed only by dividing it into two parts. The animals used as immune spleen cell donors on the 4th, 5th, and 6th days of a primary *Listeria* infection were immunized intravenously with 1.1×10^3 viable *L. monocytogenes*. Those used for cell transfer on days 7, 8, 12, and 20 received a slightly smaller immunizing dose of 0.8×10^3 viable *Listeria*. At each transfer, recipients received 2×10^8 filtered spleen cells, the viability of which varied between 79 and 84%. The challenge organism was mixed with the cell suspension and injected intravenously into recipients in a volume of 0.5 ml. The actual viable counts per inoculum varied from day to day, but fell between 0.8 and 3.0×10^4. At each day of transfer, an equal number of normal control mice was injected with the same number of *Listeria* but no cells. As there was a small but unavoidable variation from day to day in the numbers of organisms used for challenge, the experimental results have been expressed in Fig. 1 as the difference in the log viable counts found at 24 and 48 hr. The base line for this calculation was taken as the mean viable counts found in spleens and livers of both the control and cell-injected mice sacrificed 15 min after challenge. Each point of the growth curve of *Listeria* in normal mice is the mean of 35 viable counts obtained in mice which received no cells.

The results in Fig. 1 show little protective effect from cells harvested on the 4th day of an immunizing *Listeria* infection. The high 24 hr liver counts at this time were probably due to a carry over of bacteria in the spleen cell suspensions prepared from actively infected donors whose spleens would still contain large numbers of organisms at this stage of infection (3). A rapid and progressive increase occurred in the efficiency of the immunity transferred with a standard dose of immune lymphoid cells harvested between the 4th and 6th or 7th days of the immunizing infection. Beyond that time, the protective effect diminished but was still substantial in animals receiving cells from 20-day immune donors. For practical reasons, the control mice in this experiment were not injected with spleen cells from unimmunized donors. Subsequent experiments will demonstrate that equivalent numbers of normal spleen cells do not influence the growth of *Listeria* in spleen or liver.

The relative efficiencies of cells obtained at different stages of an immunizing infection are more clearly depicted in Fig. 2, in which the data from the 48 hr counts in spleen and liver have been plotted against the time of cell harvest.

Dose-Response Relationship.—Since cells obtained on day 7 of the primary

infection appeared to be optimally protective, donors were used at this time to determine the minimum number of cells needed to produce a detectable level of immunity in normal recipients. Spleen cells from a pool of immunized donors
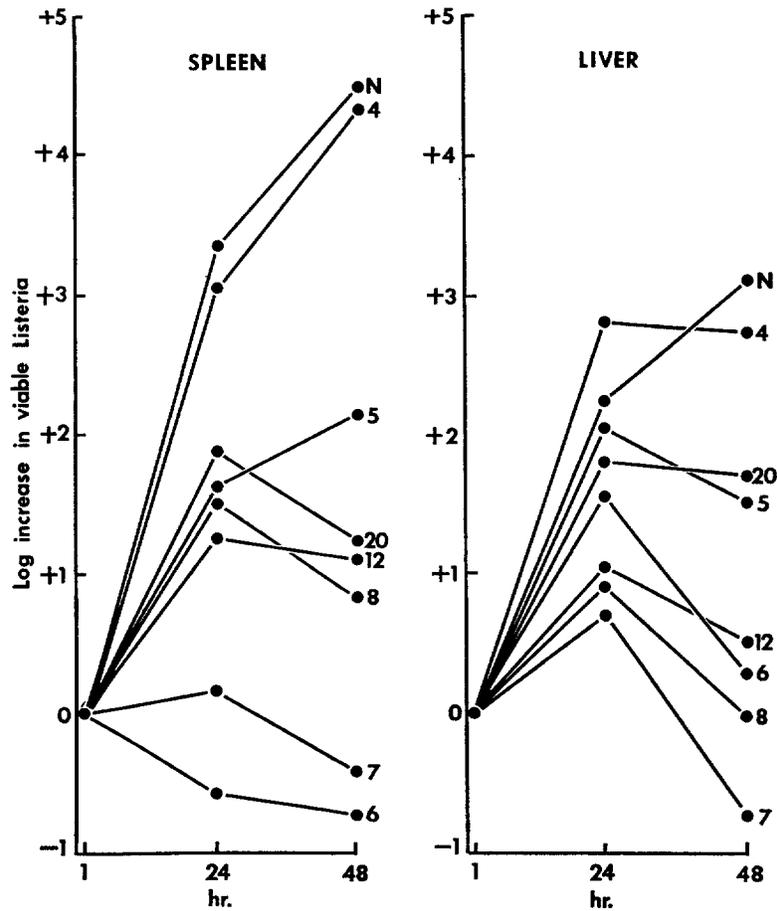

Fig. 1. Growth curves showing the inhibition of a *Listeria* challenge in the livers and spleens of mice protected with 10^8 lymphoid cells from the spleens of immunized donors. The curves are numbered for the day of the immunizing infection upon which cell transfer was made. The normal controls (N) did not received spleen cells. Means of 5.

were injected in varying numbers into recipients which had been challenged 15 min *previously* with an inoculum of 1.1 × 10^4 viable *L. monocytogenes*. The initial implantation of organisms in spleen and liver was determined in counts on five mice killed immediately prior to cell transfer. The cell suspension used

for transfer was 86% viable. Control mice received 2×10^8 spleen cells from unimmunized donors.

Fig. 3 shows the varying degree of protection conferred with graded doses of immune spleen cells. Mice injected with a large number of immune cells (2×10^8) showed progressive elimination of the challenge organism from the spleen (B) in contrast to its rapid growth in recipients of an equivalent number of normal spleen cells. Fewer immune cells produced proportionally less protection in
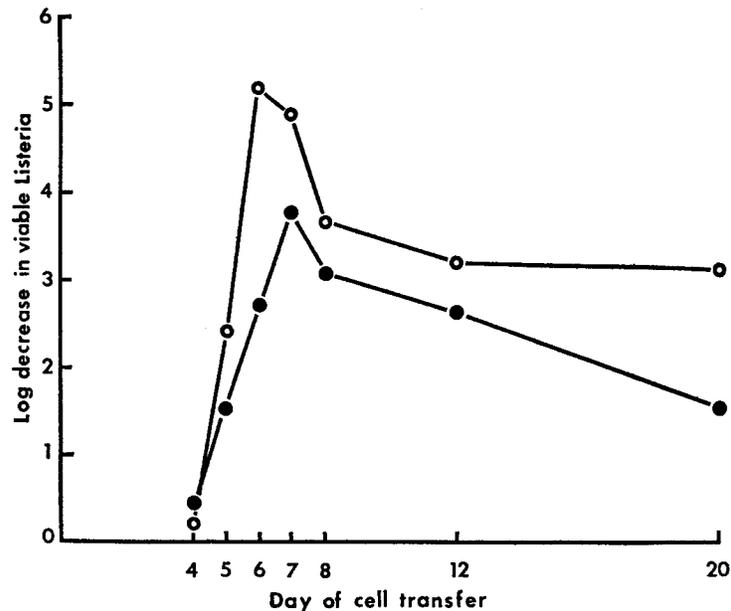

FIG. 2. Curves describing the development of a population of immunologically committed cells in the spleens of *Listeria*-infected mice. The curves were developed from the 48 hr counts recorded in Fig. 1, and show for spleen (O) and liver (●) the differences (Log_{10}) between the viable counts found in normal mice and mice injected with 10^8 cells obtained from spleens at varying stages of the immunizing *Listeria* infection.

both spleen and liver. It is not clear why the 24 hr counts in the livers of animals which received smaller numbers of immune cells should have been so high. It may have resulted from the presence of viable bacteria in the spleen cell inoculum, which lacked the countering influence of a sufficient number of immune cells to control them. Since 90% of bacteria inoculated intravenously are taken up by the liver, bacterial contamination of the spleen cell suspension would be more sharply reflected in liver than in spleen.

Comparison of Passive Immunization with Cells and Serum.—Donor mice were immunized by the intravenous injection of 6.4×10^2 living *L. monocytogenes*. 8

days later the immune mice, and a larger group of normal donors, were bled from the retro-orbital plexus. Their spleens were then used to prepare filtered
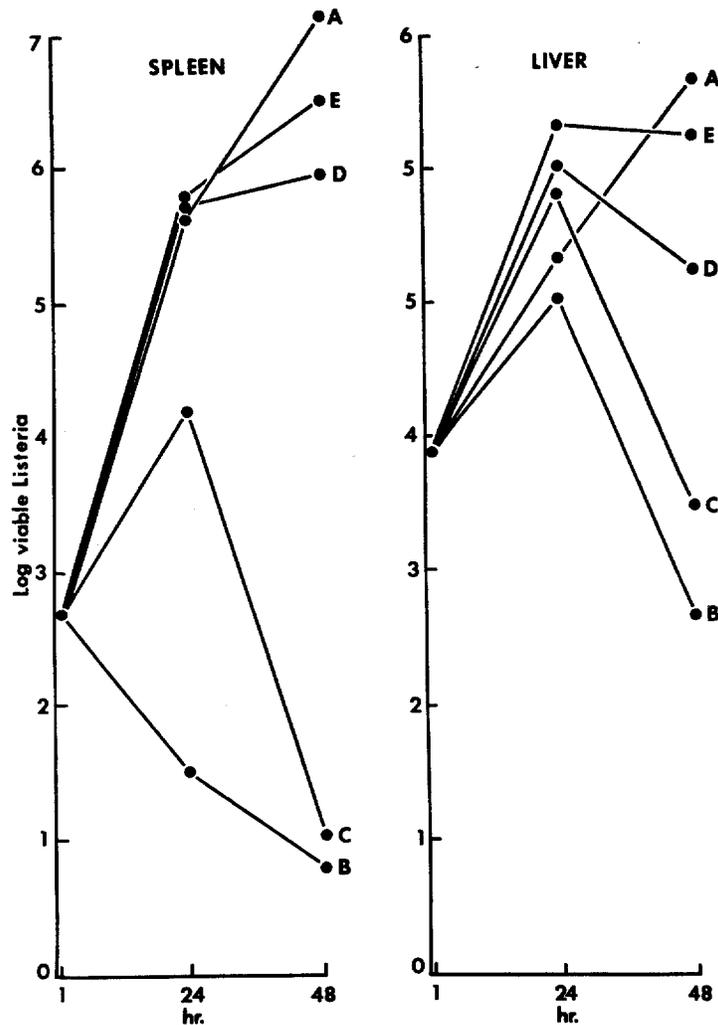

FIG. 3. Growth curves showing the protective effect of varying numbers of immune lymphoid cells from the spleens of 7-day immune donors. Recipients injected with normal cells in a dose of 2×10^8 (A) or immune cells in doses of 2×10^8 (B), 4×10^7 (C), 8×10^6 (D) and 2×10^6 (E). Means of 5.

cell suspensions. While the cells were being washed, the serum was separated and pooled. Two groups of mice were injected intravenously with 0.5 ml of suspensions containing 3.7×10^4 viable *Listeria* and 2×10^8 normal or immune

lymphoid cells of 75 and 72% viability, respectively. Two other groups of mice were injected intravenously with the same numbers of organisms suspended in 0.5 ml of serum from the same normal or immune donors. These had been incubated for 60 min at 37°C prior to injection. A third category of two groups of mice were injected with *L. monocytogenes* which had been incubated at 37°C for 1 hr in the presence of the normal or immune lymphoid cells at a concentration of 2×10^9 cells and 2×10^7 bacteria per ml; after 1:10 dilution, these mixtures were disrupted by ultrasound for 20 sec at 20 kcs. This sufficed to disrupt more than 90% of the cells without affecting the viability of the bacteria present in the mixture.

The growth curves of *L. monocytogenes* in the recipients of normal or immune spleen cells are depicted in Fig. 4A. In comparison with the recipients of normal cells, the spleens of mice receiving lymphoid cells from immune donors showed an immediate protection resulting in the death of 95% of the inoculum over a period of 48 hr. The effect in the liver was less pronounced at 24 hr, but by 48 hr, the livers showed a degree of protection similar to that found in the spleen. In almost every experiment reported here, and in an accompanying paper (7), the protection conferred by immune cells was seen immediately in the spleen but was delayed in the liver.

In contrast to the striking protection afforded by immune lymphoid cells, the transfer of 0.5 ml of immune serum had no discernible effect upon the growth pattern of *L. monocytogenes* in the spleens or livers of recipient mice even though the bacteria had been incubated in the serum for 60 min prior to injection (Fig. 4B). Moreover, incubation of the bacterial inoculum with immune lymphoid cells had no influence on the subsequent behavior of the organism, provided that cells were killed prior to transfer (Fig. 4C). This is taken as evidence that the secretion of an antibacterial antibody is not responsible for host resistance. Nonetheless, immune lymphoid cells can confer protection, but can do so only if they are viable.

Concurrent Transfer of Immunity and Specific Hypersensitivity with Immune Lymphoid Cells.—Donor mice were immunized intravenously with 1.1×10^8 viable *L. monocytogenes*. At daily intervals, 10 mice were used to measure the level of delayed hypersensitivity in the donors. On days 5, 6, and 7 of the infection, when the greatest variation had been found in the protective efficiency of spleen cells, 25 of the immunized mice were used as donors for the transfer of 10^8 spleen cells to 25 normal recipients. 10 of these were used immediately to test the level of hypersensitivity in recipients, the reactions being read after 24 hr. The other 15 recipients, together with 15 normal mice, were challenged with approximately 1×10^4 viable *Listeria*. After 15 min, and again at 24 and 48 hr, bacterial counts were made on spleens and livers from five normal and five passively immunized mice. The results are recorded in Fig. 5 as the difference in log viable counts in the spleens of normal and immune cell recipients. They show that maximum protection was achieved with cells harvested on day 6.

Fig. 5 also shows that the 6th day corresponded to the time of maximum foot-pad reactivity to *Listeria* antigen not only in the cell donors but also in cell recipients.
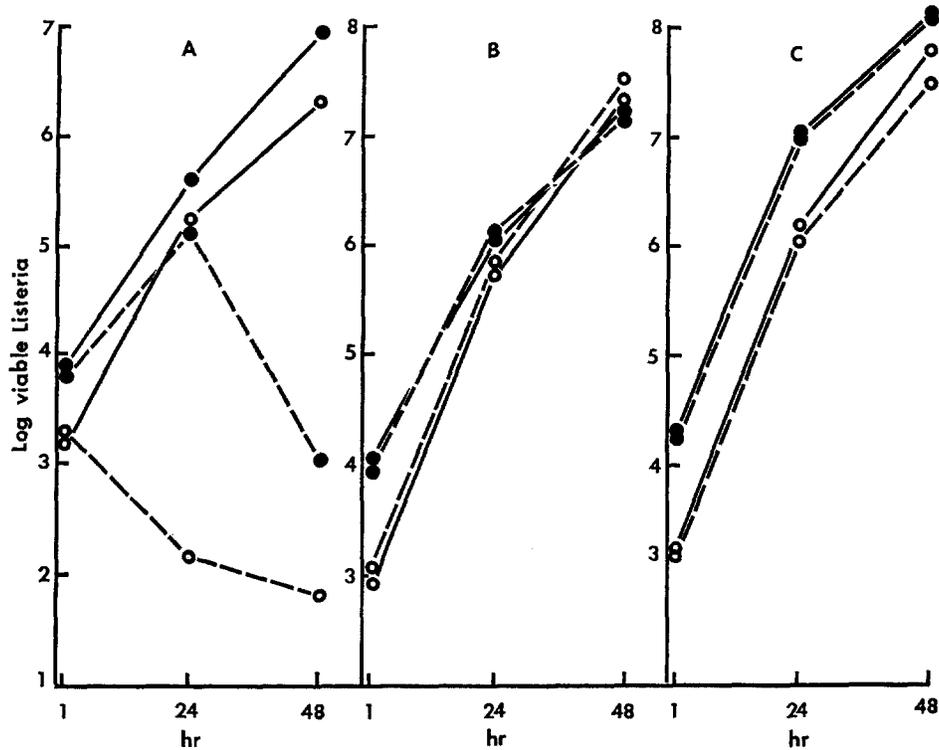

Fig. 4. A. Comparative growth curves of a *Listera* challenge in the livers (●) and spleens (○) of mice injected with 2 × 10⁸ spleen cells from normal (——) or 7-day immune donors (- - - - -). B. Growth curves of *L. monocytogenes* in animals which received only serum from the normal and immune donors described in A. The organisms were incubated with the sera before injection. Groups as designated in A. Means of 5. C. Growth curves obtained with inocula of *Listeria* which had been incubated in the presence of the normal or immune spleen cells used in A. The cells were disrupted by ultrasound immediately prior to injection. Means of 5.

The Effect of Mitomycin C on Adoptive Immunization with Cells.—It has been reported that the capacity of cells to transfer delayed-type hypersensitivity is impaired or ablated by pretreatment with mitomycin C (8). Its influence on cell-mediated protection against lethal challenge with *L. monocytogenes* was examined in the following experiments.

Filtered spleen cells from 7-day immune donors were suspended in 1% FCS.

Mitomycin C was added to half of the cells at a concentration of 1.0 μg/ml. The treated and untreated cells were then incubated for 60 min at 37°C, washed in BSS and resuspended in 1% FCS in BSS. After counting, they were diluted to contain 2.0×10^8 cells/ml. A group of control mice were injected with an equal number of cells from normal donors. These were also treated with mitomycin C. Recipients were challenged with *L. monocytogenes* 15 min prior to cell transfer.
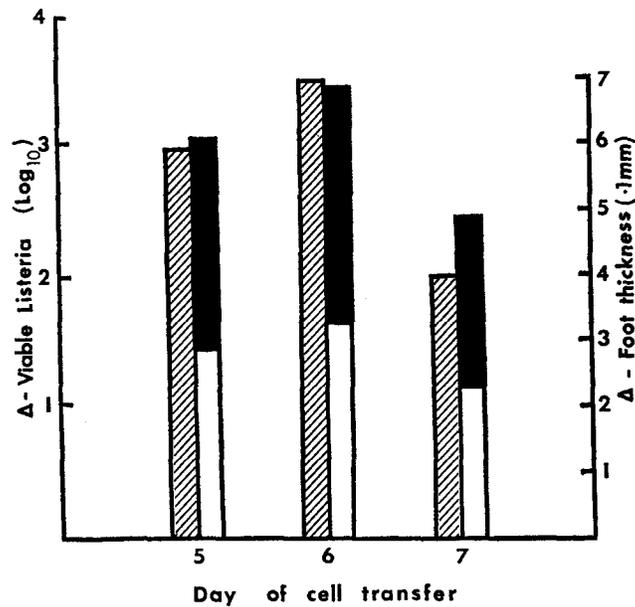

Fig. 5. Comparison between protection (hatched) and level of hypersensitivity (white) conferred with 10^8 spleen cells from 5, 6 or 7 day-immune mice. The level of hypersentitivity in the donors is shown in black. Protection is expressed as the difference (Δ) between log viable count in the spleens of normal and immune cell recipients. Recipients of normal cells gave no measurable reaction to *Listeria* antigens. Means of 5.

The effect of treating *Listeria*-immune spleen cells with mitomycin C is shown in Fig. 6. The cells were of low viability at the time of transfer (50 and 55% viable for treated and untreated cells, respectively). Those treated with mitomycin C, though no less viable, showed some reduction in their capacity to inhibit the growth of the challenge organism.

The foregoing experiment was repeated at a higher drug concentration. When exposed to mitomycin C at a concentration of 10 μg/ml, which is still low in comparison with that used by Bloom, Hamilton, and Chase (8), the cells retained their viability (82%) and some of their protective capacity remained intact (Fig. 7). It is of considerable interest that resistance increased progres-
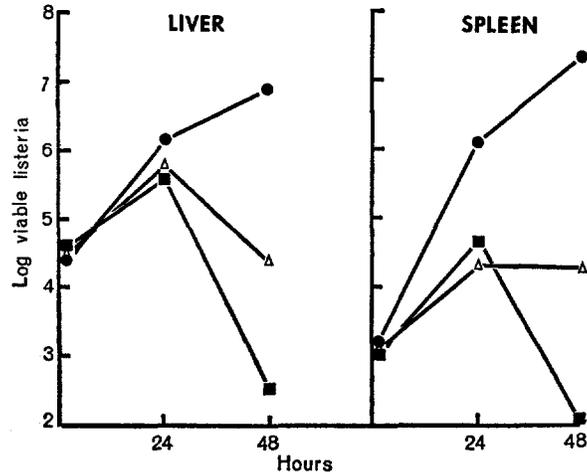
Fig. 6. Growth curves of *L. monocytogenes* in mice injected with 10^8 spleen cells from normal mice (●) or *Listeria*-immune mice. The latter were incubated in the presence (△) or absence (■) of mitomycin C (1.0 μg/ml) prior to transfer. Means of 5.
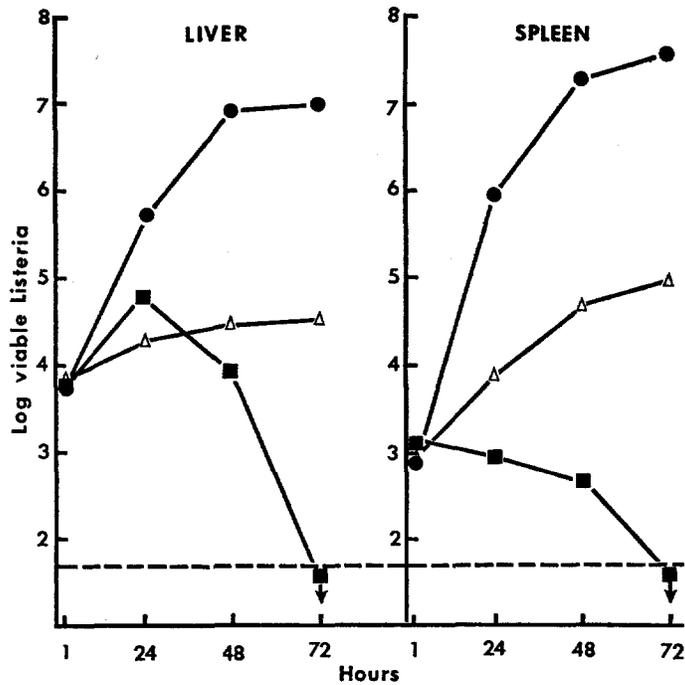

Fig. 7. Growth curves of *L. monocytogenes* in mice injected with 10^8 spleen cells from normal or *Listeria*-immune mice. Half of the immune cells (△) and the normal cells (●) were incubated in the presence of mitomycin C (10 μg/ml) for 1 hr prior to transfer. The third group (■) received untreated immune cells.

sively in the animals which received untreated cells, but not in the recipients of cells which had been treated with mitomycin C. No detectable organisms could be found at 72 hr in spleens or livers of the former, whereas the latter showed a small but progressive increase in bacterial count over the whole period.
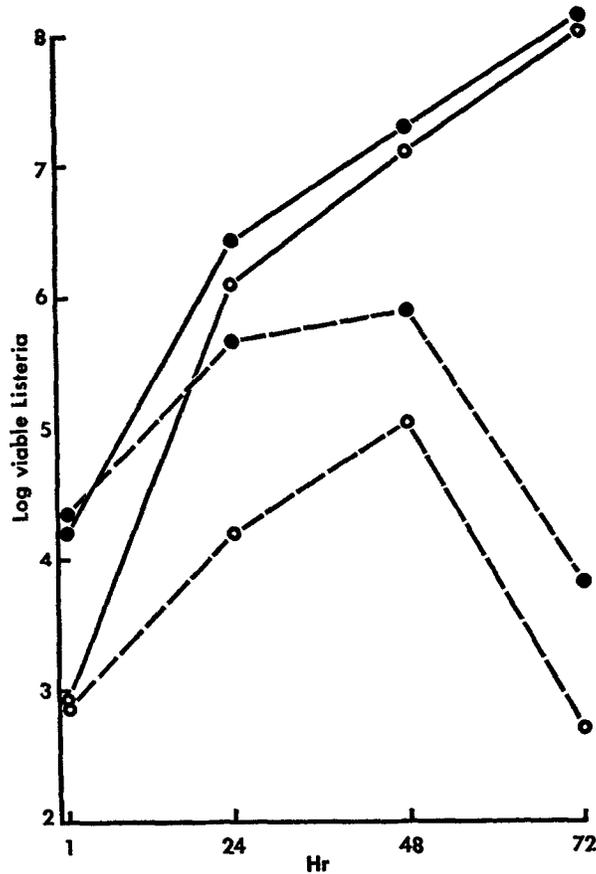

Fig. 8. Growth curves of *L. monocytogenes* in the spleens (O) and livers (●) of mice injected with 10^8 spleen cells from normal (——) or *Listeria*-immune donors (— — —) which received their immunizing infection 12 days before cell transfer. Means of 5.

The foregoing experiment suggested that mitomycin C, at the higher concentration, had prevented proliferation of donor cells in the recipient, leaving other metabolic activities intact. The degree of protection conferred by immune lymphoid cells may depend therefore upon donor cell proliferation in the recipient. This possibility receives support from an experiment (Fig. 8) in which immune cells were obtained on day 12 when the active phase of the immune response in the donors had subsided. Upon transfer to normal recipients, a

standard dose of these immune cells (10^8) gave only a low level of resistence during the first 48 hr. Between 48 and 72 hr, however, resistance mounted steeply as indicated by a dramatic fall of viable counts in spleens and livers. This could mean that some of the committed cells did not become functional until some time after transfer, or more plausibly that the committed cells became augmented in number by active proliferation in the tissues of the recipient. The latter explanation receives support from other observations[4] which show that vinblastine (a mitotic poison) effectively suppresses the immunity conferred by a population of *Listeria*-immune lymphoid cells when given to recipients at the time of cell transfer.

The Specificity of Adoptive Antibacterial Immunity.—Animals infected with BCG become highly resistant to challenge with *L. monocytogenes* (9). It was of interest to see whether this nonspecific resistance could be passively transferred with spleen cells. Evidence from a previous study (4) suggested that lymphoid cells from BCG-immunized mice would probably not protect normal recipients against a *Listeria* challenge, but that some degree of resistance might develop if the recipients were stimulated with an appropriate dose of living BCG. The following experiment was designed with this possibility in mind.

Mice were immunized by the intravenous injection of 1.2 × 10^6 living BCG. 21 days later, these animals were highly sensitive to tuberculin. Some of them, together with a group of normal mice, were used to prepare filtered spleen cell suspensions. The remainder, and an equal number of normal mice, were used to compare the growth of *L. monocytogenes* in the spleens of the normal and immune cell donors. Two groups of recipients were injected intravenously with cells from the normal or BCG-immunized donors. Together with the cells, half of each received an inoculum of living BCG (1.1 × 10^6). All animals were then challenged with *L. monocytogenes*. Spleen and liver counts were performed on five mice from each of the six groups, 24 and 48 hr after challenge.

The results recorded in Fig. 9 show that BCG-immunized donors (E) were highly resistant to challenge with *L. monocytogenes*; but recipients of lymphoid cells from these animals were no more resistant than normal mice (F) or the recipients of normal cells (C). Those receiving both immune spleen cells and living BCG (B) became partially resistant. This effect of a small inoculum of BCG was not seen in the recipients of normal cells (D). The result is interpreted to mean that resistance conferred with immune lymphoid cells is specific for the infective agent, and that the antibacterial mechanism which develops in the recipients of immune spleen cells is dependent upon some form of interaction between the specifically sensitized cells and the organism or its antigenic products.

Activation of Peritoneal Macrophages in Adoptively Immunized Mice.—It was

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[4] Tripathy, S. P., and G. B. Mackaness. 1969. The effect of cytotoxic agents on the passive transfer of cell-mediated immunity. Submitted for publication.

found in a recent study (9) that the injection of BCG into tuberculin sensitive mice causes a marked and rapid increase in resistance to challenge with *L. monocytogenes*. It was accompanied by changes in the morphology and micro-bicidal activity of free macrophages in the peritoneal cavity. A similar effect
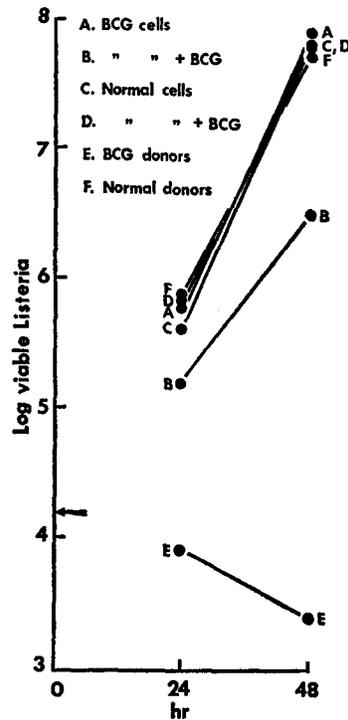

FIG. 9. The curves illustrate the behaviour of a population of *L. monocytogenes* in the spleens of mice which had received 10^8 normal (C and D) or BCG-immunized spleen cells (A and B); and in the spleens of the normal (F) or immune (E) donors. Half of the recipients of normal (D) and immune (B) cells were also injected with a small dose of BCG at the same time. The curves show that the immune donors were *Listeria* resistant (E). However, the recipients of cells from these donors were no more resistant than normal animals (F) or the recipients of normal cells (C). The injection of BCG produced a statistically significant increase in resistance only in the recipients of BCG-sensitized lymphoid cells (B). This dose of BCG had no effect on the resistance of recipients given normal cells (D).

was therefore sought in mice which had been passively sensitized with lymphoid cells from tuberculin sensitive donors.

Spleen cell donors were immunized intravenously with approximately 10^8 viable units of BCG. 14 days later, spleen cells from these and a larger group of normal mice were prepared for transfer to normal recipients in a dose of 2×10^8 cells. 10 recipients of normal or immune spleen cells were tested for tuberculin

hypersensitivity. Half of the remaining mice in each group were injected with living BCG (1×10^7). After 24 and 48 hr, five mice from each of the 4 groups were used to examine microbicidal activity in the macrophages of the peritoneal cavity as described in the methods.

No significant differences were found between groups in respect to the microbicidal activities of macrophages tested 24 hr after cell transfer. But at 48 hr, a
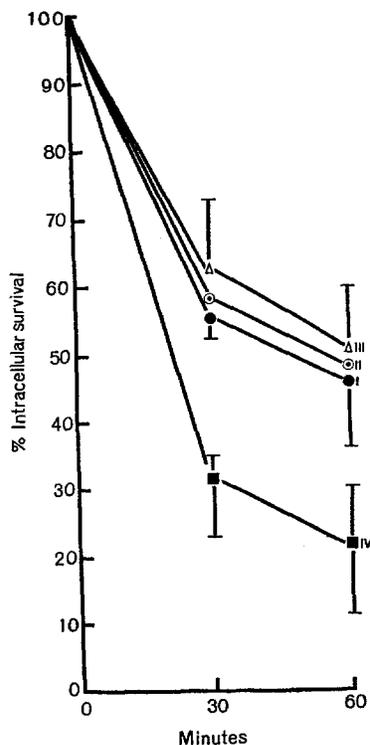

FIG. 10. Curves showing percentage survival of *L. monocytogenes* in peritoneal macrophages obtained 48 hr after injection of: I. 2×10^8 normal spleen cells. II. 2×10^8 normal spleen cells $+ 10^7$ BCG. III. 2×10^8 BCG-immunized spleen cells. IV. 2×10^8 BCG-immunized spleen cells $+ 10^7$ BCG. Brackets enclose the full range of values obtained in group IV and groups I, II, and III. Means of 5 mice.

difference had developed (Fig. 10). The macrophages of recipients of immune lymphoid cells alone were normal, but microbicidal activity was considerably enhanced in animals which had received an eliciting dose of BCG in addition to sensitized cells. Moreover, the macrophages from this group of mice spread rapidly on glass when a sample of the peritoneal washings was placed in culture, a feature characteristic of activated macrophages (9, 10).[1, 5]

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[5] North, R. J. 1969. Cellular responses leading to acquired cellular resistance. Prepared for publication.

DISCUSSION

Acquired resistance to *Listeria monocytogenes* rests finally with the activated macrophages which are so conspicuous a feature of the response to this and other facultative intracellular parasites (1), but immune macrophages can protect normal recipients only when given the opportunity to interact directly with the organisms of the challenge infection (11). Their defensive role seems therefore to be limited to their capacity to kill ingested organisms with enhanced efficiency. In other words, they lack the immunological properties which would permit the host to develop its own defense against infection. It is significant, therefore, that the immune macrophage also lacks the capacity to transfer delayed-type hypersensitivity (12). This function is subserved by cells belonging to the lymphoid series, as is indicated by their representation in regional lymph nodes (13), among the nonphagocytic components of cellular exudates (5), and in the cells of thoracic duct lymph (13, 14).

Filtered spleen cell suspensions obtained from *Listeria*-infected mice were found to contain the cellular elements necessary for the adoptive transfer of both hypersensitivity and protection against *L. monocytogenes*. Both of these important immunological modalities appeared in recipients at levels which bore a quantitative relationship, one to the other. Futhermore, antimicrobial resistance and delayed-type hypersensitivity arise concurrently in *Listeria*-infected mice (3). It is, therefore, tempting to infer that both are mediated by a single population of committed cells.

Little is known about the cells which perform these two important immunological functions. Something has been learned from the present study of the rate at which they appear in the spleen (Figs. 1 and 2), while a companion paper (7) reveals that they are extremely sensitive to inactivation by an anti-lymphocyte globulin (ALG) which does not react with macrophages. It seems, therefore, that the cells which can adoptively protect recipients against a *Listeria* infection are lymphoid in nature, even though ALG may not be uniquely specific for cells of this type (15).

When spleen cells were harvested from donors at progressive stages of an immunizing infection, the efficiency of the protection they conferred rose steeply from the 4th to the 6th or 7th day (Fig. 2). In studying the mitotic activity in the spleens of *Listeria*-infected mice, North[5] found that the percentage of cells engaged in DNA synthesis rose sharply to a briefly sustained peak at 48 hr. Most of the dividing cells were blast forms. Since transfer of resistance was not achieved with spleen cells harvested 2 days after the time of the peak mitotic response (Fig. 1), it seems that committed lymphoid cells are still few in number at this time. If the early mitotic activity is due in part, though probably not entirely[5], to cells engaged in the immune response to *Listeria*, the slow increase in the numbers of cells which can sensitize or protect normal recipients is difficult to understand. It may mean that the progeny of the dividing cells leave the

spleen soon after they are formed, or that a protracted period of maturation is necessary before they acquire full competence to perform the functions of a committed cell. The latter is unlikely in view of other evidence[5, 6] which places the onset of immunity as early as 24 hr after infection. This leaves the first alternative, which is supported by the findings of Hall et al. (16) who showed that antibody-forming cells are released from regionally stimulated nodes. If the same is true of the cells engaged in the immune response to living *Listeria*, the spleen might not acquire its greatest content of committed cells until the 6th or 7th day because it takes time for the newly formed cells to reach equilibrium with the entire pool of recirculating cells. This explanation assumes that the cells concerned in the mediation of acquired resistance are able to recirculate. Several points suggest that they can. First, they are present in thoracic duct lymph, which is a point on the pathway of recirculating cells (17). Second, cell donors show maximum reactivity to *Listeria* antigens on day 6 of a primary infection initiated with a small inoculum.[5] This corresponds to the time when committed cells are most highly represented in the spleen (Figs. 1 and 2), a fact which suggests that reactive cells have become uniformly distributed through blood and lymphoid tissues by the 6th day of infection. Finally, there is the significant finding that resistance usually appeared more rapidly in the spleens than in the livers of adoptively immunized animals.

The last point requires elaboration. The tendency of lymphoid cells to "home" on the spleen and other lymphoid organs (18) offers a possible explanation for the observed difference in the rate at which resistance appears in the spleens and livers of passively immunized mice. If transferred cells accumulate more abundantly in the spleen, this organ might be protected sooner than the liver. To investigate this possibility, *Listeria*-infected donors were injected repeatedly with tritiated thymidine during the period of peak mitotic activity in the spleen.[7] Six days later, their spleen cells were used for passive transfer to normal recipients. Approximately 24% of the transferred cells were labeled. The distribution of radioactivity in the organs of the recipients showed that the spleens acquired approximately 10 times as much radioactivity per unit weight as did the liver. This suggests that the labeled cells in the population were selectively removed by lymphoid organs. If a majority of the labeled cells in the transferred population were specifically committed to *Listeria* antigens, it would follow that committed cells can reenter the lymphoid tissue. This would make it possible for them to reach a maximum representation in the spleen at a time when their production has long since ceased. It might also explain why the spleen is immediately protected by transferred cells.

Several points can be made in regard to the manner in which immune lymph-

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oid cells accomplish the task of protecting normal recipients against a *Listeria* challenge. Three lines of evidence indicate that they do so by indirect means.

(*a*) Bacteria which had been incubated with immune lymphoid cells were uninfluenced in their behavior when injected into mice (Fig. 4C). If immune cells produce an *antibacterial* antibody, it should have been revealed in this experiment, for the cells themselves conferred immediate protection upon normal recipients (Fig. 4A). The earliest observations in the present studies were made 12 hr after transfer, at which time spleens of passively immunized animals had already inactivated most of the organisms implanted there.

(*b*) Immune lymphoid cells are devoid of protective activity when transferred to X-irradiated recipients.[4] Their defensive function must depend, therefore, upon the intervention of a radiosensitive cell supplied by the recipient. Volkman and Collins (19)[8] have shown that monocyte precursors are highly radiosensitive. If their production by the bone marrow is interrupted in animals with delayed-type hypersensitivity, reactivity to antigen disappears until monocyte production is resumed. In view of these findings, the circulating monocytes would seem to be intimately concerned not only in the classical manifestations of a delayed-type hypersensitivity reaction in the skin, but also with the expression of cell-mediated forms of acquired antimicrobial resistance. It would seem, in fact, that the immune lymphoid cells do not themselves accomplish the death of the parasite, but can influence host cells to achieve this end.

(*c*) Finally, the lymphoid cells of BCG-immunized donors were unable to protect normal recipients against a *Listeria* challenge even though the donors themselves were highly resistant to this organism (Fig. 9). Herein lies the element of specificity in acquired cellular resistance. Only when the recipients of cells from BCG-sensitized donors were injected with BCG did some degree of non-specific resistance develop against *Listeria*. The resistance mechanism must be assumed, therefore, to depend upon the interaction between the lymphoid cells and the microbial antigens with which they are reactive. It would be speculative to conclude that this interaction results somehow in the production of activated macrophages in the recipient were it not for the finding that peritoneal macrophages became altered in morphology and microbicidal activity (Fig. 10) under similar experimental conditions. When spleen cells from BCG-sensitized animals were transferred to normal recipients, no effects were seen in the free macrophages of the peritoneal cavity unless BCG was injected at the same time. Apparently, activated macrophages arise in the wake of a delayed-type hypersensitivity reaction which need not necessarily take place in their immediate vicinity. Although the effect of injecting BCG into passively immunized mice was not impressive, it was nonetheless significant, particularly as

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8 Collins, F. M., and A. Volkman. 1969. Suppression of delayed hypersensitivity and its subsequent recovery following X-radiation. Submitted for publication.

an eliciting dose of BCG takes 3 days to produce its maximum effect even in actively immunized mice (9).

The activation of host macrophages would seem from these findings to depend upon a humoral factor which is released by reactive lymphoid cells in the presence of specific antigen. A substance which circulates seems necessary to explain the effects observed in the peritoneal cavity of an animal which received sensitive cells and bacteria by intravenous injection. The migration inhibitory factor (MIF) (20, 21) could fulfill the role of the hypothetical humoral mediator which influences macrophage function. If MIF were to stimulate macrophages metabolically, it might induce in them the functional and morphological properties found in the acitvated macrophages of infected animals.

The release by lymphoid cells of a factor which causes systemic changes in macrophage activity would explain why resistance is usually slower to develop in the livers than in the spleens of adoptively immunized animals. If most of it were produced in lymphoid tissues, because reactive cells are more abundant there, its effects would appear more slowly in the liver and still more slowly in the peritoneal cavity.

Consideration must be given to an alternative explanation for the protective properties of immune lymphoid cells. They could secrete a cytophilic antibody which is so avidly bound by cells that it is effectively absent from serum. It can be imagined that specific antibody on the surface of macrophages would render them sensitive to antigen. It is already known that anti-mouse cell antibodies in newborn calf serum stimulate pinocytosis and raise the synthetic activity of macrophages in vitro (22). It is conceivable that antibody on the cell surface and microbial antigen in the cell's environment would have a similar effect. There is as yet no evidence that acquired cellular resistance depends upon cytophilic antibody. If it does, the activation of macrophages would require that free antigen be available and disseminated widely enough to influence cells in secluded areas such as the peritoneal cavity. That such things can happen is unequivocally attested by the macrophage disappearance reaction of Nelson and Boyden (23).

When immune lymphoid cells were transferred in small numbers (Fig. 3), or were harvested late in the immunizing infection (Fig. 8), low levels of resistance were found in recipients during the first 24 or 48 hr after transfer. A little later, the level of resistance rose sharply as indicated by a sudden drop in viable bacteria. This delayed onset of resistance did not occur with cells which had been treated with mitomycin C (Fig. 7). As mitomycin C can interfere with cell division without abolishing protein synthesis (24), these findings imply that immunologically committed cells can augment their contribution to host defenses by proliferation, so that the cells involved may be antigen-sensitive in a manner analogous to that of the memory cells which become engaged in a secondary antibody response. A similar conclusion was reached by Bauer and

Stone (25) in regard to the passive transfer of tuberculin sensitivity in guinea pigs.

SUMMARY

It has been shown that the immune response of mice to infection with *L. monocytogenes* gives rise to a population of immunologically committed lymphoid cells which have the capacity to confer protection and a proportionate level of delayed-type hypersensitivity upon normal recipients. The cells were most numerous in the spleen on the 6th or 7th day of infection, but persisted for at least 20 days. Further study revealed that the immune cells must be alive in order to confer protection, and free to multiply in the tissues of the recipient if they are to provide maximum resistance to a challenge infection. The antibacterial resistance conferred with immune lymphoid cells is not due to antibacterial antibody; it is mediated indirectly through the macrophages of the recipient. These become activated by a process which appears to depend upon some form of specific interaction between the immune lymphoid cells and the infecting organism. This was deduced from the finding that immune lymphoid cells from BCG-immunized donors, which were highly but nonspecifically resistant to *Listeria*, failed to protect normal recipients against a *Listeria* challenge unless the recipients were also injected with an eliciting dose of BCG. The peritoneal macrophages of animals so treated developed the morphology and microbicidal features of activated macrophages. It is inferred that acquired resistance depends upon the activation of host macrophages through a product resulting from specific interaction between sensitized lymphoid cells and the organism or or its antigenic products. Discussion is also made of the possibility that activation of macrophages could be dependent upon antigenic stimulation of macrophages sensitized by a cytophilic antibody.

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