CELLULAR KINETICS ASSOCIATED WITH THE DEVELOPMENT OF ACQUIRED CELLULAR RESISTANCE*

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There is good evidence for believing that acquired immunity against those bacterial species which can survive and multiply inside phagocytic cells is not mediated by humoral antibody. Instead, it depends on the ability of the host to acquire, during the living infection, mononuclear phagocytic cells with enhanced microbicidal activity (1–3). This form of acquired resistance is not specific in its expression, because it enables the host to resist infections caused by antigenically unrelated bacteria, but a specific immunological basis seems to exist, since resistance can be regenerated only by reinfecting the host with the homologous organism (4), and animals which acquire this type of immunity invariably develop a state of delayed hypersensitivity which is specific for antigens of the infecting organism (2). However, the way in which a specific immune response can cause changes in the intrinsic bactericidal mechanisms of mononuclear phagocytic cells is still unexplained.

The aim of this study was to determine whether the host response to infections with facultative intracellular bacterial parasites includes a proliferation of lymphoid cells, as would be expected of any mechanism with an immunological basis, and to find temporal relations between this lymphoid cell response and those changes in macrophage physiology upon which resistance to the infectious agent depends. One objective was to reveal the types of cells involved in the host's immune response and the stages when they participate. The two parasites employed in the study were *Listeria monocytogenes* and bacillus Calmette-Guérin (BCG).

Materials and Methods

Animals.—Specific pathogen-free adult mice were employed. The colony was obtained from Charles River Beeding Laboratories, North Wilmington, Mass. and was maintained in an infection-free environment and fed on a sterile, vitamin-enriched diet.

Bacteria.—*L. monocytogenes* (strain EGD) was maintained in a virulent state by repeated passage in mice. The organism had an LD_{50} of 5×10^8 when injected intravenously. It was grown in Trypticase soy broth for 12–18 hr, washed and diluted in 0.9% sodium chloride solution, and

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injected intravenously. The number of viable organisms injected was obtained by plating out the inoculum on nutrient agar. The dose varied between 1.5 and 3.0 $\times$ 10^8.

The two strains of BCG employed were BCG Tice and BCG Montreal. They were obtained as 7–9 day old cultures in Tween albumin medium from the Trudeau Institute Mycobacterial Culture Collection, Saranac Lake, N. Y. They were prepared for injection in the same way as *L. monocytogenes*. Approximately 5 $\times$ 10^7 viable units were injected intravenously.

The number of viable bacteria in the spleens of infected mice was obtained by plating out 10-fold serial dilutions, of homogenates of spleens on nutrient agar. Five mice were used for each time point studied.

Delayed Hpyersensitivity.—Delayed reactions to antigens of the parasites were measured in the hind footpad. *Listeria* antigens were prepared from the culture filtrate of a 4 day culture of the organism in Trypticase soy broth (5). The mice were injected subcutaneously into the right hind footpad with 0.02 ml of the antigen solution containing 20 μg protein. Reactions were measured 24 hr later with dial gauge calipers and compared with measurements of the uninjected footpad (measured to 0.05 mm).

BCG-infected mice were tested with 10 μg of purified protein derivative—PPD (Parke Davis and Co., Detroit) in the same way as described above.

Cellular Spreading.—Peritoneal cells were harvested in 2 ml of Puck's saline (6) containing 10 IU heparin/ml. The cell suspension was adjusted to 5 $\times$ 10^5/ml, and 0.8 ml was added to each of a number of simple culture chambers. The chambers were placed in an incubator at 37°C for 20 min and gently washed out with Puck's saline. The macrophages adhering to the floors of the chambers were fixed with 2.5% glutaraldehyde solution. Cellular spreading was measured according to a method described previously (7).

Radioautography.—Cells in the spleen and peritoneal cavity performing DNA synthesis at different stages of the infection were labeled radioactively by an intravenous injection of 10 μc tritiated thymidine (3 c/mmole) dissolved in 0.9% sodium chloride solution. The mice were sacrificed 30 min after the injection.

Smears of peritoneal cells were obtained by reflecting the abdominal skin and inserting a capillary tube into the peritoneal cavity. The peritoneal fluid (obtained by capillary action) was blown onto glass slides, smeared, air-dried, and fixed in methanol.

Spleens were removed, immersed in Earle's balanced saline solution (BSS), and cut into small pieces with a new scalpel blade. The cells were dissociated, and the suspension was passed through several layers of sterile gauze. A small aliquot was centrifuged in the cold for 30 min at 3000 rpm. The resulting pellet of cells was thoroughly resuspended in a drop of fetal calf serum, smeared onto slides with a camel hair brush, air-dried, and fixed in methanol.

All preparations were extracted with cold trichloroacetic acid for 30 min and washed thoroughly before radioautography (8). The preparations were exposed for 3 wk, and after developing and fixing were stained with 1% toluidine blue solution made alkaline with 1% Tris buffer.

Microscopy.—A Zeiss EM 9A electron microscope was used to examine peritoneal cells which had been labeled in suspension in vitro for 30 min at 37°C in medium 199 in Earle's BSS containing 0.5 μc tritiated thymidine (^{3}H-TdR)/ml. The cells were washed in BSS, spun into a pellet, and fixed in 2.5% glutaraldehyde containing 0.1 M sodium cacodylate, pH 7.4. They were then postfixed in 1% osmium tetroxide dissolved in Earle's BSS (pH 7), dehydrated in ethanol, and embedded in Araldite. Sections were cut on an LKB ultramicrotome and radioautographed for electron microscopy (8).

Small pieces of spleen were fixed in 10% neutral formaldehyde solution and embedded in glycol methacrylate (9) or wax. Sections 1–2 μ thick were radioautographed as described above and stained with either toluidine blue solution or methyl green–pyronin.

RESULTS

This study compares certain aspects of the cellular response of mice to infections caused by two intracellular parasites with different growth characteristics. *L. monocytogenes* divides rapidly in the mouse, and in sublethal doses causes a relatively short-lived acute infection. BCG, on the other hand, grows slowly and gives rise to a protracted infection. The results show that both infections cause similar cellular responses, consisting of lymphoid cell proliferation, macrophage proliferation, and a change in the physiological state of macrophages. The host response to each infection was investigated on three separate occasions and proved to be highly reproducible.

Growth of the Parasites in the Spleen—The growth of *L. monocytogenes* in the spleen is shown in Fig. 1, where it can be seen that an intravenous injection of 2×10^3 organisms resulted in the uptake of about 10% of this number by phagocytic cells in the spleen. In this organ there was an initial rapid multiplication of the parasite, resulting in population of about 10^5 at the end of 24 hr, followed by a slower increase to a peak population of 10^6 at the end of 48 hr. Beyond this time the number of viable organisms in the spleen began to decrease progressively until the 9th day of infection, when viable bacteria could no longer be detected. Mice therefore were capable to acquiring a high level of resistance against *L. monocytogenes* within 48 hr of infection.

The growth curve of BCG was not obtained. However, the experimental results of other workers in this laboratory show that this organism multiplies very slowly in the spleens of mice similarly infected: it increases about 10-fold in the organ during the first 12–14 days and then declines slowly during the ensuing weeks.

Incorporation of Tritiated Thymidine by Cells in the Spleen.—The proportion of mononuclear cells engaged in DNA synthesis at any one time during a primary *Listeria* infection is shown in Fig. 1. At the beginning of the infection less than 2% of the mononuclear cells were labeled. The proportion doubled at the end of 24 hr and reached 12% by 48 hr. This high level of labeling was sustained until the 4th day of the infection. The labeling rate then declined progressively to approach normal values by the 9th day. Thus, during this infection, the initial phase of bacterial multiplication was associated with a large increase in the number of mononuclear cells synthesizing DNA in the spleen.

In BCG-infected mice there were two well-resolved peaks of labeling in the spleen (Fig. 2). The first increase in the proportion of cells labeled took place over the first 48–72 hr. It was followed by a decrease and then a second phase of proliferation, which began on the 7th or 8th day. The second response was much more intense than the first and reached a peak on the 11th or 12th day, when an average of nearly 16% of mononuclear cells were labeled by a single

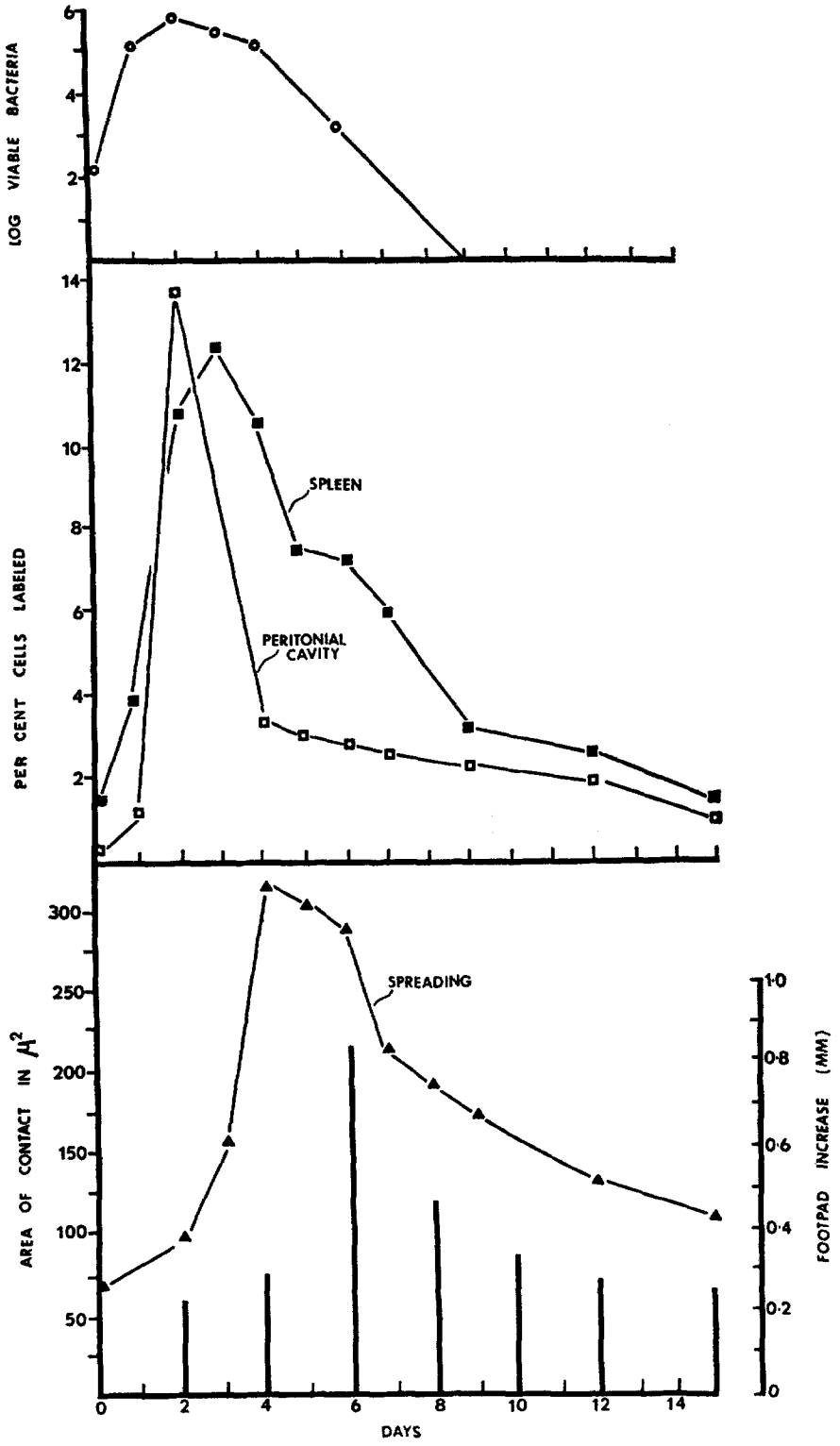

FIG. 1. Changes in the proportion of mononuclear cells in the spleen and peritoneal cavity which incorporate ^{3}H-TdR from a single pulse at different stages of a *Listeria* infection. Included are changes in the spreading ability of peritoneal macrophages (average area of contact of 100 macrophages in 20 min) and the deveopment of delayed hypersensitivity during the infection. The results were obtained from a single group of mice.

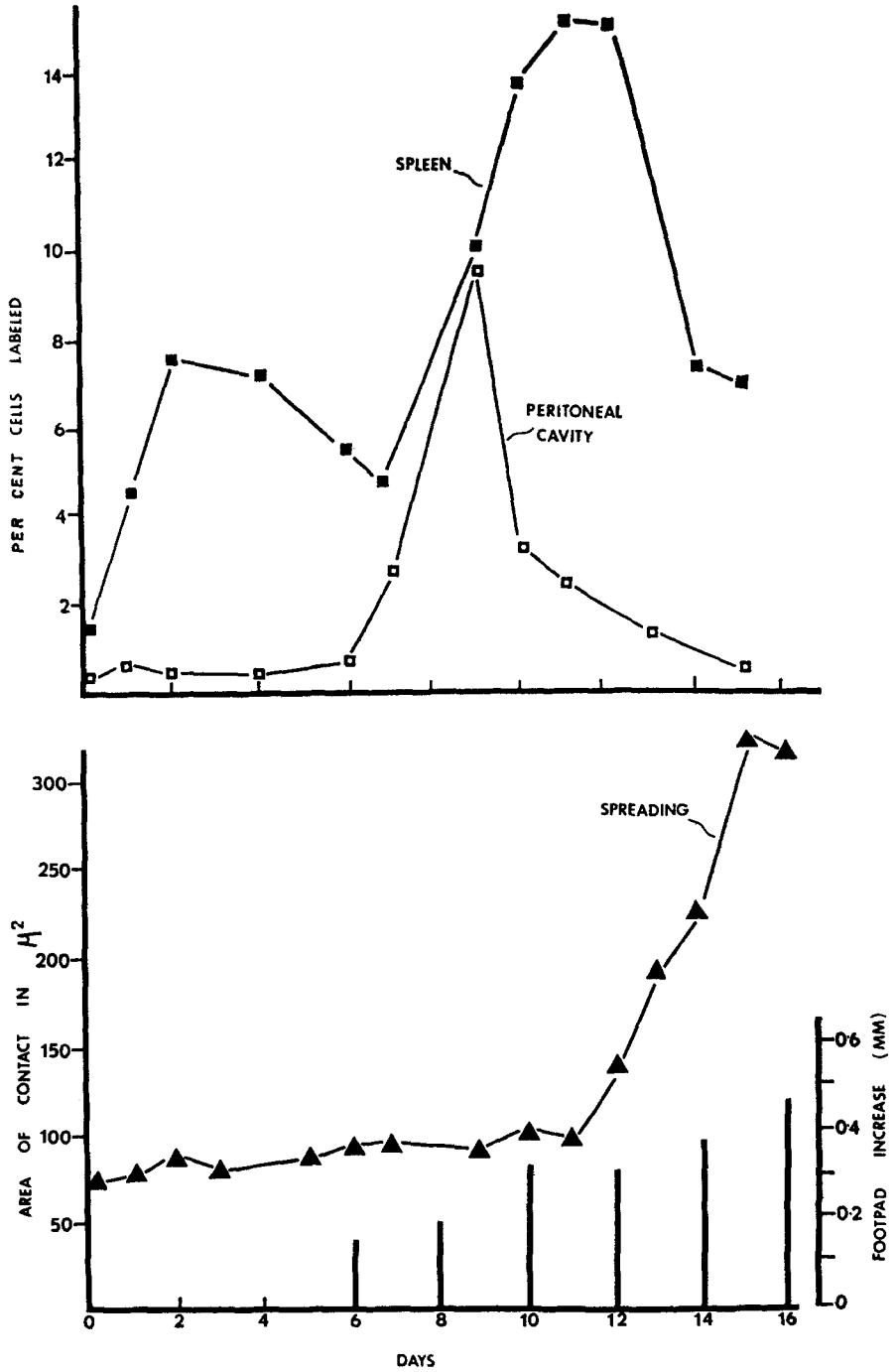

FIG. 2. Cellular changes during a BCG infection. The spleen showed two peaks of ^{3}H-TdR incorporation into mononuclear cells. Peak labeling of peritoneal mononuclear cells was coincident with the onset of the second splenic response. The spreading ability of peritoneal macrophages increased after the second mitotic response in the spleen, as did delayed reactivity to PPD.

pulse of ^{3}H-TdR. The proportion of cells labeled dropped sharply between the 12th and 14th days.

In both infections those cells in the spleen which incorporated tritiated thymidine were predominantly large cells with a dense basophilic cytoplasm (Fig. 4). Other labeled cells were medium and large lymphocytes and fixed macrophages.

Incorporation of Tritiated Thymidine by Cells in the Peritoneal Cavity.—Sooner or later, each infection resulted in a sharp increase in the proportion of mononuclear cells engaged in DNA synthesis in the peritoneal cavity. In *Listeria*-infected mice the proportion of incorporating cells did not change greatly until the 2nd day of infection (Fig. 1). From a value of less than 0.5% at the beginning, the proportion increased only slightly over the first 24 hr, but jumped to 14% by 48 hr. This sudden increase in the proportion of labeled cells was followed by an equally sudden decrease between 48 and 72 hr. Labeling decreased more slowly thereafter.

In contrast, BCG-infected mice did not show a significant increase in the proportion of their peritoneal mononuclear cells synthesizing DNA until after a relatively long delay (Fig. 2). A significant increase occurred between the 6th and 7th days, but it was not until the 9th day of the infection that peritoneal labeling reached a briefly sustained peak of 10%.

It will be apparent that in each infection the proliferation of mononuclear cells in the peritoneal cavity corresponded to the onset of a proliferative response of lymphoid cells in the spleen. In mice infected with *L. monocytogenes* both the splenic and peritoneal responses were coincident and occurred with greatest intensity in the interval between 24 and 48 hr. In BCG-infected mice the increase in the proportion of cells synthesizing DNA in the peritoneal cavity occurred much later and corresponded to the onset of the second and more intense of the two proliferative responses in the spleen. Thus, there was a temporal correlation between splenic lymphoid cell proliferation and the proliferation of cells in the peritoneal cavity. Morphological examination suggested that a majority of the labeled cells in the peritoneal cavity were differentiated macrophages, a view which is substantiated by the following experiments.

Evidence That Cells Which Synthesize DNA in the Peritoneal Cavity Are Resident Macrophages.—It was necessary to determine whether or not those cells which synthesize DNA in the peritoneal cavity during infections with either *L. monocytogenes* or BCG could be defined physiologically as mature resident macrophages. To answer this question, mice were injected intraperitoneally with 0.1 ml of a 1% suspension (w/v) of polystyrene spheres (Dow Chemical Co., Midland, Mich.) in distilled water. 2 wk later the mice were injected intravenously with either 2×10^8 viable *L. monocytogenes* or 5×10^7 BCG, and were given pulse-labels intravenously with 10 μc of ^{3}H-TdR at differ-

ent time intervals during the infections. Smears of peritoneal cells were radioautographed and counts were made of the number of radioactively labeled cells containing polystyrene. The results of one of two experiments employing *Listeria*-infected mice are summarized in Table I. They show that some 57% of the peritoneal mononuclear cells contained polystyrene 2 wk after it was injected. When animals with marked macrophage populations were infected with *L. monocytogenes*, the proportion of cells synthesizing DNA increased from 1.4% to 15% between 24 and 48 hr. Of the cells which incorporated ^{3}H-TdR on the 2nd day of the infection, 84% contained polystyrene particles. It can be concluded, therefore, that at least this proportion of the dividing cells were differentiated macrophages which had been resident in the peritoneal

TABLE I

Proportion of Peritoneal Mononuclear Cells Which Incorporate ^{3}H-TdR at Different Times during a Listeria Infection and Which Contain Polystyrene Particles Ingested In Vivo 3 wk before Infection

Day of infection	Cells containing polystyrene	Cells labeled with ^{3}H-TdR	Cells containing polystyrene and labeled with ^{3}H-TdR
	% ± SD	% ± SD	% ± SD
1	57.4 ± 12.4	1.1 ± 0.5	0.81 ± 0.2
2	31.32 ± 6.7	15.0 ± 3.0	12.6 ± 4.5
3	43.6 ± 4.8	3.5 ± 1.4	3.0 ± 1.2
4	37.7 ± 9.8	2.9 ± 1.1	2.5 ± 0.9

cavity for some time. The same conclusions were reached after examining preparations from BCG-infected mice.

An electron microscopic examination of peritoneal cells labeled in vitro on the 2nd day of a *Listeria* infection provided further evidence that the majority of labeled cells were resident macrophages (Fig. 6). Labeled cells which did not show ingested polystyrene spheres displayed the same ultrastructural characteristics as those which did. Most of the other cells which labeled were medium in size and contained a nucleus with abundant chromatin. Their most characteristic feature was a large number of free ribosomes in the cytoplasm.

Emergence of Delayed Type Hypersensitivity.—The development of delayed hypersensitivity during a *Listeria* infection is shown in the histogram of Fig. 1. Reactivity to *Listeria* antigen could be detected as early as the 2nd day of the infection and reached a maximum level on the 6th day. Thereafter it decreased progressively until the experiment was terminated.

On the other hand, delayed skin reactivity to PPD was first detected on the 6th day of a BCG infection. Thereafter reactivity increased progressively until the experiment was terminated (Fig. 2).

The results show, therefore, that a state of delayed hypersensitivity, like

other events in the host response, occurred early during a *Listeria* infection but later during a BCG infection.

Emergence of Macrophages with Enhanced Spreading Activity.—One of the characteristics of the activated macrophages which possess enhanced microbicidal activity is the rate at which they spread in contact with a glass surface (10). This property developed in the peritoneal macrophages of *Listeria*-infected mice at a rate illustrated in Fig. 1. Increased spreading activity was detected as early as the 2nd day of the infection. It increased rapidly over the next 48 hr and reached a maximum on the 4th day of the infection, when macrophages from infected animals established an area of contact with the substratum 5 times greater than did control cells in the same interval of time.

In contrast, the spreading ability of macrophages from mice infected with BCG was not greater than normal until the infection had progressed for 12 days (Fig. 2). Spreading ability reached a maximum on the 15th day, when the macrophages of infected mice spread 4 times more than control macrophages in the same time interval. This increased spreading ability was sustained for at least 6 more days.

Thus both infections resulted in the emergence of a population of peritoneal microphages with a greatly increased potential to increase their area of contact with a foreign surface. Furthermore, although these cells appeared at a different time during each infection, in both infections they emerged after peak proliferation of peritoneal macrophages and in parallel with the development of delayed hypersensitivity.

Phagocytic and Spreading Potential of Host Macrophages on the 4th Day of a Listeria Infection.—It has been argued (7) that the spreading of macrophages on a glass surface represents an attempt on the part of the cells to ingest the glass surface. According to this interpretation, macrophages with increased spreading potential would be expected to display increased phagocytic potential. This possibility was tested by comparing the spreading and phagocytic activities of cells from normal and infected mice.

Peritoneal cells were harvested from six normal mice and from six mice which had been infected 4 days previously with 2×10^8 *L. monocytogenes*. The cells of each group were pooled, washed in Puck's saline, and resuspended at 5×10^6/ml in Puck's saline containing 10% fetal calf serum. Some of the cells were used for studying spreading, and some for studying phagocytosis.

Spreading capacity was measured in culture chambers to which 0.8 ml of the cell suspension was added. The average area of contact was estimated at intervals during incubation at 37°C. Phagocytosis was studied with the cells adhering to glass. Cultures similar to those used above were incubated for 30 min at 37°C in the case of normal control cells, and for 10 min at 25°C in the case of cells from infected animals. In this way the cells of each group were comparable in respect to the extent of spreading when used to assess their respective

phagocytic abilities. After washing, 0.8 ml of a 2% suspension (w/v) of 1.88 μ diameter polystyrene spheres in 10% fetal calf serum was added in Puck's saline. The preparations were then incubated at 37°C. At intervals two chambers of

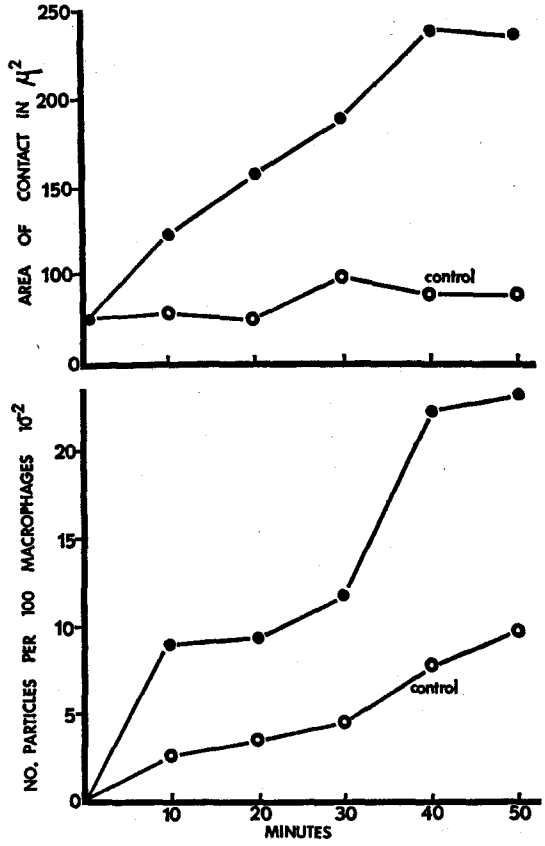

FIG. 3. Comparison of the spreading and phagocytic ability of macrophages from normal mice and from mice infected with *L. monocytogenes*. The ability to ingest polystyrene spheres and to spread on glass was much greater in the cells of infected mice.

each group were removed to assess phagocytosis according to a method described previously (7).

The results in Fig. 3 show that macrophages taken from mice infected 4 days previously with *L. monocytogenes* were able to spread on a planar surface and to phagocytose polystyrene particles at a much greater rate and to a much greater extent than macrophages from normal mice, which showed little ability either to spread or to ingest particles under the conditions employed. The steplike nature of the graph for phagocytosis was due to the method employed and has

been explained elsewhere (7). It should be realized that both the spreading and phagocytic activities of macrophages were tested on inert surfaces in the presence of homologous serum. It is almost certain, therefore, that increased spreading and increased phagocytic activity are reflections of an intrinsic change in the cells from infected animals. This conclusion was supported by the unreported finding that both activities were almost completely inhibited in the presence of iodoacetate (5×10^{-4} M).

DISCUSSION

The results of these studies have shown that in the mouse infection with either *L. monocytogenes* or BCG results in a pattern of cellular responses consisting of an intense proliferation of lymphoid cells in the spleen, a coincident proliferation of resident macrophages in the peritoneal cavity, and a subsequent emergence of a population of macrophages with a greatly increased ability to phagocytose inert particles and to spread on a foreign surface. The timing of these different cellular activities indicates the possibility that there is a causal relation between lymphoid cell division and macrophage division, and that the division of both types of cells is a prerequisite for the changes which occur in macrophages. Thus, in spite of the fact that cellular proliferation and an increase in the phagocytic activity of macrophages occur early during a *Listeria* infection, but only after a long delay in BCG-infected mice, the temporal relations between cellular proliferation and the production of activated macrophages are the same. It would appear, therefore, that the rates of initiation and development of these components of the host response are determined by the metabolic characteristics of the parasite.

In BCG-infected mice there are two periods of lymphoid cell proliferation in the spleen. Macrophage proliferation always coincides with the second period, suggesting that the latter corresponds to the single period of proliferation observed in the spleens of mice infected with *L. monocytogenes*. The significance of the two periods of proliferative activity in the spleens of BCG-infected mice is not known, but the possibility is being investigated that the first is due to antibody production in response to free antigen released from dead bacteria, and is independent of the cellular events leading to delayed type hypersensitivity. This view is based on the finding (11) that an intravenous injection of heat-killed BCG fails to induce this form of immune response and is not accompanied by changes in host macrophages. It is possible, therefore, that it is the second period of proliferation which represents the cellular response to living organisms resident within phagocytic cells and is the one concerned with the development of delayed sensitivity. Consistent with this view is a preliminary finding which indicates that only the first period of mitotic activity occurs in mice injected intravenously with heat-killed BCG. Since no distinguishing differences could be found in the morphology of the DNA-synthesizing

blast cells of the first and second mitotic peaks in BCG-infected mice, it seems that in both responses the cells are similar in appearance even though they may be concerned with different types of immunity (12–14).

The possible significance of the mitotic responses in the spleen and peritoneal cavity in relation to host immunity is best seen in mice infected with *L. monocytogenes*, where the simultaneous division of splenic lymphoid cells and peritoneal macrophages immediately precedes the onset of efficient antibacterial immunity. Although the growth curve of BCG was not followed, similar experiments by other workers in this laboratory (11)[1] have shown that BCG stops multiplying in the spleen on or about the 14th day of infection. Hence, the onset of the host's antibacterial mechanisms against BCG is similar to that found in *Listeria*-infected mice, since it occurs soon after a phase of proliferation affecting both splenic lymphoid cells and peritoneal macrophages.

The foregoing observations suggest that the development of acquired resistance to the infectious agents studied is directly dependent upon the lymphoid cell response. Evidence to support this contention has been obtained recently in studies which show that specific cellular resistance to *L. monocytogenes* can be transferred efficiently from immune mice to normal recipients with splenic lymphoid cells (5), and that the transfer can be blocked by antilymphocyte globulin (15). Since it is well established that immunity against *L. monocytogenes* depends ultimately on the acquisition by the host of macrophages with increased bactericidal properties (16), it is apparent that the altered physiological state of the phagocyte is accomplished through the influence of sensitized lymphoid cells.

These studies have shown that the host's acquired control over bacterial multiplication becomes evident at a time when macrophages with increased spreading and phagocytic ability appear in the peritoneal cavity. There is evidence that both of these cellular activities are performed by the same cellular mechanism and that they consume metabolic energy (7). This implies that *Listeria* and BCG infections give rise to macrophages which are capable of increased metabolic activity. Furthermore, it has been shown (10, 16) that macrophages with increased spreading ability have a greatly increased bactericidal capacity. It seems likely, therefore, that the enhanced microbicidal activity of activated macrophage is coupled to changes in cellular metabolism.

It was of interest to find that the macrophages which divide in the peritoneal cavity during a BCG or a *Listeria* infection are mature cells which were resident in the peritoneal cavity prior to the initiation of infection. In view of the large number of macrophages which became labeled by a single injection of ^{3}H-TdR, it is probable that all of the cells which show increased metabolic potential are

[1] Collins, F. M. 1969. Virulence of *M. tuberculosis* for the mouse and guinea pig. Prepared for publication.

recently divided cells. This may mean that macrophages must undergo division before they acquire enhanced antibacterial properties. The survival value to the infected host of macrophages with increased phagocytic and microbicidal properties is obvious. The fact that these changes are accompanied by intense mitotic activity adds a further advantage, in that greater numbers of cells become available for the host's defenses. The magnitude and systemic nature of this change in the phagocytic elements of the tissues are revealed in the following paper (17), which demonstrates that the Kupffer cells of the liver share in the changes described for the free phagocytes of the peritoneal cavity.

SUMMARY

Mice infected with either BCG or *Listeria monocytogenes* display the same type of cellular response pattern. In both infections there is an intense proliferation of splenic lymphoid cells, a coincident proliferation of differentiated macrophages, and a subsequent production of macrophages with increased metabolic potential. The temporal relations between these events and the onset of host resistance indicate that lymphoid cell proliferation and macrophage proliferation are essential events in the response which leads to cellular resistance against facultative intracellular parasites.

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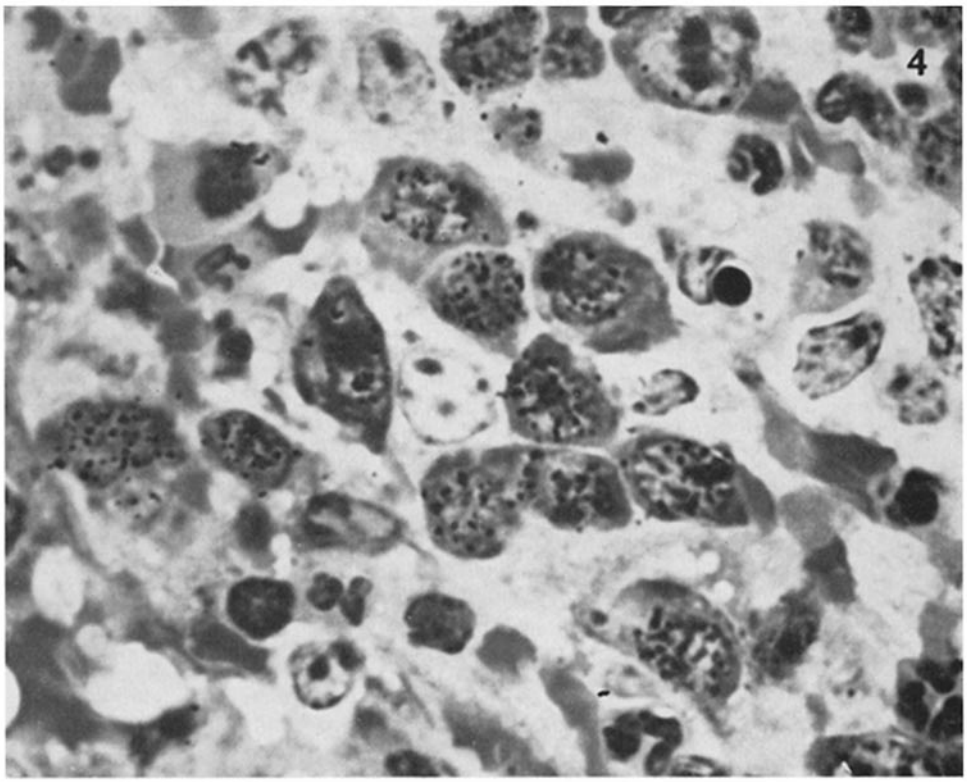
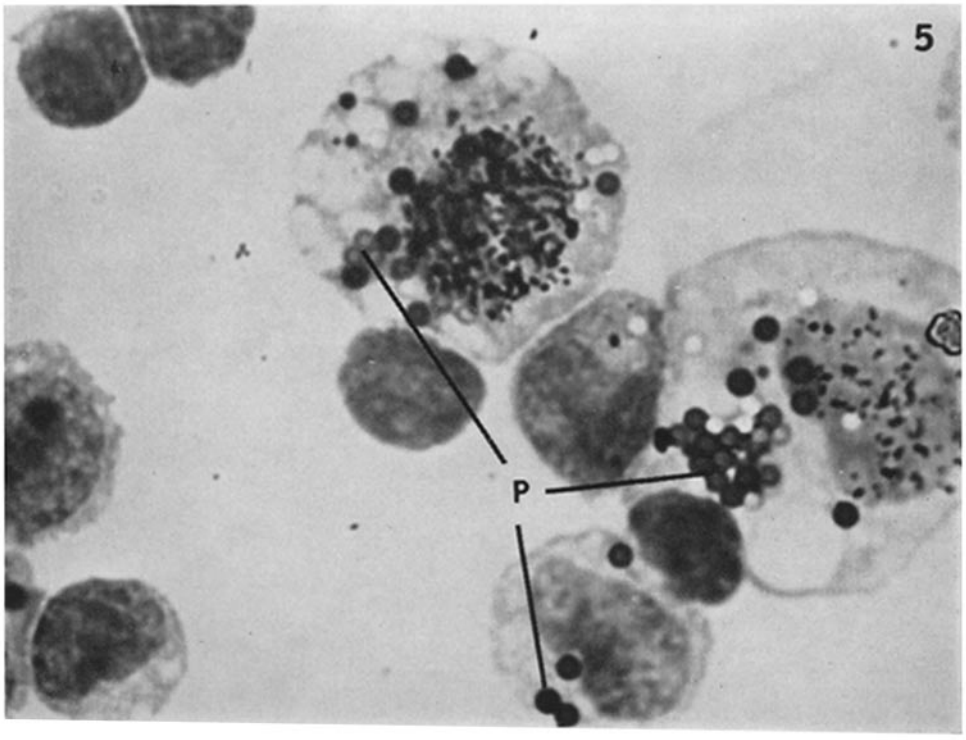
FIG. 4. Radioautograph of section of plastic-embedded spleen of a *Listeria*-infected mouse, showing that large basophilic cells in the red pulp incorporate ^{3}H-TdR during the infection. The cells were labeled by a single pulse of ^{3}H-TdR. Toluidine blue, × 2200.

FIG. 5. Peritoneal macrophages containing polystyrene (*P*) and labeled by a single pulse of ^{3}H-TdR on the second day of a *Listeria* infection. The host was injected with polystyrene spheres intraperitoneally 2 wk before infection. × 4700.





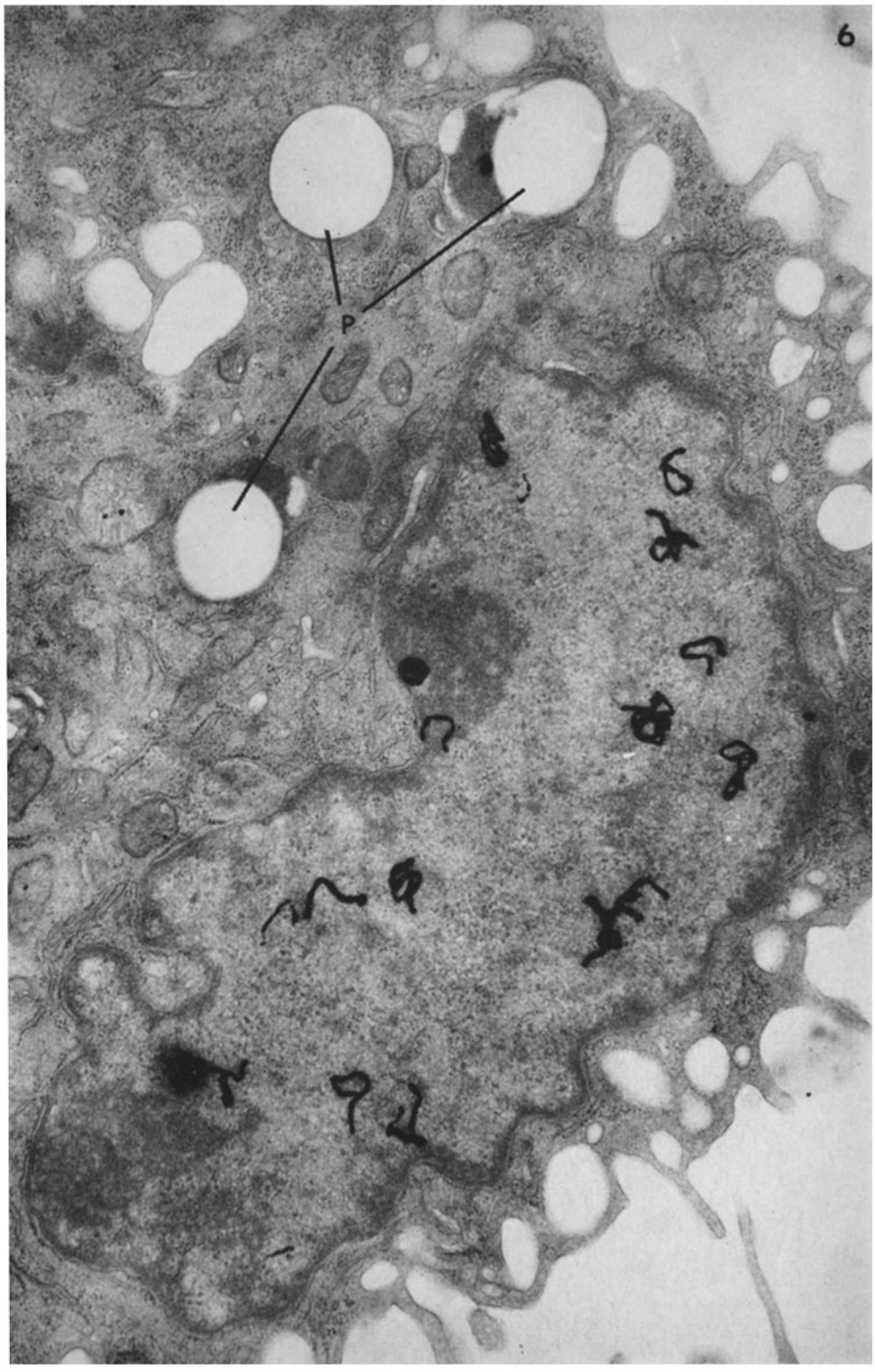


FIG. 6. Peritoneal macrophage harvested and labeled in vitro with ^{3}H-TdR on the second day of a *Listeria* infection. It was allowed to ingest polystyrene spheres (*P*) in in vivo 2 wk before infection. × 21,900.