Activity of Macrophage and Neutrophil Cellular Fractions from Normal and Immune Sheep Against Listeria monocytogenes

P. M. OUTTERIDGE, J. W. OSEBOLD, AND Y. C. ZEE

Department of Veterinary Microbiology, School of Veterinary Medicine, University of California, Davis, California 95616

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Cellular immunity to Listeria monocytogenes infection was studied by assaying for antibacterial activity in fractions of leucocytes collected from the peritoneal cavity, lungs, and mammary glands of immunized sheep. The cells were collected in populations that were largely either macrophages or neutrophils. Mechanically disrupted cells were divided into nuclear, lysosomal, and supernatant fluid fractions and then subjected to freezing and thawing. Comparison with similarly treated rabbit cells showed that greater fragility exists in the lysosomes of sheep cells, as indicated by the amount of acid phosphatase activity released. Inhibition of bacterial growth was assayed in a broth medium at pH 4.6. As expected, nuclear and lysosomal fractions from neutrophils were inhibitory. Some antibacterial activity was found in nuclear fractions of macrophages. The lysosomes of macrophages collected from the peritoneal cavity and the mammary gland did not inhibit the growth of L. monocytogenes. Peritoneal macrophages were allowed to interact with sensitized lymphocytes and an avirulent strain of L. monocytogenes for 4 hr prior to disruption and fractionation, but antibacterial activity was not detected. Pulmonary alveolar macrophages from 5 out of 16 sheep contained Listeria inhibitory activity in their lysosomes. The mechanism was inhibitory but not bactericidal.

The term "cellular immunity" may be used to describe the increased capacity of macrophages to kill or inhibit the growth of intracellular microorganisms. Diseases in which this occurs include those caused by facultative intracellular bacteria such as Mycobacterium, Brucella, Listeria, and Salmonella species (8, 17, 21, 32).

The mechanism of cellular immunity remains undefined, but it has been attributed to a nonspecific hyperactivation of macrophages in lesion sites (6), to cytotoxic antibodies on macrophages (13, 31), and to delayed hypersensitivity (19). In vitro studies on the interaction of macrophages and Listeria monocytogenes have shown that the "immune cells" resist the destructive effects of the organism and also suppress bacterial growth (18, 21). More recent investigations have indicated that mice can be stimulated by infection with Listeria to exhibit cross-resistance to Mycobacterium, suggesting that cellular immunity may not be immunologically specific (5). However, the same study also showed that mice had greater resistance against the organism to which they had been immunized. Youmans (36) has described evidence to show that macrophages from immunized animals possess both a nonspecific resistance and a specifically directed immune cell mechanism for intracellular killing.

It has been noted that macrophages from both normal and immune animals have increased numbers of lysosomes in their cytoplasm during in vitro incubation (3, 10) and following infection with Listeria monocytogenes (23). The neutrophil granule has been shown to fuse with phagocytic vacuoles (40), and this is followed by the killing of bacteria inside the vacuole (2). It has been suggested by Zeya and Spitznagel (37) that the intracellular killing by neutrophils is due to the release of bactericidal cationic proteins from granules into the phagocytic vacuole. North (22) showed that mouse macrophage lysosomes fuse with phagocytic vacuoles containing Listeria with the release of lysosomal contents.

1 Present address: Commonwealth Scientific and Industrial Research Organization, Division of Animal Health, Parkville, Victoria 3052, Australia.
However, there is a notable lack of reports on the bactericidal properties of macrophage granules. It seemed important to investigate the interaction of these granules with bacteria and, in particular, the granules from those cells exhibiting the increased resistance associated with cellular immunity. Studies are reported here using macrophages from normal sheep and _Listeria_ immunized sheep. Sheep were chosen as the experimental animal since they are commonly subject to natural _Listeria_ infections and cellular immunity has been demonstrated in vitro with the peritoneal exudate cells of immunized sheep (21). Macrophages derived from the peritoneal cavity, lungs, and mammary glands were investigated.

Cellular fractions from macrophages were compared with fractions obtained from the neutrophils of sheep and rabbits.

**MATeRIALS AND METHODS**

**Animals.** The sheep were of cross-bred Hampshire-Rambouillet stock. Animals were obtained from premises where listeriosis had never been encountered, and they were serologically tested to demonstrate an absence of mercaptoethanol-resistant agglutinating antibodies to _L. monocytogenes_ (24). Leukocytes were obtained from the mammary glands of virgin ewes that were free of mastitis. New Zealand White rabbits were also used as a source of leukocytes.

**Bacteria.** A virulent strain of _L. monocytogenes_ (strain 3-54, serotype 4b) was used in antibacterial assays and for the immunization of sheep. The strain was originally isolated from the brain of a naturally infected sheep and was frequently passaged through mice to maintain its virulence.

**Immunization of sheep with _Listeria._** Immune macrophage production was stimulated in sheep by subcutaneous and intraperitoneal inoculations of living _L. monocytogenes_. The bacteria were grown for 18 hr in Trypticase soy broth (Baltimore Biological Laboratory), centrifuged at 15,000 x g for 25 min, washed once in Zobell's solution (39) (pH 7.0), and then resuspended in Zobell's solution. Numbers of bacteria were estimated photometrically, and bacteria were inoculated in three or more doses ranging from 2 x 10^6 to 4 x 10^9 per kg of body weight at approximately 7-day intervals (actual doses were 1.1 x 10^10 to 2.7 x 10^10).

**Leukocytes.** All leukocytes were harvested in sterile 0.25 M sucrose solution to which sodium heparin had been added at a concentration of five units per ml. Cells were counted on a hemocytometer. Differential cell counts were made on Giemsa-stained preparations.

**Neutrophils.** Neutrophils were obtained from the nonlactating mammary glands of ewes after the infusion of 5 μg of bacterial lipopolysaccharide (Escherichia coli 055:B5, Difco) into each gland via the streak canal (26). The migration of leukocytes into the glands was thus stimulated, and the cells were washed out after 6 hr with 10 ml of sterile 0.25 M sucrose solution and collected in sterile centrifuge tubes.

Neutrophils were also obtained from the peritoneal cavities of rabbits 6 hr after the infusion of shellfish glycogen (Mann Research Laboratories). The glycogen was sterilized with ethylene oxide and prepared in a concentration of 1 mg per ml in sterile 0.25 M sucrose solution. A 300-ml amount of this solution was injected intraperitoneally per rabbit. The cell-laden fluid was obtained by paracentesis from the rabbits under ether anesthesia.

**Macrophages.** Macrophages from the mammary glands were collected from the same ewes that provided neutrophils by again washing out the glands with 10 ml of sterile 0.25 M sucrose solution 5 days after the infusion of bacterial lipopolysaccharide (26). Peritoneal macrophages were collected from the ewes 5 days after injecting 200 ml of sterile mineral oil (Drakeol 35VR, Pennsylvania Refining Co.) intraperitoneally. The peritoneal exudate cells were washed out in cold 0.25 M sucrose solution after slaughter of the animal.

Lung alveolar macrophages were obtained after removing the lungs from sheep which had been exsanguinated. The lungs were suspended by a clamp attached to the trachea, and sterile 0.25 M sucrose solution was infused into the bronchi. Endobronchial lavage was repeated several times, and the cell-laden liquid was collected by pouring the lung fluid into a sterile flask.

Peritoneal macrophages were obtained from rabbits 5 days after intraperitoneal injection of 50 ml of sterile mineral oil. The cells were obtained from anesthetized rabbits by introducing 300 ml of sterile 0.25 M sucrose solution into the peritoneal cavity. The cell-laden fluid was collected by paracentesis into a sterile flask.

**Cell fractionation.** All cell types were washed twice in sterile, chilled 0.25 M sucrose solution and were then disrupted with a Teflon pestle homogenizer (Tri-R Instrument Co.) by using the method of Cohn and Hirsch (4). The progress of cell disintegration was followed by examining samples of cell homogenates under a phase-contrast microscope. Nuclei and large cytoplasmic fragments were centrifuged into a pellet at 3,000 x g for 20 min at 4 C (nuclear fraction). After the nuclear fraction was removed, lysosomes and some other subcellular particles were collected as a pellet by centrifuging at 15,000 X g for 10 min at 4 C (lysosomal fraction). The remaining supernatant fluid was also collected and retained. Samples were frozen and stored at -20 C.

The protein content of cellular fractions was measured by using the method of Lowry et al. (16).

**Acid phosphatase assay.** An assay for acid phosphatase activity was carried out on 0.5 ml of cell fraction to which was added 0.2 ml of 0.125 M β-glycerophosphate and 0.3 ml of 0.3 M acetate buffer (pH 5.0). The reaction was stopped after 15 min at 37 C by adding 0.2 ml of 25% trichloroacetic acid. The inorganic phosphorus released was measured by the method of Chen et al. (1), modified so that 1 ml of assay solution was added to the enzyme-substrate mixture. After an additional 2 hr at 37 C, the tubes
were centrifuged to remove the protein precipitate, and the supernatant fraction was transferred to cuvettes and read in a spectrophotometer at a wavelength of 660 nm. The value obtained was then adjusted according to the total volume of the fraction and was also related to the acid phosphatase content of 10⁶ leukocytes.

Antibody response in immunized sheep. The synthesis of immunoglobulin G (IgG) antibodies to *L. monocytogenes* was used as an index of antigenic stimulation in the sheep which were the source of immune macrophages. A somatic agglutination test for mercaptoethanol-resistant antibodies was used according to the technique of Osebold and Aalund (24).

Antibacterial assay against *L. monocytogenes*. A growth inhibition assay was used similar to that described by Muschel and Treffers (20). Twofold dilutions were made of the cell fractions in 1 ml of sterile Zobell's solution (pH 7.0). *Listeria* were grown in Trypticase soy broth on a shaker at 37 C for 18 hr. A 1/10 dilution of this culture was then made in fresh broth and incubated until a bacterial optical density (OD) of 0.15 ± 0.01 at 640 nm was reached. The bacteria, which were assumed to be in the log phase, were washed twice in Zobell's solution (pH 7.0), and 0.3 ml of this bacterial suspension was added to the dilutions of cell fractions followed by an additional 0.7 ml of Zobell's solution (pH 4.6). The pH of the test mixture in some samples was as high as 5.5 in the first tube. After the first few dilutions of the cell fractions, the acidity increased to pH 4.6. After incubation for 1 hr at 37 C, 5 ml of Trypticase soy broth was added to each tube. The initial OD of the tubes was measured at 640 nm and the tubes were returned to the water bath. When the OD in control tubes (bacteria alone) reached 0.4 to 0.45, the OD of all tubes was again read. A measure of bacterial growth was obtained by subtracting initial OD from the final OD. The antibacterial titer was the highest dilution of the fraction at which bacterial growth was 50% or less than that of the controls.

Fixation and embedding of cells for electron microscopy. Cells were fixed in gluteraldehyde and postfixed with osmium tetroxide (30). They were then stained with uranyl acetate and embedded in Maraglas 665 (Marblette Corp., Long Island City, N.Y.) for sectioning. Sections were examined with an A.E.I. EM 6B electron microscope.

**RESULTS**

Distribution of acid phosphatase among the subcellular fractions of sheep and rabbit neutrophils and macrophages. A comparative study of the effects of leukocyte disruption was carried out with neutrophils and macrophages from both sheep and rabbits. Data from the work of others on rabbit cells was also considered in reference to the results obtained here with sheep leukocytes. Because of the close association between acid phosphatase and lysosomes, the amounts of the enzyme activity among the subcellular components were used as an index of lysosomal content distribution (7).

Cell counts for each type of leukocyte are shown in Table 1. Cell preparations were identified according to the major cell type. The amount of acid phosphatase activity was considerably greater in the sheep macrophages than in the other cells. Table 2 indicates that the enzyme content was significantly greater in sheep macrophages at <5% level of probability when compared to the other cell types.

As expected from the findings of other investigators, the per cent of acid phosphatase activity associated with the lysosomes of rabbit neutrophils was relatively high (56.4%). More of the enzyme activity remained with the lysosomes from rabbit cells than remained with the sheep lysosomes (Table 2). Only 20.6% of the activity remained in this fraction of the sheep macrophages. The results suggested that there was leakage of contents from lysosomes after mechanical disruption of the cells.

**Table 1. Counts of macrophages and neutrophils obtained from peritoneal cavities of rabbits and mammary glands of sheep**

<table>
<thead>
<tr>
<th>Predominant cell type</th>
<th>Mean cell count</th>
<th>Percentage of cells in differential counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td><strong>Sheep mammary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>macrophages</td>
<td>2.256 × 10⁶ ± 1.712</td>
<td>1.05 ± 0.68</td>
</tr>
<tr>
<td><strong>Rabbit peritoneal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>macrophages</td>
<td>1.90 × 10⁶ ± 0.79</td>
<td>10.97 ± 0.32</td>
</tr>
<tr>
<td><strong>Sheep mammary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>1.456 × 10⁶ ± 0.296</td>
<td>98.36 ± 0.21</td>
</tr>
<tr>
<td><strong>Rabbit peritoneal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>1.14 × 10⁶ ± 0.58</td>
<td>84.29 ± 13.31</td>
</tr>
</tbody>
</table>

*a Cell counts and their standard errors calculated as the mean of cell collections from six animals.

*b Standard error not calculated.
tion of the cells. The values for standard errors denote that the variation in acid phosphatase activity was less among sheep cells from which the release of acid phosphatase was more consistently encountered. The finding that a major portion of the lysosome contents was released influenced the design of subsequent experiments. Attempts to detect antibacterial activity in the leukocyte fractions required the testing of both lysosomes and the lysosome-free supernatant fluid.

The release of acid phosphatase by the lysosomes appeared to parallel the release of antibacterial activity by the lysosomes of rabbit and sheep neutrophils. Preliminary experiments showed that both the fractions containing the lysosomes and the post-15,000 × g supernatant fluid contained antibacterial activity toward *L. monocytogenes* and *E. coli*. This antibacterial activity was distributed between the lysosomes and the supernatant fluid in approximately the same proportion as the acid phosphatase activity.

**Antibacterial activity of leukocyte fractions**

<table>
<thead>
<tr>
<th>Predominant cell type</th>
<th>Mean acid phosphatase activity per 10⁶ cells</th>
<th>Probability that acid phosphatase of sheep macrophages exceeds other cells</th>
<th>Percentage of activity among cellular fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep mammary macrophages</td>
<td>1,314.9a</td>
<td>—</td>
<td>6.94 ± 3.53a 72.43 ± 7.26 20.63 ± 10.66</td>
</tr>
<tr>
<td>Rabbit peritoneal macrophages</td>
<td>287.6</td>
<td>0.05</td>
<td>6.20 ± 3.96 52.40 ± 15.21 41.40 ± 16.54</td>
</tr>
<tr>
<td>Sheep mammary neutrophils</td>
<td>63.2</td>
<td>&lt;0.05</td>
<td>0.88c 76.72 ± 11.90 22.50 ± 12.36</td>
</tr>
<tr>
<td>Rabbit peritoneal neutrophils</td>
<td>155.2</td>
<td>&lt;0.05</td>
<td>2.16c 41.40 ± 17.00 56.44 ± 18.10</td>
</tr>
</tbody>
</table>

* Nanomoles of inorganic phosphorus released.
* Standard errors calculated as the mean of determinations from six animals.
* Standard error not calculated

**Fig. 1.** Antigenic stimulation of sheep by *Listeria monocytogenes* as indicated by the synthesis of mercaptoethanol-resistent agglutinating antibodies. Titers are expressed as the reciprocal of the serum dilution. Closed circles = immunized sheep. Open circles = nonimmunized sheep.
from sheep immunized with L. monocytogenes. The cellular immune state was induced by the subcutaneous inoculation of live virulent L. monocytogenes. Synthesis of mercaptoethanol-resistant (IgG) antibodies was used as an index of antigenic stimulation (24). Figure 1 shows the serum agglutinating antibody response in sheep receiving three inoculations of the organism. The response curve is in contrast to the absence of IgG agglutinating activity plotted for three nonimmunized sheep. Previous studies had shown that the peritoneal exudate cells of sheep immunized in this way demonstrated the phenomenon of cellular immunity in vitro (21).

Mammary neutrophils. Mammary neutrophils were collected at weekly intervals for a period of 7 weeks from six sheep. Neutrophils constituted nearly 90% of the cells. Three animals received immunizing inoculations during this period, and three remained unimmunized.

The results of assays for antibacterial activity from the neutrophil fractions are shown in Table 3. As expected, there was anti-Listeria activity in all three cellular fractions. The activity was greatest in the nuclear fractions. There was a reduction of antibacterial activity from the lysosomes and supernatant fluid of neutrophils collected late in the experiment after the animals had served repeatedly as cell donors. Conceivably this might have been related to the lipopolysaccharide used to induce the neutrophils. Wiener et al. (34) found that lipopolysaccharide did not produce degranulation of neutrophils in vitro, but it did cause some loss of acid phosphatase from mouse peritoneal leukocytes if it was administered to the intact animal. The progressive loss of antibacterial activity in the sheep mammary neutrophils may have been due to increasing levels of locally produced antibody to the lipopolysaccharide resulting in phagocytosed antigen-antibody complexes which could cause neutrophil degranulation (27).

Mammary macrophages. Mammary macrophages were also obtained from the sheep at weekly intervals. The samples contained nearly 80% macrophages and less than 4% neutrophils. Electron micrographs of the lysosomal cellular fraction revealed considerable numbers of lysosomes as electron-dense bodies. There were many vesicles of different sizes. Many of the empty vesicles were thought to be lysosomal membranes which remained after the loss of their contents. The heterogeneous nature of the preparations was also shown by the presence of mitochondria with their recognizable internal cristae and by clumps of ribosomes.

The mean antibacterial titers are recorded in Table 4. Antibacterial assays showed a small, variable amount of activity in the nuclear fractions from both immunized and normal animals. Antibacterial activity was not detected in the lysosomal fractions from either group. Thus, there was no indication that the immunizing pro-
TABLE 4. Inhibitory titers for Listeria monocytogenes by macrophages obtained from the mammary glands of sheep

<table>
<thead>
<tr>
<th>Day</th>
<th>Immunized</th>
<th>Nonimmunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclei</td>
<td>Supernatant fluid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−7*</td>
<td>5b</td>
<td>0.55^4</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>0.72</td>
</tr>
<tr>
<td>42</td>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>62</td>
<td>0</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Days from the first immunizing inoculation of L. monocytogenes. Negative value equals days before inoculation.

b Geometric mean antibacterial titer per milliliter of undiluted cell fraction obtained from three immunized ewes. Titers expressed as the reciprocal of fraction dilutions.

c Geometric mean antibacterial titer per milliliter of undiluted cell fraction obtained from three nonimmunized ewes. Titers expressed as the reciprocal of fraction dilutions.

d Numbers in italic equals the mean protein concentration in milligrams per milliliter of undiluted cell fraction.

procedure had altered the antibacterial capacity of the mammary macrophages.

**Peritoneal macrophages.** Peritoneal macrophages from nine immunized sheep were tested. The samples contained approximately 60% macrophages, 14% lymphocytes, and 23% neutrophils. A peritoneal macrophage obtained 5 days after intraperitoneal injection of oil is shown in Fig. 2. A feature of the peritoneal macrophages was the extensive development of the golgi area and the convolutions and projections of the cytoplasmic membrane, suggesting activation of the cells. There was a relative paucity of recognizable lysosomes in many of these macrophages.

The antibacterial titers from each cellular fraction are shown in Table 5. There was a small amount of anti-Listeria activity associated with the nuclear fractions from three of the animals. No antibacterial activity was detected in the lysosomal and supernatant fractions.

**Lung alveolar macrophages.** Lung alveolar macrophages were obtained from nine immunized sheep. The samples contained 93% macrophages, 5% lymphocytes, and 1% neutrophils. The ultrastructure of lung alveolar macrophages is shown in Fig. 3. Considerable numbers of lysosomes and other electron-dense bodies were seen. It was observed, however, that many of the lung macrophages were in various stages of degeneration. This was in contrast to the structural integrity of macrophages obtained from the peritoneal cavity and the mammary gland.

Antibacterial assays with lung macrophages yielded unexpected results. Four of the animals had high antibacterial titers in all subcellular fractions (Table 5). Peritoneal macrophages from the same animals were inactive or showed only minimal activity in the nuclear fractions. Mammary macrophages had been tested from three of the four positive sheep, and those cells had also been nearly devoid of antibacterial activity. Lung macrophage samples from the five remaining sheep demonstrated no antibacterial activity. Some samples from these nonreacting animals were tested, both at pH 4.6 and at pH 7, but no activity could be demonstrated. In addition, tests were performed with lung alveolar macrophages from seven nonimmunized sheep. One of the specimens had a high level of activity in the lysosomal fraction (titer of 512). That specimen and three others had antibacterial activity in both the nuclear and supernatant fluid fractions.

The antibacterial fractions from immunized sheep number 8 were used in an assay to test for their effect on Listeria colony formation in an agar medium. Colonies formed in numbers comparable to the controls, indicating that the mechanism was not bactericidal, although the lung macrophage fractions inhibited Listeria replication in broth medium.

**Peritoneal exudate cells incubated with avirulent Listeria organisms prior to fractionation.** Living avirulent Listeria cells (antigen) were allowed to interact with living host cells for the purposes of
stimulating the release of molecular mediators from lymphoid cells and to promote the formation of secondary lysosomes in macrophages. Sheep 7 and 8 were the source of peritoneal exudate cells (2 \times 10^8 cells) which were incubated with an avirulent isolate of *L. monocytogenes* (25) in the presence of fresh autologous serum. The leukocytes and bacteria were mixed in a ratio of 40 bacteria to 1 peritoneal cell, which yielded a phagocytic index of 6 bacteria per phagocyte. The mixtures were incubated in rubber-stoppered tubes at 37°C for 4 hr in a slowly revolving drum. The appearance of the parasite-host cell interaction is shown in Fig. 4. Phagosomal membranes surrounded groups of ingested bacteria in which there were varying stages in the loss of internal bacterial structure. Active phagocytosis attested to the viability of the cells and the adequacy of the techniques for cell collection and handling. Electron-dense granular material was adjacent to the *Listeria* cells. Similar material was seen by Leake et al. (14) in rabbit alveolar macrophages after the ingestion of *L. monocytogenes*.

After incubation, the leukocytes were sedimented at 75 \times g, washed in Zobell's solution at pH 7, homogenized with a Teflon pestle, and fractionated as described previously.

The avirulent isolate replicated so slowly that its presence in the antibacterial assays did not interfere with the interpretation of the growth of the virulent assay strain. Two experiments were carried out, but no antibacterial activity could be demonstrated in any of the cell fractions.

**DISCUSSION**

The nature of the microbicidal system of macrophages has not been revealed. Various studies on cellular immunity have demonstrated the enhanced antimicrobial activity of living macrophages and the apparent interactions between macrophages and lymphocytes (33). Ultimately, however, investigators of cellular immunity wish to explain the phenomenon on a molecular basis. This investigation was an attempt to get beyond studies with living cells and search for inhibitory or bactericidal substances of the lysosomes of immune macrophages. Macrophages from immunized animals were used on the assumption that they might contain more inhibitory factors than normal macrophages or that they would contain additional factors. It was theorized that active substances of the lysosomes might bind with cellular components in whole-cell homogenates, thus rendering them inactive for an antibacterial assay. Therefore, the subcellular granules were separated from the remainder of the cell components, and their contents were then released by freezing and thawing. Assays were performed at acid pH to mimic the environment of the phagocytic vacuole. A pH of 4.6 was suitable since it did not affect the viability of *L. monocytogenes*, whereas further increases in acidity were inhibitory to the bacterium.

The search was not for increased amounts of acid hydrolases, but for a functional inactivating system that might best be detected in the macrophages of an antigenically stimulated host when tested against the homologous microorganism. It seemed likely that an inactivating system in macrophages would arise in consort with molecular mediators released in vivo by specifically sensitized lymphoid cells. Lymphocytes in considerable numbers were in association with the macrophages collected from the mammary gland (16%) and the peritoneal cavity (15%). However,
Listeria antigen was not added to the suspensions until after the cells were disrupted. This could have blocked the role of the lymphoid cells. Nonetheless, live macrophages, free of lymphocytes, have been shown to function as "immune" cells in vitro. In the technique of Mackaness (18), explants of peritoneal cells from immunized mice were washed free of lymphocytes, cultivated in vitro for 24 hr, and then exposed to L. monocytogenes. It was reported that virtually every cell in monolayers prepared from such mice was capable of inactivating ingested Listeria. This was a circumstance that would require transfer of a chemical mediator at a time prior to intimate interaction with the bacterium in the assay. Consequently, it seemed likely in our studies that the macrophages could have either stored or synthesized antibacterial components when collected as early as 5 days after the sheep were stimulated with living L. monocytogenes.

The lysosomal fractions of immune macrophages from three tissue sites were found to be generally inactive in assays for growth inhibition of L. monocytogenes. Antibacterial activity was encountered in some nuclear fractions and in the lysosomal fractions of sheep and rabbit neutrophils. These results were thought to be due to histones in the nuclei and basic proteins in the lysosomes, respectively. Zeya and Spitznagel (38) have associated antibacterial activity with the basic proteins in the specific granules of neutrophils, but similar proteins could not be demonstrated in the lysosomes of rabbit macrophages (12). Thus, the factors responsible for intracellular killing in macrophages were not revealed by the isolation of the subcellular particles of the cells and release of their contents.

The presence of a factor inhibitory to the growth of Listeria in the cellular fractions from some of the sheep lung macrophages was not in accord with the general pattern of experimental results. The possibility exists that an additional mechanism for microbial inhibition may be present in lung macrophages. There was no obvious evidence of pneumonitis which could be correlated with the active lung macrophages. The role of nuclear histones might be considered as the basis for the activity because of the degenerated appearance of many lung macrophages observed by electron microscopy. But this seemed unlikely in view of the results with the peritoneal macrophages and several of the other lung cell preparations. It was

<table>
<thead>
<tr>
<th>Sheep no.</th>
<th>Days&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peritoneal macrophages</th>
<th>Lung alveolar macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nuclei</td>
<td>Supernatant fluid</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>2.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.36</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>30.00</td>
<td>16.19</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>3.99</td>
<td>2.98</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>6</td>
<td>6.56</td>
<td>1.80</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>36.75</td>
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<td>9</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>21.50</td>
<td>10.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of days between last immunizing inoculation and macrophage collection.
<sup>b</sup> Antibacterial titer expressed as the reciprocal of fraction dilution.
<sup>c</sup> Numbers in italic equal the protein concentration in milligrams per milliliter of undiluted cell fraction.
<sup>d</sup> Not done.

TABLE 5. Inhibition titers for Listeria monocytogenes by peritoneal macrophages and lung alveolar macrophages obtained from immunized sheep

PHAGOCYTE FRACTIONS AGAINST LISTERIA
reported by Holub and Hauser (11) that some lung alveolar histiocytes in the rabbit produce antibody. However, by immuno-electrophoretic examination we were unable to detect immunoglobulin of any class in the three cell fractions of lung macrophages. Perhaps the substance we have demonstrated in some of the lung macrophage homogenates is comparable to "mycosuppressin" found by Youmans in homogenates of the lungs of guinea pigs and rabbits immunized with BCG. Mycosuppressin inhibited the respiration and growth of Mycobacterium tuberculosis. The inhibitor to L. monocytogenes replication found in the alveolar macrophages of sheep was not bactericidal, and there was no apparent correlation of its presence with the immune
status of the host since it was not detected in the cells of all immunized sheep and was also encountered in one of seven nonimmunized specimens.

Our finding that not all of the acid phosphatase activity in rabbit and sheep macrophages is associated with the lysosomes is in conformity with the work of Leake and Myrvik (15) with rabbit alveolar macrophages. They also used the Teflon pestle method to disrupt the cells and found that from 34.1 to 49.8% of the total acid phosphatase activity was associated with the cellular fraction containing the lysosomes. These figures agree with those found for acid phosphatase activity associated with the granules of rabbit peritoneal macrophages (41.4%, ±16.54) in our experiments. In the sheep mammary macrophages, however, only 20.63%, ±10.66, was found to be associated with the pellet containing the lysosomes. As a matter of comparative biology it appeared that the lysosomes of sheep macrophages and neutrophils were more easily disrupted than those of the rabbit.

One set of experiments was designed to enhance the opportunity for the participation of molecular mediators from lymphocytes. Peri-

**FIG. 4.** Phagocytosed *Listeria monocytogenes* in a peritoneal macrophage of sheep. Electron-dense granular material surrounds the groups of ingested bacteria within phagosomal membranes. Nucleus (N). × 21,000.
toneal exuate cells (60% macrophages, 14% lymphocytes) from \textit{L. monocytogenes}-immunized sheep were allowed to interact with a live, avirulent mutant of \textit{L. monocytogenes}. The purpose was to induce the release of lymphokines and to stimulate secondary lysosome formation in the macrophages. Although this milieu of interacting cellular components did not reveal a microbial inhibitory system, the works of other investigators (9, 28) indicate the role of the “immune lymphocyte” in cell-mediated immunity to infectious agents.

Explanations of the mechanisms for microbial inactivation by neutrophils are emerging from recent studies. The cationic proteins of the lysosomes (38) and the myeloperoxidase system appear to be central in importance (29). It seems a perversity that the macrophage has been so difficult to understand. The dissimilarity of antimicrobial mechanisms between the neutrophil and the macrophage appears to point to the uniqueness of the macrophage and its need to play a different biological role. The results of the experiments reported here emphasize the complexity of the antimicrobial system and the need to search for novel interactions between lymphocytes and macrophages in the state of cellular immunity.

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LITERATURE CITED