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Cellular Immunity of Mice Infected with *Listeria monocytogenes* in Diffusion Chambers

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The importance of bringing live bacteria into intimate contact with macrophages as a prerequisite for establishing cellular immunity was investigated. The bacterium *Listeria monocytogenes* was shown to replicate and survive in diffusion chambers implanted in the peritoneal cavities of mice. Humoral substances accruing from host responses to diffusing soluble antigens of the microorganism were unable to inactivate the bacteria. The resistance of mice immunized by subcutaneous inoculation of the live organism always exceeded the resistance of mice with *Listeria* diffusion chamber implants. Animals with sham diffusion chambers were more resistant to a challenge by *L. monocytogenes* than were normal mice. Host resistance was not significantly different between *Listeria* diffusion chamber implant groups and sham diffusion chamber implant groups. The results suggested that direct involvement of macrophages with the parasite is necessary to achieve cellular immunity.

The term "cellular immunity" has been applied to host-parasite relationships characterized by an increased capacity for intracellular destruction by macrophages in the immune state. Induction of this increase in resistance has been associated with the use of viable microorganisms. Thus, many studies have shown that immunization with killed bacteria induces an antibody response without increasing the level of resistance to that obtained following immunization with live organisms (2, 5, 15). It has been suggested that labile antigens, vital to immunogenicity, are lost when the organisms are inactivated. According to this concept, if the antigens could be preserved, killed organisms would immunize as readily as live organisms (8).

Thus far, the concept of cellular immunity has been associated with host-parasite relationships involving extensive exposure of macrophages to the parasite, with the assumption that this is followed by some intracellular replication and even survival of the microorganisms in the host cells in certain infection systems. It is probably necessary for the organisms to be viable and metabolically active in the host for a period of time. However, the importance of bringing the live organism into intimate contact with the cytoplasm of the macrophage, as a prerequisite for establishing a state of cellular immunity, has not been determined. In this study, *Listeria monocytogenes* was allowed to replicate in vivo, but the bacteria were excluded from macrophages. Thus, the requirements for the exposure of antibody-forming cells to labile antigens and exposure of the host to the products of metabolizing bacteria were fulfilled, but microorganisms were not within the cytoplasm of metabolically active macrophages.

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MATERIALS AND METHODS

Three experiments, referred to as A, B, and C, were performed at different times with the same experimental design. Each experiment compared the host resistance of four groups of mice to a challenge inoculation of *L. monocytogenes* in 10-fold graded doses. Diffusion chambers containing *L. monocytogenes* were implanted in the peritoneal cavities in one animal group while another group received the chambers without the bacteria. A group immunized by subcutaneous inoculation and a normal control group completed the sets of animals.

Diffusion chambers. Diffusion chambers were constructed on specially prepared plexiglass rings (20 mm outer diameter, 16 mm inner diameter and 5 mm high). Filter membranes (Millipore Corp., Bedford, Mass.) with a 0.22-μ pore size were attached with Millipore cement. Each chamber was carefully examined for good bonding between the plastic and the membrane, and was "rimmed" with a second application of cement. A hole in the wall of the ring, large enough to admit a needle, was used for loading the chambers. A piece of Parafilm "M" (Marathon Division, American Can Co., Neenah, Wis.) was shaped over the

loading pore and extended over the membrane surface so that the chamber could be held in the fingers without touching it directly. The completed chambers were sterilized with propylene oxide vapor and were loaded with the appropriate number of *L. monocytogenes* in a volume of 0.5 ml. Special procedures were adopted to minimize the possibility of working with leaking chambers, or those with inoculum contaminating the surfaces. Loading procedures were performed in a bacteriological safety hood. Inoculum was delivered from a Cornwall continuous pipetting apparatus automatic syringe (Becton, Dickinson and Co., Rutherford, N.J.), the needle of which was flamed between each chamber injection. The syringe needle perforated the parafilm seal and then entered the chamber through the loading pore. After injecting the inoculum, the chamber was carefully moved away from the needle, the parafilm shield was removed, and the pore was then sealed with Acryloid B-7 cement (Industrial Polychemical Service, Gardena, Calif.). After 30 min, each chamber was aseptically transferred into 25 ml of tryptose broth and was allowed to stand for approximately 10 min. The chambers were then removed from the broth and implanted in mice. The "wash broth" for each chamber was incubated at 37 C for 5 days. If *L. monocytogenes* grew in the broth, the animal receiving that chamber was sacrificed and discarded.

Bacterial culture. L. monocytogenes strain 3-54, serotype 4b, originally isolated from the brain of a naturally infected sheep, was used throughout the studies. Bacteria for chamber inocula, mouse immunization, and challenge were first passed through mice and then subcultured from fatally infected animals. Organisms for chamber inocula were grown in tryptose broth for 18 hr at 37 C, and then diluted in broth to the desired concentration; a sample was used for viable bacterial counts. Organisms cultivated for immunizing or challenge purposes were washed and diluted in Zobell's solution (18) and were also subjected to viable bacterial counting.

Agglutination tests. Tests for somatic agglutinating antibodies were performed with whole serum and 2-mercaptoethanol-reduced serum according to the technique described by Osebold and Aalund (12).

Pooled mouse sera were frozen at −20 C until tested. Samples from mice containing diffusion chambers were prepared from two to four mice. Sera tested from subcutaneously immunized mice were pooled from 10 or more animals.

Animal groups. Outbred lines of young adult, female mice of the Swiss-Webster strain (approximately 25 g in weight) were used. Mice were procured from two sources. At the beginning of each experiment, the animals were randomly arranged into four groups.

(i) *Listeria chamber implant group.* Diffusion chambers containing approximately 6×10^3 *Listeria* cells suspended in tryptose broth were implanted in the peritoneal cavities of mice. Surgery for the implantation was performed with chloropromazine tranquilization or ethyl chloride induction followed by ether anesthesia. Each laparotomy was closed with separate suturing of peritoneum and muscle from the skin closure. After the chambers had been present for 30 days, the resistance of the animals was challenged by subcutaneous inoculation of viable *L. monocytogenes*.

The bacterial population in the chambers was determined periodically with animals killed prior to the challenge and those dying after the challenge. Chambers were removed from the peritoneal cavities, and the contents were aspirated with a syringe and needle. The volume of fluid recovered from each chamber was recorded (usually about 0.5 ml), and a 0.1-ml sample was used to estimate the number of viable organisms by the drop-plate method (14).

(ii) *Sham chamber implant group.* Diffusion chambers containing sterile tryptose broth were implanted in the same manner as described for the *Listeria* chamber implant group. Thirty days after chamber implantation, the animals were challenged with *L. monocytogenes*.

(iii) *Immune group.* Mice in the immunized group received two subcutaneous inoculations of living *L. monocytogenes*. The initial dose of 10^4 organisms was followed 10 days later by a second dose of 10^5 organisms. The second dose was administered at the same time that the chambers were implanted in animals of the other groups. Therefore, the period between the last immunizing inoculation and the challenge was the same for all treated groups.

(iv) *Normal control group.* Untreated mice were housed under the same conditions as the treated groups, and their natural resistance to *L. monocytogenes* was challenged in the same manner.

Data analysis. Levels of resistance were measured by the host response to 10-fold graded doses of *L. monocytogenes*, expressed in 50% end points determined by the method of Karber (9). Standard error was calculated by the procedure of Irwin and Cheeseman (7). The difference between the effects of the challenge on animal groups within an experiment were evaluated by the formula:

$$Z = \frac{X - Y}{\sqrt{\delta_x^2 + \delta_y^2}}$$

The Z value represents the difference between two variables (X and Y, the two LD_{50} logs) divided by the standard error of the difference of the two variables. For example, a Z value of 2 would indicate a significant difference in the two groups at the 5% level assuming that the animal response followed a normal distribution.

However, Irwin and Cheeseman (7) encountered heterogeneity of response in mice to *Salmonella typhimurium* infection; they suggested that a value of 3 could more safely be considered a significant difference than a value of 2, when analyzing host responses in animals containing the degree of heterogeneity of those used in their study. In ascribing significance to data reported here, interpretations were arbitrarily made; Z values of 3.0 and greater were considered significant, 2.5 to 2.9 were possibly significant, and 2.4 and less were not significant.

The 50% death times (t_{50}) were plotted as the cumulative percentage dead on a probability scale against time in days on an arithmetic scale (3).

RESULTS

Growth characteristics of *L. monocytogenes* in the diffusion chambers are plotted in Fig. 1. Data from all of the experiments were used to make the graph. The bacterial counts were assumed to be representative of the *Listeria* population in animals carried through to challenge. The *Listeria* cells increased rapidly from a population of 6×10^3 to more than 10^8 organisms within 24 hr. By the third day, the count had decreased by a factor of ten and then remained at approximately 10^7 throughout the 30-day period prior to challenge. The count following challenge fluctuated somewhat, but the bacterial numbers never receded below 10^4 organisms, and most counts remained high. For example, a chamber removed 173 days after implantation contained 6×10^7 organisms.

Although the growth of bacteria in the chambers was accompanied by the release of soluble antigens (6), the synthesis of agglutinating antibodies was surprisingly limited. As shown in Fig. 2, the highest titer obtained was a partial reaction at a 1:25 serum dilution 19 days after chamber implantation. The antibodies were sensitive to 2-mercaptoethanol, indicating that they were probably of the IgM class.

A very limited antibody response was obtained in animals immunized with two sublethal inoculations of living *L. monocytogenes*. Table 1 shows that trace reactions could be obtained in serum diluted 1:25. In these animals, there was also evidence that most of the antibodies were IgM molecules.

The data on 50% death time (t_{50}) indicated that death following inoculation of *L. monocytogenes* did not vary greatly between the different treated groups within an experiment (Table 2). However, the t_{50} values between experiments showed that mice in experiment C died in little more than half the time required to kill in experiment B. When the t_{50} values for the four challenged groups in experiment C were accumulated, the total value was 9.0 days, as compared to 16.8 days in experiment B. The mice in experiment C were obtained from a different animal supplier than those used in the other two experiments. These C mice were observed to be more heterogenous in their response to the bacterial infection, in addition to dying at an accelerated rate, which suggested greater susceptibility.

An example of mouse mortality patterns is shown in Fig. 3, a resume of experiment A. In all three experiments, the resistance of immunized animals was greater than the natural resistance of normal animals. The degree of enhanced

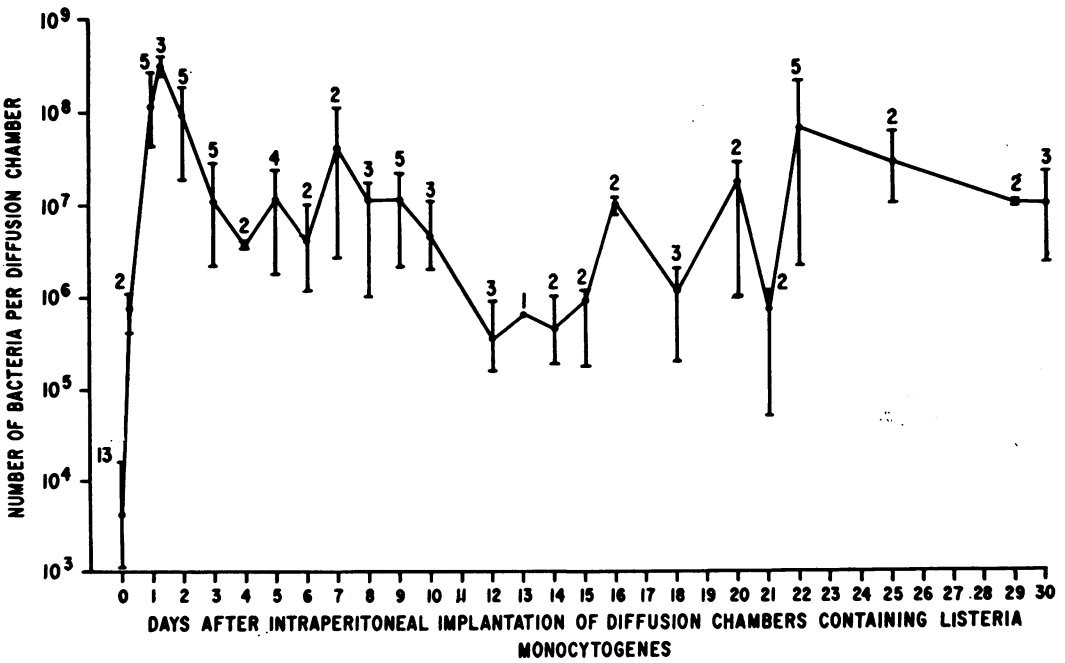

FIG. 1. *Growth and survival of Listeria monocytogenes within diffusion chambers implanted in the peritoneal cavities of mice. Numbers on bars represent number of chamber counts averaged to determine the graph point. Vertical bar indicates the range of counts.*

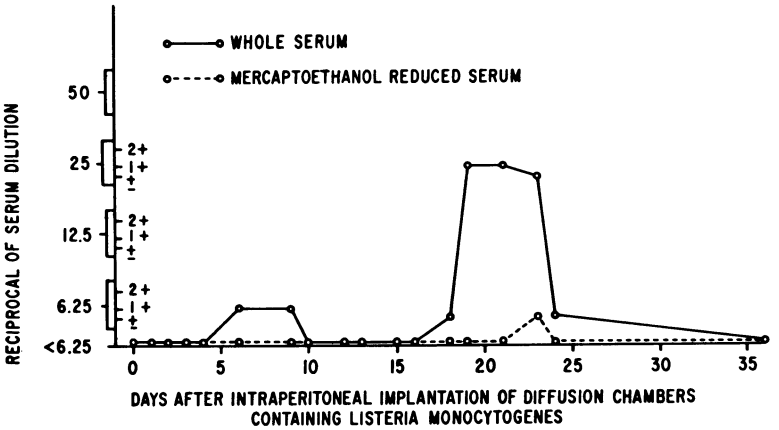

FIG. 2. *Agglutinating antibody response of mice to Listeria monocytogenes in serum following implantation of diffusion chambers. Each point represents a determination on serum pooled from two to four mice. The points were compiled from several experimental trials.*

TABLE 1. *Agglutinating antibodies in mouse serum following immunization by subcutaneous inoculation of living Listeria monocytogenes*[a]

Titer	No. of sera reacting	
	Whole serum	Mercapto-ethanol-reduced serum
1:25 (trace agglutination).....	1	
1:12.5 (trace agglutination)....	3	2
1:6.25 (trace agglutination)....	1	
1:6.25 (no agglutination).....		3

[a] Pooled sera were tested from five groups immunized at different times. Sera were collected 13 to 15 days after the last inoculation.

resistance varied, however, since immunized animals in experiment C withstood only 13 times the dose that killed normal mice, whereas the comparable figure in experiment A was 1,000 times that dose (Table 2). Thus, the difference between immunized and control groups in experiments A and B was clearly significant, whereas the difference in experiment C was considered possibly significant (Table 3).

In five out of six animal groups containing diffusion chambers (*Listeria* implant and sham implant groups in the three experiments), there was an increased resistance over that of normal animals. Furthermore, the four values obtained from experiments A and B were clearly significant (Table 3).

Sustained exposure of the host to viable bacteria in the diffusion chambers was never able to enhance the resistance to the level achieved by inoculating live organisms into the subcutis. The value derived from comparison of immunized groups and *Listeria* implant groups was not clearly significant in any of the experiments. However, this must be viewed in light of the resistance-enhancing effects of the sham implant, which also made those groups significantly different from immunized groups in only one of the three experiments (Table 3). The differences between *Listeria* implant and sham implant groups were never significant.

DISCUSSION

Animals containing diffusion chambers were exposed to antigens diffusing from approximately 10^7 organisms, sustained over a 30-day period. The limited antibody response of the mice could not be readily explained. A wide range of vertebrates are known to contain demonstrable levels of agglutinating antibodies to *L. monocytogenes* in the serum of apparently normal subjects. In fact, these ubiquitous antibodies have confounded efforts at serodiagnosis (16). The normally occurring antibodies were shown to be of the IgM class by Osebold and Aalund (12), who proposed that they be depolymerized with 2-mercaptoethanol in order that IgG antibodies might be detected following antigenic stimulation from the bacterium. However, the mice did not contain the expected levels of IgM antibodies as normal animals, and synthesis of antibodies after stimulation by live *L. monocytogenes* was absent or feeble, regardless of whether the bacteria were confined in chambers or inoculated subcutaneously. Immunized animals received 110,000

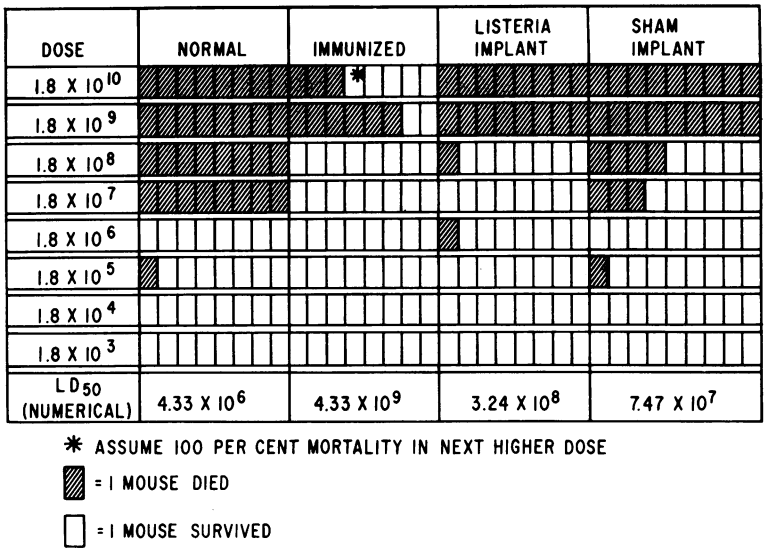

FIG. 3. *Mortality patterns in four animal groups following subcutaneous inoculation of Listeria monocytogenes (experiment A).*

TABLE 2. *Comparisons of mortality data among four groups of mice following subcutaneous inoculation of Listeria monocytogenes*

Mouse group	Expt A				Expt B				Expt C			
	LD_{50}		Treated/normal[a]	t_{50}[b] (days)	LD_{50}		Treated/normal	t_{50} (days)	LD_{50}		Treated/normal	t_{50} (days)
	Log ± SE	Numerical			Log ± SE	Numerical			Log ± SE	Numerical		
Normal.....	6.64 ±0.266	4.3 × 10^6	—[c]	4.2	7.15 ±0.330	1.4 × 10^7	—	4.0	6.37 ±0.317	2.4 × 10^6	—	2.6
Immunized..	9.64 ±0.285	4.3 × 10^9	1,000	3.0	9.28 ±0.275	1.9 × 10^9	134	3.9	7.50 ±0.277	3.1 × 10^7	13	1.7
Listeria implant....	8.51 ±0.299	3.2 × 10^8	74.8	3.7	8.57 ±0.322	3.7 × 10^8	26	5.2	7.25 ±0.309	1.8 × 10^7	7.5	2.6
Sham implant....	7.87 ±0.312	7.5 × 10^7	17.2	3.0	8.60 ±0.271	4.0 × 10^8	28	3.7	6.10 ±0.429	1.3 × 10^6	0.5	2.1

[a] LD_{50} treated group/LD_{50} normal group.
[b] Death time (in days) for 50% of the fatally infected animals. Accumulated t_{50} : experiment A = 13.9 days; experiment B = 16.8 days; experiment C = 9.0 days.
[c] Not applicable.

living organisms, and, of course, it was uncertain how much that dose might have been increased by in vivo replication. The limited antibody response was reminiscent of results obtained by Mackaness (10); mice immunized with one LD_{50} dose of live *Listeria* cells produced no demonstrable antibodies, whereas a second dose of 100 LD_{50} produced an agglutinating antibody titer in pooled sera of only 1:62. Only after the injection of large doses of heat-killed *Listeria* cells could Mackaness obtain a titer of 1:1,000 in pooled mouse sera. Hasenclever and Karakawa (4) also reported a lack of antibody response in mice following immunization with living *L. monocytogenes.*

However, the fact that the antibody response in the mice in the current experiment was poor, in a situation where acquired immunity could be demonstrated, suggested that a resistance mechanism, in addition to humoral antibody, might be involved. Thus, the finding offered indirect evidence on the role of cells in acquired resistance.

TABLE 3. *Statistical significance of difference in mortality between mouse groups following challenge with Listeria monocytogenes*

Mouse groups compared	Expt		
	A	B	C
Immunized to normal	7.68[a] (significant)	4.93 (significant)	2.67 (possibly significant)
Listeria implant to normal	4.69 (significant)	3.06 (significant)	1.97 (not significant)
Sham implant to normal	3.02 (significant)	3.38 (significant)	0.52 (not significant)
Immunized to *Listeria* implant	2.73 (possibly significant)	1.67 (not significant)	0.59 (not significant)
Immunized to sham implant	4.17 (significant)	1.74 (not significant)	2.75 (possibly significant)
Listeria implant to sham implant	1.48 (not significant)	0.08 (not significant)	2.17 (not significant)

[a] Calculated as the value Z: $Z = (X - Y)/\sqrt{\delta_x^2 + \delta_y^2}$. Interpreted as: 3.0 and greater = significant; 2.5 to 2.9 = possibly significant; 2.4 and less = not significant.

The failure of humoral factors to destroy the *Listeria* population in the chamber was of interest, and it contrasted with the death of *Escherichia coli* in diffusion chambers as shown by Steward et al. (17). In the current study, bacteria persisted in the chambers for 30 days prior to challenge and for prolonged periods after the subcutaneous inoculation of a challenge dose of *L. monocytogenes*. Immunoglobulins, the components of complement, and other macromolecules had access to the contents of the chambers. This was assured both on the basis of membrane pore size and the observation that many of the chambers contained a small fibrin clot. Thus, it was demonstrated that humoral substances alone were unable to inactivate the parasite.

In five out of six comparisons, the dose required to kill *Listeria* implant or sham implant groups was larger than that required to kill normal mice. This was statistically significant in four of the six comparisons. Although the LD_{50} of the immunized groups was always larger than that of the *Listeria* implant and sham implant groups, the statistical difference between them was clearly significant in only one of six comparisons. Furthermore, none of the experiments indicated significant differences between the *Listeria* implant and the sham implant groups. The fact that animals containing diffusion chambers, with or without bacteria, were more resistant than normal mice indicated a nonspecific increase in resistance associated with the presence of the foreign body in the peritoneal cavity. This effect of the chambers complicated interpretations regarding the role in resistance of the chamber-confined bacteria. Adler and Fishman (1) reported an adjuvant effect from diffusion chambers which enhanced antibody response to hemocyanin and ferritin. Although the chambers did not enhance antibody response in our studies, the change in the resistance status of the animals was apparent. All immunological studies with diffusion chambers would appear to require careful evaluation of this phenomenon.

The likelihood that cellular immunity is important in the resistance of mice to *L. monocytogenes* was suggested by Osebold and Sawyer (13). Their studies in mice demonstrated that passively administered antibody did not alter the course of infection, but immunization with sublethal inoculations of the live organism did enhance resistance. In 1962, Njoku-Obi and Osebold (11) first demonstrated cellular immunity against *L. monocytogenes* in in vitro studies with sheep macrophages. In the same year, Mackaness (10) presented evidence of cellular resistance to the same organism in mice.

In the experiments described here, the results in animals containing listeriae in diffusion chamber implants and those containing sham diffusion chambers generally fell between the responses of normal and immunized animals. Consequently, when mice containing diffusion chambers were found to be significantly more resistant than normal mice, it was difficult to demonstrate that they were also significantly different from immunized mice because of the limited range of animal response. It is uncertain from these results whether the viable bacteria in the chamber had any resistance-enhancing effect. Certainly, the organisms remained viable in high concentration for prolonged periods. This demonstrated

that exchange of nutrients into the chamber and escape of metabolic products continued at an effective rate. The diffusion of soluble antigens and their distribution was sustained for 30 days prior to challenge of the animals, but the effect of this on immunity was not apparent. One might have supposed that antigenic stimulation superimposed upon the nonspecific resistance enhancement caused by the chamber itself would produce an animal far more resistant than those inoculated subcutaneously with only 110,000 living organisms, but this was never the case. Thus, the results suggest that direct involvement of macrophages with the parasite is necessary to achieve cellular immunity.

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LITERATURE CITED

1. Adler, F. L., and M. Fishman. 1962. Adjuvant effect of diffusion chambers on soluble antigens. Soc. Exptl. Biol. Med. **110**:691–695.
2. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exptl. Med. **124**: 585–600.
3. Donovick, R., C. M. McKee, W. P. Jambor, and G. Rake. 1949. The use of the mouse in a standardized test for antituberculous activity of compounds of natural or synthetic origin. II. Choice of mouse strain. Am. Rev. Tuberc. **60**:109–120.
4. Hasenclever, H. F., and W. W. Karakawa. 1957. Immunization of mice against *Listeria monocytogenes*. J. Bacteriol. **74**:584–586.
5. Holland, J. J., and M. J. Pickett. 1958. A cellular basis of immunity in experimental brucella infection. J. Exptl. Med. **108**:343–360.
6. Houser, E. D., and L. J. Berry. 1961. The pathogenesis of staphylococcus infections. I. The use of diffusion chambers in establishing the role of staphylococcus toxins. J. Infect. Diseases **109**:24–30.
7. Irwin, J. O., and E. A. Cheeseman. 1939. On an approximate method of determining the median effective dose and its error, in the case of a quantal response. J. Hyg. **39**:574–580.
8. Jenkin, C. R., and D. Rowley, 1963. Basis for immunity to typhoid in mice and the question of "cellular immunity." Bacteriol. Rev. **27**:391–404.
9. Kärber, G. 1931. Beitrag zur kollektiven behandlung pharmakologischer Reihenversuche. Arch. Exptl. Pathol. Pharmakol. **162**:480–483.
10. Mackaness, G. B. 1962. Cellular resistance to infection. J. Exptl. Med. **116**:381–406.
11. Njoku-Obi, A., and J. W. Osebold. 1962. Studies on mechanisms of immunity in Listeriosis. I. Interaction of peritoneal exudate cells from sheep with *Listeria monocytogenes in vitro*. J. Immunol. **89**:187–194.
12. Osebold, J. W., and O. Aalund. Interpretation of serum agglutinating antibodies to *Listeria monocytogenes* by immunoglobulin differentiation. J. Infect. Diseases, *in press*.
13. Osebold, J. W., and M. T. Sawyer. 1957. Immunization studies on listeriosis in mice. J. Immunol. **78**:262–268.
14. Pomales-Lebrón, A., and C. Fernández. 1952. A method for estimating the number of bacteria in liquids and tissues. J. Bacteriol. **64**:837–839.
15. Saito, K., T. Akiyama, M. Nakano, and D. Ushiba. 1960. Interaction between *Salmonella enteritidis* and tissue cultured macrophages derived from immunized animals. Japan. J. Microbiol. **4**:395–407.
16. Seeliger, H. P. R. 1961. Listeriosis, p. 224–252. Hafner Publishing Co., New York.
17. Steward, J. P., L. R. Collis, and R. J. Roantree. 1963. Effects of active immunization and of total body X-irradiation upon the humoral bactericidal system of the guinea pig as measured with strains of enteric bacilli. J. Immunol. **92**:616–625.
18. Zobell, C. E., and M. H. Zobell. 1932. Metabolism studies on the *Brucella* group. III. Viability in aqueous solutions. J. Infect. Diseases **50**: 538–541.