INFECTION AND IMMUNITY, Feb. 1983, p. 779–784
0019-9567/83/020779-06$02.00/0

Vol. 39, No. 2

Bactericidal Activity of Alveolar and Peritoneal Macrophages Exposed In Vitro to Three Strains of *Pasteurella multocida*

FRANK M. COLLINS,[1]* CAROL J. NIEDERBUHL,[1] AND S. GORDON CAMPBELL[2]

Trudeau Institue, Inc., Saranac Lake, New York 12983,[1] and Department of Microbiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14850[2]

Received 2 August 1982/Accepted 15 November 1982

Normal ICR mice were infected intravenously, intraperitoneally, or aerogenically with *Pasteurella multocida* strains isolated from a turkey (S68), calf (V90), or rabbit (J20) lung. Both the turkey and calf isolates were highly virulent for mice and multiplied logarithmically in the lungs, liver, and spleen, resulting in death of the animals in 18 to 36 h. The rabbit strain was avirulent for mice, but repeated passage in mice did result in some increased virulence. All three strains of *P. multocida* were inactivated rapidly by normal mouse peritoneal macrophages, provided that the organisms were opsonized with specific hyperimmune serum before being exposed to the macrophage monolayers. *P. multocida* was slowly inactivated by normal mouse alveolar macrophages when the organisms were preopsonized. However, the surviving organisms later multiplied extensively in vitro. Macrophages harvested from hyperimmunized mice were no better at inactivating opsonized *P. multocida* cells than were normal mouse cells. The relative importance of the different phagocytic cell populations in the uptake and killing of opsonized *P. multocida* cells is discussed in relation to immunity to this important animal pathogen.

Pasteurella multocida is responsible for several economically important diseases of domestic animals (2). Cultivation of nasal swabs routinely taken from apparently normal cattle indicates that *P. multocida* can also colonize the nasopharyngeal mucosae of adult animals without the subsequent development of an acute phase of respiratory or systemic disease (2). Overt disease in such animals may occur after some form of stress (transport or dehydration) or exposure to an intercurrent viral infection (14). These animals then develop a rapidly fatal hemorrhagic septicemia or acute fibrinous pneumonia (shiping fever). Protection studies carried out in birds, cattle, and mice indicate a predominantly humoral type of immunity in appropriately vaccinated animals (2, 4, 8), and attempts to adoptively protect mice with immune spleen cells have been generally unsuccessful (6). This does not mean, however, that phagocytic cells (both polymorphonuclear and mononuclear) are not involved in the immune response (7), provided that the appropriate specific opsonins are also present (15).

The rapidity and severity of lung involvement seen in animals suffering from fibrinous pneumonia raises questions about the ability of the normal resident alveolar macrophages to phagocytose and kill this parasite within the lung. The present study makes use of an aerogenically infected mouse model (6) to compare the phagocytic and bactericidal abilities of alveolar and peritoneal macrophages exposed in vivo and in vitro to *P. multocida* strains of different virulence for mice.

MATERIALS AND METHODS

Animals. Specific-pathogen-free TRU:ICR mice were obtained from the Trudeau Animal Breeding Facility, Saranac Lake, N.Y. They were maintained under the conditions described elsewhere (5). Five-week-old female mice (22 to 25 g) were used throughout.

Organisms. *P. multocida* S68 was isolated from an infected turkey lung by M. A. Soltys (University of Guelph, Ontario). The organism was serotyped as a 5A strain and had an intravenous median lethal dose (LD_{50}) of about 10 viable organisms for chickens and 1 to 2 organisms for ICR mice (4). *P. multocida* V90 was isolated at the Cornell Veterinary College from the lungs of a 6-week-old calf suffering from acute fibrinous pneumonia. The organism was serotyped at the National Animal Disease Center, Ames, Iowa, as a type 1A strain. It had an intravenous mouse LD_{50} of 60 viable bacilli. *P. multocida* J20 was isolated from New Zealand white rabbits suffering from snuffles and was kindly provided by G. R. Carter (Virginia Polytechnic Institute, Blacksburg, Va.); it was a serotype 12A strain with low rabbit virulence and an intravenous LD_{50} for ICR mice of $>10^9$ viable bacilli. After repeated passage in mice, this value dropped to approximately 10^8 viable organisms (see Table 1). The rabbit

TABLE 1. Virulence tests of *P. multocida* strains in ICR mice challenged by different routes

P. multocida strain	Serotype (somatic/capsule)	Primary host species	Primary host virulence	Mouse virulence (LD_{50}) (no. of organisms)			
				Intravenous	Intraperitoneal	Subcutaneous	Aerosol
S68	5A	Turkey	Very high	1–2	1–2	2	5–10
V90	1A	Cattle	High	60	100	50	280
J20[a]	12A	Rabbit	Low	10^8	10^8		$>10^6$

[a] Mouse-passaged strain.

isolate grew poorly in liquid medium but could be maintained effectively in the laboratory by alternating passage in normal ICR mice (with large intravenous inocula) followed by plating on 10% sheep blood agar. Spleen homogenates were harvested after 2 to 3 days of infection and plated to check for purity.

Infecting inocula. (i) Intravenous challenge. *P. multocida* S68 and V90 cells were inoculated into heart-brain infusion (HBI) broth (Difco Laboratories, Detroit, Mich.) and incubated at 37°C until late logarithmic growth phase (usually 6 h). The culture was diluted with an equal volume of fresh medium and stored in 1-ml ampoules at −70°C until required (3). Thawed suspension (0.1 ml) was inoculated into 10 ml of sterile HBI broth and incubated at 37°C for 4 to 6 h. The logarithmic-phase suspension was diluted appropriately in fresh HBI broth and used immediately. Infecting inocula of *P. multocida* J20 were prepared from blood agar cultures by emulsifying several overnight colonies in Hanks balanced salt solution (HBSS) plus 1% fetal calf serum (FCS) and standardizing the suspension by counting with a Petroff-Hauser chamber under phase-contrast illumination. The viability of the challenge inocula was checked by plating 10-fold dilutions on blood agar and counting the resulting colonies 24 h later.

(ii) Aerogenic challenge. Groups of mice were exposed to an aerosol generated in a Middlebrook chamber as described elsewhere (6). The infective dose was estimated from dose-response curves prepared earlier. At 30 min after the challenge five mice were killed, and the lung homogenates were plated on HBI agar to check the size of the infectious inoculum within the lung.

(iii) Intraperitoneal challenge. The bacterial suspension in 0.1 ml HBSS plus 1% FCS was injected to one side of the midline with a 26-guage needle. The viability of the suspension was checked immediately after injection.

Bacterial enumeration in vivo. Groups of five randomly selected mice were killed at increasing time intervals, and the lungs, spleens, and livers were homogenized separately in cold HBSS plus 1% FCS. When appropriate, the peritoneal cavity was washed out with 2 ml of HBSS as described previously (7). The counting errors were usually less than 20% of the mean.

In vitro macrophage culture methods. (i) Peritoneal macrophage culture. Mice were killed by a cervical dislocation, and the peritoneal cavity was washed out with 2.5 ml of cold HBSS containing 5% FCS and 10 IU of heparin, as described by Spitalny and North (13). Wash fluid that was visibly contaminated with blood was discarded. The cells from several mice were pooled, counted with a hemacytometer, and adjusted to 5×10^6 cells per ml. Viability was checked by dye exclusion. The cells were allowed to adhere to cover slips to form monolayers by incubating them in 5% CO_2-enriched air at 37°C for 2 h. Nonadherent cells were removed by washing the cover slips with a jet of saline containing 1% FCS (13).

(ii) Alveolar macrophage cultures. Normal mouse lungs were cannulated and washed out five times with 1 ml of saline as described by Senior et al. (11). The cells were washed once by centrifugation ($500 \times g$ for 10 min) and suspended at RPMI 1640 medium plus 5% FCS, counted with a hemacytometer, and standardized at 2×10^6 cells per ml. The cells were allowed to adhere to small cover slips to form monolayers by incubating them in petri dishes in a 5% CO_2-enriched atmosphere at 37°C for 3 h. The nonadherent cells were removed by gentle washing with culture fluid. The cover slips were replaced in warm fresh medium and recultured in RPMI 1640 plus 5% FCS for 1 h before being challenged.

Phagocytosis and killing rates in vitro. A log-phase culture of *P. multocida* was counted with a Petroff-Hauser chamber and diluted to approximately 10^6 bacteria per ml of RPMI 1640 medium plus 5% FCS. The suspension was diluted with an equal volume of RPMI 1640 containing 5% normal or *P. multocida*-immune mouse serum (15). The bacteria-serum mixture was incubated at 37°C for 30 min, diluted 1:4 with fresh RPMI 1640 medium, and exposed to sonic vibration for 3 s (50% power output) to break up any agglutinated clumps of bacteria. The macrophage culture fluid was aspirated and replaced with 1 ml of the bacterial suspension, and the culture was returned to the CO_2 incubator for 20 min. Excess unphagocytosed bacteria were removed by washing the cover slips with saline and placing the macrophage monolayers in fresh medium before returning them to the CO_2 incubator. Three randomly selected cover slips were removed at time zero and thereafter at 15-min intervals. Each cover slip was placed in 10 ml of HBSS containing 0.1% WR1339 detergent and subjected to sonic vibration for 5 s to rupture the phagocytes and release the bacteria (13). The supernatant fluid was plated on HBI agar, and the average number of viable bacteria was determined (3). The results were also expressed as percentages of the number of viable bacteria present at time zero.

RESULTS

Growth of the *P. multocida* strains in ICR mice. The virulence of the *P. multocida* strains for normal mice varied depending upon the strain and the route of challenge (Table 1). Virulence also appeared to be somewhat lower after an

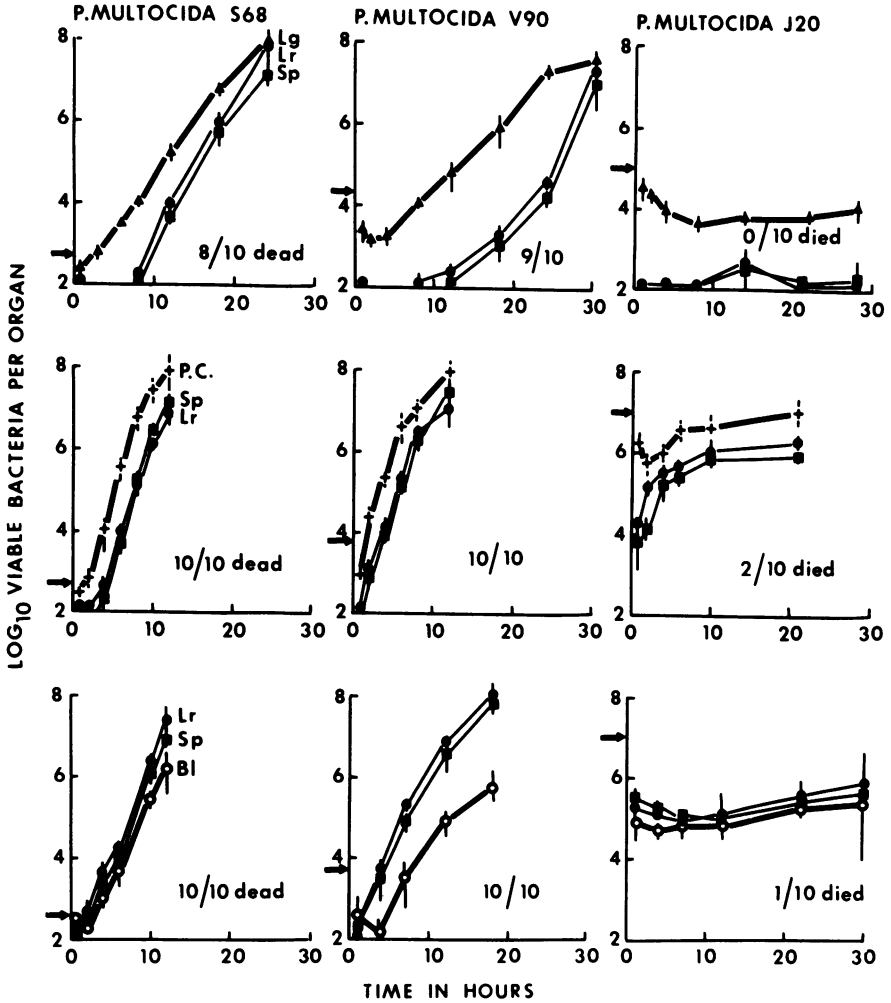

FIG. 1. Growth curves for three strains of *P. multocida* in normal ICR mice after intravenous (bottom), intraperitoneal (middle), or aerogenic (top) challenge. Arrows indicate the size of the challenge inoculum. Symbols: ●, liver; ■, spleen; ▲, lungs; ○, blood; and +, peritoneal cavity. The vertical bars represent the standard error of the mean for five determinations.

aerogenic than an intravenous or intraperitoneal challenge, as judged from the in vivo growth curves (Fig. 1). Both the turkey and bovine strains of *P. multocida* multiplied rapidly within the liver and spleen after intravenous inoculation, and all of the challenged mice died within 18 h. On the other hand, the rabbit strain (J20) tended to multiply in vivo relatively slowly (Fig. 1), so that death did not occur unless very large challenge inocula were employed (Table 1).

Phagocytosis and killing in vitro of *P. multocida* by normal peritoneal macrophages. The normal peritoneal wash fluid contained 40% phagocytic, esterase-positive cells and only 1% polymorphs; the remaining cells resembled small lymphocytes (Table 2). Peritoneal macrophage monolayers usually contained between 1×10^5 and 2×10^5 macrophages per cover slip. Pretreating the mice with 0.5 ml of 10% proteose peptone 3 to 6 h before harvest markedly increased the relative proportion of polymorphonuclear leukocytes (45 to 50%), with corresponding reductions in the relative proportion of macrophages and lymphocytes (Table 2). The 3-h exudate cells showed no detectable shift in in vitro activity compared with the normal resident macrophage preparations regardless of whether the monolayers were exposed to *P. multocida* S68 cells pretreated with 5% normal mouse serum or to the corresponding mouse hyperimmune serum (Collins and Niederbuhl, unpublished data).

The specific opsonin(s) increased the uptake

TABLE 2. Number and type of cells recovered from the lungs and peritoneal cavities of normal ICR mice

Cells recovered	No. (%) of following cell type			
	White blood cells (total)	Polymorphonuclear leukocytes	Macrophages	Lymphocytes
Peritoneal wash fluid				
Normal	$4.71 (\pm 1.11) \times 10^6$	$4.7 (\pm 0.5) \times 10^4$ (1)	$1.88 (\pm 0.11) \times 10^6$ (40)	$2.78 (\pm 0.12) \times 10^6$ (59)
Proteose-peptone treated (3 h)	$2.6 (\pm 0.18) \times 10^6$	$1.22 (\pm 0.15) \times 10^6$ (47)	$7.0 (\pm 0.9) \times 10^5$ (27)	$7.0 (\pm 1.1) \times 10^5$ (26)
Alveolar lavage (normal)	$5.1 (\pm 0.9) \times 10^6$	$2.6 (\pm 0.40) \times 10^4$ (0.5)	$4.6 (\pm 0.11) \times 10^6$ (90)	$5.08 (\pm 0.2) \times 10^5$ (10)

of *P. multocida* by the peritoneal macrophages approxiamtely 10-fold compared with the uptake of bacteria pretreated with normal mouse serum (Fig. 2). The subsequent rate of inactivation of the opsonized bacteria was also increased. The inactivation of a control suspension of unopsonized *Listeria monocytogenes* cells by the normal mouse macrophages was consistent with earlier reports by Spitalny and North (13). Comparative studies of the rate of inactivation of the three strains of opsonized *P. multocida* indicated that a rapid initial kill occurred in all three preparations (Fig. 3), although the rabbit strain (J20) appeared to be inactivated slightly more rapidly than the other two, more highly mouse-virulent strains. However, it was doubtful that this difference was great enough to be considered significant.

Phagocytosis and killing of *P. multocida* by mouse alveolar macrophages. Lavage of normal mouse lungs resulted in recovery of 1×10^6 to 2×10^6 nucleated cells per mouse, of which 80 to 90% were esterase positive and actively phagocytic (Table 2). Very few polymorphs were present in lavage fluids taken from normal mice. Most of the remaining cells appeared to be small lymphocytes or epithelial cells. When the lavaged cells were cultured in vitro, a substantial proportion were lost from the cover slips when they were subjected to the vigorous washing procedure normally used to prepare peritoneal macrophage monolayers. As a result, many of the cover slips failed to produce continuous alveolar macrophage monolayers at the time of infection. The severity of the washing procedure was therefore modified slightly, and the initial incubation period was increased to 3 h to allow better retention of the alveolar macrophages to the cover slips.

Opsonized *P. multocida* S68 cells were readily phagocytosed by the alveolar macrophage monolayers, and almost 90% of the bacilli were killed within 60 min (Fig. 4). However, relatively few bacilli were taken up in the absence of the specific opsonin, and substantial extracellular growth was then observed over the subsequent 90-min incubation period.

Killing by macrophages harvested from immunized mice. Groups of mice were hyperimmunized by twice-weekly subcutaneous injections of 10^8 heat-killed *P. multocida* S68 or V90 cells for 4 weeks. The initial dose of vaccine was suspended in Freund complete adjuvant (Difco), but all subsequent injections were given as saline suspensions. Peritoneal and alveolar macrophages were harvested from the vaccinated mice, but no difference was observed in the rate of phagocytosis or killing of the preopsonized *P. multocida* S68 cells regardless of whether the cell donors had been vaccinated (Fig. 5). The

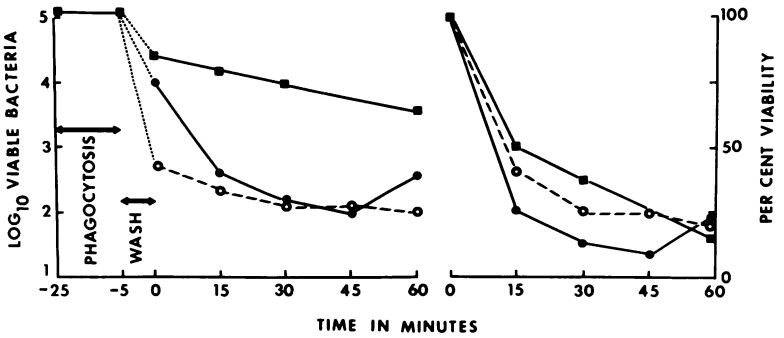

FIG. 2. Viability (left) of *P. multocida* S68 cells pretreated with specific hyperimmune mouse serum (●) or normal mouse serum (○) before being exposed to normal mouse peritoneal macrophage monolayers in vitro. The decline in viability relative to the time zero counts is shown on the right. A control consisting of *L. monocytogenes* cells (■) pretreated with normal mouse serum was included.

immune cells did not phagocytose unopsonized *P. multocida* S68 cells substantially better than did normal mouse phagocytes.

DISCUSSION

Strains of *P. multocida* isolated from different host species vary extensively in their virulence for white mice (2, 4). In the present study, both the turkey and bovine isolates were highly virulent for mice. Their virulence can be correlated with the presence of a well-defined capsular layer, which inhibits phagocytosis and killing by the normal host phagocytes (2). Unopsonized pasteurellae multiply freely in the extracellular fluids of the normal host at rates little different from those seen in shaken broth cultures (3). Thus, it might be considered on the basis of these studies that the capsular antigenic makeup of the *P. multocida* strains was the most important determinant of mouse pathogenicity (2). Murata et al. (10) compared a swine pneumonia isolate (serotype 1A) with a fowl cholera strain (5A) and found both to be virulent for mice. Similarly, those cat isolates that could be typed were A capsular types, whereas the corresponding dog isolates were generally of other capsular types and of much lower virulence for mice (12). On the other hand, the rabbit strain, which was an A capsular type, was of low virulence for mice (Fig. 1). To date, too few rabbit isolates have been investigated in sufficient detail to indicate whether this is a general finding (9, 12).

Although there were substantial differences in the virulence (LD_{50}) for mice of the three *P. multocida* strains tested in the present study (Table 1), it should be noted that they were all inactivated rapidly by normal mouse peritoneal macrophages at almost identical rates over the first 15 min (Fig. 3). This was somewhat surprising in view of the fact that the rabbit strain (J20) was almost totally avirulent for ICR mice, whereas the other two strains were highly virulent. *P. multocida* J20 was difficult to maintain in

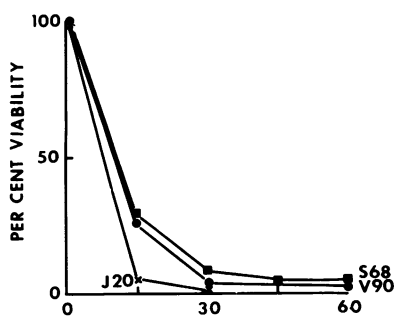

FIG. 3. Killing curves for opsonized *P. multocida* S68, V90, and J20 cells after phagocytosis by normal peritoneal macrophages in vitro.

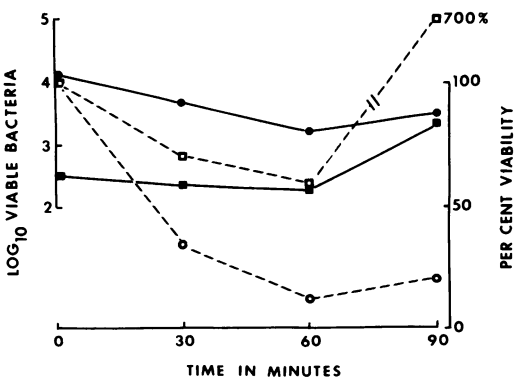

FIG. 4. Killing curves for opsonized *P. multocida* S68 cells exposed in vitro to monolayers of normal mouse alveolar macrophages. Immune mouse serum (■, □) or normal mouse serum (●, ○) was used to treat the *P. multocida* cells before their addition to the macrophage monolayers. The broken lines represent the percent viability relative to that at time zero.

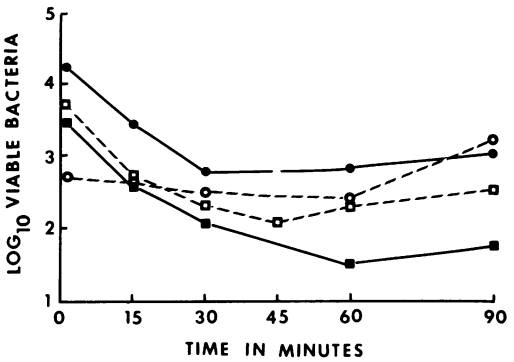

FIG 5. In vitro inactivation of specifically opsonized *P. multocida* S68 cells by peritoneal or alveolar macrophages harvested from immunized or normal mice. Symbols: ○, normal alveolar macrophages; ●, immune mouse alveolar macrophages; □, normal peritoneal macrophages; ■, immunized mouse peritoneal macrophages.

vitro without alternating passage in normal mice. When transfer was continued in this way over a considerable period of time, the level of virulence gradually increased slightly until an intravenous LD_{50} dose was stablized at about 10^8 viable bacilli (Table 1). The mouse-passaged strain produced larger, more mucoid colonies on blood agar than did the original culture, but other cultural and biochemical characteristics appeared to be unchanged. There was a progressive reduction in colony size and growth vigor when this organism was continuously maintained in vitro for any length of time. The nature of the in vivo-induced change to the J20 strain remains unclear, however.

In passive protection studies carried out in *P. multocida*-challenged animals, only the presence of specific immune antibodies (opsonins) directed against the capsular antigen appeared to offer substantial immune protection (1, 2, 15). Such a conclusion seems to be consistent with the fact that splenic and peritoneal lymphocyte suspensions harvested from actively immunized animals are unable to adoptively protect normal syngeneic recipients unless the specific opsonin(s) is also present (4). Similarly, it is clear that both normal peritoneal and alveolar macrophages are as effective as those harvested from hyperimmunized donors at inactivating this highly virulent pathogen, provided that they are pretreated with specific opsonic antibody before being exposed to the macrophage monolayer.

Alevolar and peritoneal macrophages took up about the same number of opsonized *P. multocida* cells in these in vitro cultures. They then killed 80 to 90% of these bacteria over the following 60 min. (Fig. 2 and 4). However, the surviving organisms multiplied extensively, and complete sterilization was never achieved in these preparations. The surviving bacteria may have been cell associated rather than fully phagocytosed, and thus were not exposed to the bactericidal action of the lysosomal enzymes. However, the phagocytosis and killing rates for the alveolar as compared with the peritoneal macrophages were never sufficiently different to explain the great susceptibility of the host to aerogenic challenge.

ACKNOWLEDGMENTS

We thank George Spitalny, Trudeau Institute, for his frank and helpful discussions throughout these studies.

The work was supported by Public Health Service grant HL-19774 from the National Heart, Blood, and Lung Institute, grant AI-14065 administered by the U.S.-Japan Cooperative Medical Sciences Program for the National Institute of Allergy and Infectious Diseases, and grant RR-05705 from the General Research Support Branch, Division of Research Resources, National Institutes of Health.

LITERATURE CITED

1. **Bain, R. V. S.** 1963. Hemorrhagic septicemia, p. 1–78. Food and Agriculture Organization of the United Nations publ. no. 62, Rome, Italy.
2. **Carter, G. R.** 1967. Pasteurellosis: *Pasteurella multocida* and *Pasterurella hemolytica*. Adv. Vet. Sci. **11**:321–379.
3. **Collins, F. M.** 1973. Growth of *Pasteurella multocida* in vaccinated and normal mice. Infect. Immun. **8**:868–875.
4. **Collins, F. M.** 1977. Mechanisms of acquired resistance to *Pasteurella multocida* infection: a review. Cornell Vet. **67**:103–138.
5. **Collins, F. M., N. E. Morrison, and V. Montalbine.** 1978. Immune response to persistent mycobacterial infection in mice. Infect. Immun. **20**:430–438.
6. **Collins, F. M., and J. B. Woolcock.** 1976. Immune responses to *Pasteurella multocida* in the mouse. RES J. Reticuloendothel. Soc. **19**:311–321.
7. **Collins, F. M., and W. H. Woodruff.** 1979. Inactivation of *Pasteurella multocida* within the mouse peritoneal cavity. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **245**:106–113.
8. **Heddleston, K. L., and L. P. Watko.** 1967. Fowl cholera: susceptibility of various animals and their potential as disseminators of the disease. Adv. Vet. Sci. **11**:247–251.
9. **Lu, Y. S., D. H. Ringler, and J. S. Park.** 1978. Characterization of *P. multocida* isolates from the nares of healthy rabbits and rabbits with pneumonia. Lab. Anim. Sci. **28**:691–697.
10. **Murata, M., T. Horiuchi, and S. Namioka.** 1964. Studies on the pathogenicity of *Pasteurella multocida* for mice and chickens on the basis of O groups. Cornell Vet. **54**:293–307.
11. **Senior, R. M., E. J. Campbell, and B. Villiger.** 1981. Obtaining and culturing human and animal alveolar macrophages, p. 69–83. *In* D. D. Adams, P. J. Edelson, and H. S. Koren (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.
12. **Smith, J. E.** 1958. Studies on *Pasteurella septica*. II. Some cultural and biochemical properties of strains from different host species. J. Comp. Pathol. Ther. **68**:315–323.
13. **Spitalny, G. L., and R. J. North.** 1981. Destruction of *Listeria monocytogenes in vitro*, p. 685–691. In D. O. Adams, P. J. Edelson, and H. S. Koren (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.
14. **Thomson, R. G., M. L. Benson, and M. V. Savan.** 1969. Pneumonic pasteurellosis of cattle: microbiology and immunology. Can. J. Comp. Med. **33**:194–206.
15. **Woolcock, J. B., and F. M. Collins.** 1976. Studies of the immune mechanism in *Pasteurella multocida*-infected mice. Infect. Immun. **13**:949–958.