INFECTION AND IMMUNITY, Oct. 1980, p. 254-262
0019-9567/80/10-0254/09$02.00/0
Vol. 30, No. 1

# Kinetics of Interaction and Fate of *Pasteurella hemolytica* in Bovine Alveolar Macrophages

S. K. MAHESWARAN,[1]* K. A. BERGGREN,[1] R. R. SIMONSON,[1] G. E. WARD,[1] AND C. C. MUSCOPLAT[2]

*Departments of Veterinary Pathobiology[1] and Large Animal Clinical Sciences,[2] College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108*

To study the role of pulmonary alveolar macrophages (PAMs) in phagocytizing *Pasteurella hemolytica*, we developed an in vitro cultivation method for preparing them. This procedure provided an adherent monolayer of PAMs which were nonspecific esterase-positive and phagocytized latex beads. The phagocytosis and fate of *P. hemolytica* (biotype A, serotype 1) by PAMs in suspension were studied. The kinetics of phagocytosis were determined by quantitatively measuring the uptake of 24-h [$^3$H]thymidine-labeled bacteria by the PAMs in the presence of opsonins. Results showed that the uptake of *P. hemolytica* was enhanced in the presence of normal serum or antiserum. A total of 90% of the bacteria were phagocytized in the presence of normal adult bovine serum, and up to 95% were phagocytized in the presence of an antiserum. These studies also showed that normal serum, but not fetal calf serum, contained heat-stable natural antibodies which readily initiated the opsonization of *P. hemolytica*. The heat-labile complement system was also involved in the opsonization. The fate of *P. hemolytica* inside the PAMs was investigated by transmission electron microscopy and by the viable plate count method. Approximately 90% of the normal serum- or antiserum-opsonized *P. hemolytica* were phagocytized by PAMs at a bacteria/PAM ratio of 20:1 and were completely degraded after 60 min of exposure. Prolonged incubation of this mixture of bacteria and PAMs resulted in cytotoxic changes and destruction of PAMs. At a low bacteria/PAM ratio (10:1 or less), there was phagocytosis and killing of bacteria but no cytotoxic changes on the PAMs. The exact mechanism which initiated this phenomenon was not demonstrated. Perhaps toxic substance(s) released by the excess unphagocytized bacteria caused the cytotoxic changes to the PAMs.

Bovine respiratory disease, also called pneumonic pasteurellosis, affects all age groups of cattle and continues to be one of the most serious disease problems plaguing the cattle industry in North America. Most investigators to date indicate that bovine respiratory disease is caused as a result of the interaction of environmental factors (such as prolonged transportation, crowding, mental stress, physical stress, extremes of cold, atmospheric pollutants) and several etiological agents, such as viruses and bacteria (13). Pneumonia and death associated with bovine respiratory disease is caused by *Pasteurella hemolytica* or *P. multocida*. In the United States and Canada, *P. hemolytica* is most often isolated from pneumonic lesions (3).

Although considerable research has been performed on various aspects of bovine respiratory disease, there is a complete lack of understanding pertaining to the interactions of the pulmonary defense systems and *P. hemolytica*. Previous investigations in laboratory animals (11) have established the importance of the mucociliary apparatus and phagocytosis by the pulmonary alveolar macrophages (PAMs) in their initial defense against inhaled bacteria. Any impairment in these functions results in lung infections with bronchopneumonia. Studies with murine models suggest that this abnormality, i.e., impairment in phagocytic function by PAMs, is the most important (22). In cattle, studies have shown that infection with either parainfluenza-3 virus or infectious bovine rhinotracheitis virus predisposes them to pulmonary infection with *P. hemolytica* (10, 14). However, it is not known whether these viruses cause impairment of the phagocytic function of PAMs.

Relatively little is known about the virulent factors of *P. hemolytica*. Since this organism is encapsulated, it may possess surface determinants which are antiphagocytic. In earlier publication, we reported on the effect of encapsulation on phagocytosis of two strains of *P. multocida* (types A and B) by bovine neutrophils (15). In this investigation, we extended these studies to *P. hemolytica* and evaluated the kinetics of

phagocytosis and bacterial killing by bovine PAMs collected from healthy cattle. Since the rate of bacterial ingestion by PAMs had not been determined, the relative significance of the contribution of the phagocytic component to the overall defense of the bovine lung against bacterial invasion was unknown. Comparisons of rates of ingestion and inactivation of *P. hemolytica* by PAMs should help delineate, quantitatively, the maximum proportion of inhaled bacteria susceptible to destruction by lung phagocytes.

## MATERIALS AND METHODS

**Strain of *P. hemolytica* and radioactive labeling.** *P. hemolytica* strain 10056 was isolated from a yearling that died of pneumonia-associated bovine respiratory disease in a feedlot in Minnesota. The virulence of this organism, which belonged to biotype A, serotype I (5, 18), was maintained by one mouse passage (4), and an 8-h broth culture of this organism was lyophilized. For each experiment, one lyophilized culture was rehydrated with sterile distilled water and propagated overnight at 37°C on brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.). Radioactive labeling was achieved by inoculating several colonies into 5 ml of brain heart infusion (Difco), containing 0.1% yeast extract (Difco)-1% N-Z Amine AS (Sheffield Chemical, Union, N.J.), and allowing it to grow for 4 h on a shaker; 0.5 ml was then inoculated into 5 ml of the same broth containing 100 μCi of [$^3$H]thymidine (specific activity, 60 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) and 1.37 mg of deoxyadenosine (Sigma Chemical Co., St. Louis, Mo.). After 24 h of incubation with gentle shaking at 37°C, the bacteria were centrifuged at 3,000 × *g* for 10 min, washed three times in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% gelatin, and suspended in PBS to a concentration of $10^9$ colony-forming units per ml. This was determined spectrophotometrically and confirmed by the pour-plate colony count method.

**Opsonic sources and bacterial opsonization.** Fetal calf serum was obtained from fetuses removed from cattle slaughtered at a local abattoir. Normal adult bovine serum was collected from a healthy 6-year-old cow. To study opsonization with immune serum, we immunized calves with either a live vaccine or a bacterin. Antiserum against a live vaccine was produced by injecting two calves intradermally with 2 ml of an 18-h broth culture containing $1 \times 10^7$ organisms/ml once a week for 2 weeks. Sera were collected 2 weeks after immunization was completed. Bacterin (killed vaccine) was prepared by inactivating a bacterial suspension containing $1 \times 10^{14}$ organisms/ml with 0.3% formaldehyde overnight. An equal volume of this bacterin was absorbed onto an aluminum hydroxide-gel adjuvant (Alhydrogel; Accurate Chemical and Scientific Corp., Hicksville, N.Y.). Antiserum against the bacterin was produced by injecting two calves with 2-ml doses administered intramuscularly at the precapsular region once a week for 2 weeks. Sera were collected 2 weeks after immunization. All sera were stored in small portions at −70°C and thawed shortly before use. Heat-inactivated serum was prepared by heating at 56°C for 30 min. Serum was diluted to a final concentration of 10% in Hanks balanced salt solution containing 0.1% gelatin (HBSSG). Specific antibody levels against *P. hemolytica* found in fetal calf serum, normal adult bovine serum, and serum from calves immunized with either a live vaccine or bacteria were measured by the indirect bacterial agglutination test described by Friend et al. (6). The endpoint dilution was recorded as the indirect bacterial agglutination titer.

Bacterial opsonization was accomplished as previously described (15, 21). Briefly, 1-ml amounts of the opsonic source in HBSSG and 0.1 ml of the bacterial suspension containing $10^9$ colony-forming units per ml were incubated at 37°C in polypropylene vials (Biovials; Beckman Instruments Inc., Chicago, Ill.) on a shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 200 rpm for various time periods. Each mixture was washed with 2 ml of sterile PBS and centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was then discarded, and the bacterial pellet was suspended in sterile HBSSG.

**Preparation of PAMs.** Lungs from freshly slaughtered finished steers were obtained from a local abattoir. These lungs were grossly normal and in retrospect bacteriologically sterile. The right and left diaphragmatic lobes of the lungs were lavaged gently three times in situ with a total of 3,000 ml of sterile saline containing streptomycin (100 μg/ml) and penicillin (100 U/ml). If the lavage fluid appeared bloody, it was discarded. The lavage fluid was pooled and centrifuged at 1,020 × *g* for 15 min. The supernatant was decanted, and the cellular pellet was suspended in a complete medium of RPMI-1640 containing 2 mM L-glutamine, streptomycin (100 μg/ml), penicillin (100 U/ml), and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). The total number of cells was determined with a hemocytometer, and the PAM concentration was adjusted to $1 \times 10^7$ per ml of complete medium. A 1-ml amount of this was inoculated into tissue culture-treated plastic petri dishes (100 by 20 mm, Falcon, Division of Becton, Dickinson & Co., Cockeysville, Md.) together with 200 ml of complete medium, and cells were allowed to attach for 3 h at 37°C in a humid atmosphere containing 5% $CO_2$. After incubation, the nonadherent cells were decanted, and adherent cells were replenished with complete medium containing nystatin (5 μg/ml) and 20% fetal calf serum and further incubated overnight. This procedure provided a well-defined adherent cell population in a monolayer. Lung lavage products aspirated from over 50 animals were used in these studies. The number of cells recovered from the lung lavage ranged between $1.03 \times 10^8$ to $1.67 \times 10^8$ which contained 78 to 95% PAMs, 2 to 17% lymphocytes, 2 to 8% neutrophils, and 0 to 6% ciliated columnar cells. After purification by the adherence technique, the percent PAMs recovered ranged between 54 and 65% of the original cell population. PAMs were identified by microscopic examination after staining with Wright-Giemsa stain, and utilizing other criteria, such as whether or not they were nonspecific esterase positive (17) and their ability to engulf latex beads (8). The PAMs were maintained as monolayers at 37°C in a humidified

atmosphere containing 5% $CO_2$ for 1 day until use. For phagocytosis studies, PAMs were used as a suspension which was accomplished by the method of Holt (9). The media were decanted from each petri dish, the PAM monolayer was overlayed with RPMI-1640 containing 20% fetal calf serum–9 mM lidocaine hydrochloride (Astra Pharmaceutical Products, Worcester, Mass.) and incubated for 30 min, and the cells were gently scraped off with a rubber policeman. They were then decanted into centrifuge tubes and washed twice with RPMI-1640 containing 25 mM HEPES to remove all traces of lidocaine. Viability was determined by trypan blue exclusion, and the cell concentration was adjusted in the same medium to $5 \times 10^6$ viable PAMs per ml. Viability constantly ranged between 80 to 90%. All phagocytosis experiments were performed with PAMs that had been cultured overnight.

**Determinations of phagocytosis and killing.** The percentage of phagocytized (uptake) bacteria was determined by a previously described method (15), and results were expressed as a percentage of the total radioactivity taken up by PAMs. A total of $1 \times 10^7$ CFUs of bacteria were presented to $5 \times 10^5$ PAMs, yielding a bacteria-to-PAM ratio of 20:1. The number of viable intracellular bacteria present after ingestion by the PAMs was determined by viable counts from lysed PAMs. Briefly, after intervals of 30, 60, 90, 120, 150, and 180 min of incubation, the mixture was centrifuged and washed three times with sterile Hanks balanced salt solution to remove nonphagocytized bacteria. The washed pellet containing the PAMs was suspended in 2.5 ml of sterile water and shaken vigorously to lyse the PAMs. Viable counts were performed to determine the number of surviving bacteria. The total number of bacteria was measured by adding 0.2 ml of the phagocytosis mixture at time zero to 2.5 ml of sterile water, and viable counts were made. The percentage of bacteria killed by the PAMs at different time intervals was calculated by the formula described by Verhoef et al. (21).

**Electron microscopy.** PAMs exposed to *P. hemolytica* were processed for examination by transmission electron microscopy as previously described by Kishimoto et al. (12). For comparison, unexposed PAMs were processed simultaneously. All preparations were examined with a Zeiss EM 95-2 electron microscope.

**Statistics.** Analysis of the differences between means was performed by analysis of variance of a two-way classification.

## RESULTS

**Serology.** Fetal calf serum did not contain any antibodies against *P. hemolytica* as revealed by a zero indirect bacterial agglutination titer. The normal adult bovine serum gave an indirect bacterial agglutination titer of 128. Prevaccinated calves gave titers of 4 against *P. hemolytica*. Calves vaccinated with either a live vaccine or bacterin and sera collected 2 weeks postimmunization had identical indirect bacterial agglutination titers of 512.

**Phagocytosis of *P. hemolytica*.** All experimental data on the kinetics of phagocytosis were based upon the mean of five replicates done with triplicate samples in each experiment.

To study the maximum uptake of *P. hemolytica* by PAMs, bacteria of different concentrations were opsonized for 30 min in 10% fresh normal serum and added to PAMs in bacteria-to-PAM ratios of 10:1, 20:1, 30:1, 40:1, 50:1, and 80:1. Phagocytosis mixtures were constituted, and the uptake of the bacteria by PAMs was measured at 30 min. Maximal uptake of bacteria took place at bacteria-to-PAMs ratios (data not shown) of 10:1, 20:1, and 30:1. All further assays were carried out at a bacteria-to-PAMs ratio of 20:1.

After opsonization of the *P. hemolytica* for 30 min in 10% fresh normal serum or antiserum against bacterin or fetal calf serum, phagocytosis mixtures were constituted and uptake of PAMs was measured at 30 min. Figure 1 shows that, in the absence of serum or fetal calf serum, the bacteria were poorly phagocytized (18 to 20%). When normal serum was used as the opsonic source, there was 90% phagocytosis. However, 95% of the organisms were phagocytized when antiserum was used as the opsonic source.

Figure 2 compares the results of normal serum and antiserum produced by a bacterin with their heat-inactivated counterparts. Approximately 94.6% uptake occurred when antiserum was used as the opsonin, and there was 89.8% uptake when normal serum was the source of opsonin. When their heat-inactivated counterparts were used as opsonic sources, the percent uptake were 86.2% and 82.6%, respectively. The differences in the percent uptake of *P. hemolytica*, when normal serum or its heat-inactivated counterpart was used as the opsonin, was statistically significant

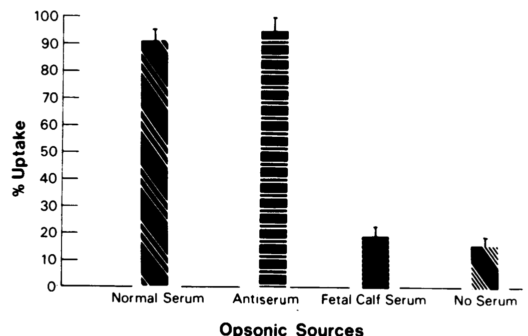


FIG. 1. *Phagocytosis of P. hemolytica by PAMs after opsonization for 30 min in 10% normal bovine serum, antiserum or fetal calf serum. The uptake by the PAMs was determined at 30 min. Each vertical bar is the mean ± standard error of the mean of five experiments.*

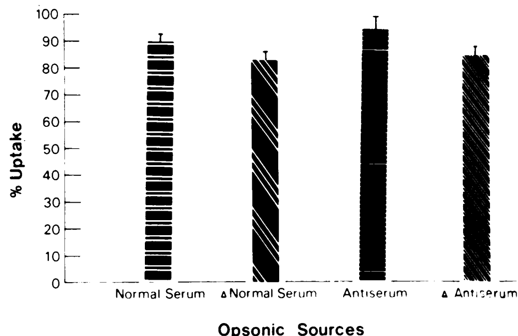


FIG. 2. *Effect of various opsonic sources on phagocytosis of P. hemolytica. The bacteria were opsonized for 30 min in 10% normal bovine serum, antiserum, or their heat-inactivated counterparts (Δ). The uptake by PAMs was determined at 30 min. Each vertical bar is the mean ± standard error of the mean of five experiments.*

($P < 0.05$). Likewise, the differences in the percent uptake, when antiserum or its heat-inactivated counterpart was used as the opsonin, was also statistically significant ($P < 0.025$).

To study the effect of opsonization time on the percent of the organisms phagocytized, bacteria were incubated for 15-, 30-, 45-, and 60-min periods in normal serum or heat-inactivated serum before phagocytosis mixtures were constituted. Uptake was measured after 30 min of incubation. Maximal uptake took place after 15 min or more of incubation (data not shown). For convenience, a 20-min opsonization period was used for all further experiments.

For studies on the optimal time needed for phagocytosis, *P. hemolytica* were opsonized for 20 min with normal serum, mixtures were constituted, and uptake by PAMs was measured at 0, 5, 10, 15, and 25 min. In this experiment, tubes containing phagocytized mixtures were removed at 5-min intervals and placed in an ice bath until the last 25-min tube was completed. All samples were then processed at the same time for determination of percent phagocytosis. Zero minutes represented the results obtained after adding opsonized bacteria to PAMs and immediately placing the mixture in an ice bath. Results showed (Fig. 3) that optimal uptake (88%) took place after 10 min or more of incubation. A 50% uptake indicates actual uptake and no background counts. All further studies on uptake were done after 15 min of incubation. It should be emphasized that this assay does not distinguish between phagocytosis and adherence to the PAMs; however, the TEM studies have shown that uptake is always associated with phagocytosis.

**Fate of ingested *P. hemolytica*.** The fate of ingested *P. hemolytica* in PAMs was studied by two methods. In these experiments *P. hemolytica* was opsonized for 20 min in 10% normal serum or antiserum produced by a bacterin or live vaccine, or their heat-inactivated counterparts, were exposed to PAMs at a ratio of 20:1 (bacteria/PAM). At 5, 30, 60, 90, 120, 150, and 180 min time intervals, duplicate samples were taken from the mixture: one was processed for transmission electron microscopy, and the other was used to determine the percent killed bacteria by the viable plate count.

Ultrastructural studies of PAMs revealed fine structural characteristics in control preparations (Fig. 4). A nucleolus was observed in the deeply indented nucleus, and the chromatin was clumped around the nuclear periphery in an irregular dense band. The cytoplasm contained many phagocytic vacuoles, many of which were presumably pinocytotic. The contents of larger phagocytic vacuoles contained membrane profiles and myelin-like configurations which suggested that they may be secondary lysosomes, such as residual bodies. Other organelles identified in the cytoplasm were pleomorphic lysosomes, sparse rough endoplasmic reticulum, abundant mitochondria, numerous free ribosomes, and a prominent Golgi apparatus.

The results described below from transmission electron microscopy studies and the subsequent conclusions drawn in this study were based upon the mean of four replicates for each sample time. A total of 200 PAMs together with unphagocytised bacteria from different fields were carefully counted. Examination of ultrathin sections by

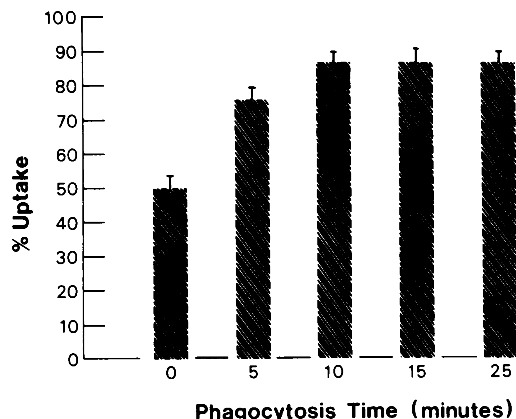


FIG. 3. *Influence of time of phagocytosis on the percent uptake of P. hemolytica by PAMs. The bacteria were opsonized for 20 min in normal bovine serum, and the uptake by PAMs was determined at 0, 5, 10, 15, and 25 min. Each vertical bar is the mean ± standard error of the mean of five experiments.*

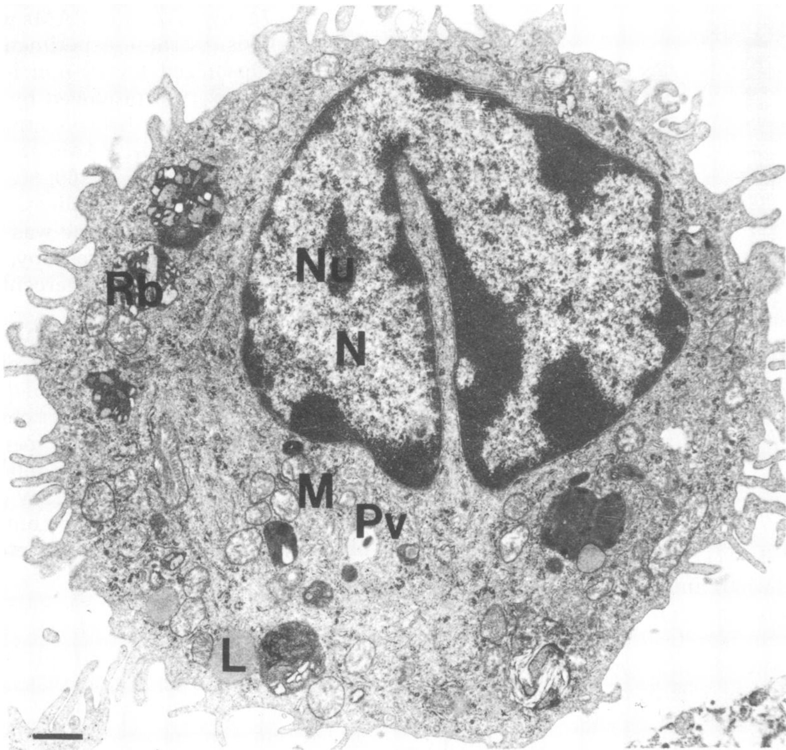


FIG. 4. *An electron photomicrograph of a typical bovine PAM. N, Nucleus; Nu, nucleolus; L, primary lysosomes; Rb, residual bodies; Pv, phagocytic vacuoles; M, mitochondria, ×6,919. Bar = 1 μm.*

transmission electron microscopy revealed that normal serum or antiserum-opsonized *P. hemolytica* were ingested by the PAMs immediately after exposure. After 30 min of exposure, most of the bacteria were found inside the phagosomes of PAMs and were found in various stages of degradation (Fig. 5). Many degraded bacteria showed condensation of the cytoplasmic material, and in some the cytoplasm was entirely absent. There were also abundant cellular debris and remnants of cell wall fragments inside some phagosomes. A number of unphagocytized bacteria were also found extracellularly (10%). After 60 min of exposure, all bacteria inside the phagosomes of PAMs were completely degraded (Fig. 6). Very few intact or identifiable bacteria were seen. These results were the same regardless of the source of serum used to opsonize the bacteria. A small percentage of the PAMs (<10%) showed cytotoxic changes after 30 min of exposure. After 60 min of exposure, about 30% of the PAMs showed cytotoxic changes when compared with the numbers in control preparations. After 90 min of incubation, almost all PAMs showed drastic cytotoxic changes with evidence of rupture (Fig. 7). The cytotoxic changes seen in the PAMs were loss of cytoplasmic ground substances, less electron-dense cytoplasm, and less electron-dense and evenly dispersed chromatin material in the nucleus.

When the ratio of bacteria to PAMs was reduced to 10:1 or less and the mixtures were incubated up to 180 min, all organisms were completely phagocytized and degraded. Furthermore, the PAMs showed no evidence of cytotoxic changes. These results suggested that with a high bacteria-to-PAM ratio (20:1 or more), excess bacteria which were not phagocytized by the PAMs may have secreted toxic substances which were responsible for causing cytotoxic changes on the PAMs.

Results from transmission electron microscopy studies were complemented by studying the fate of ingested bacteria by the viable plate count. Data generated from these studies showed that a high percentage (85 to 92%) of the organisms were killed inside the PAMs at 60 min or more exposure (data not shown). This observation was the same regardless of the source of serum used to opsonize *P. hemolytica.*

## DISCUSSION

*P. hemolytica* is an important etiological agent in the bovine respiratory disease complex and is responsible for pneumonia and death in cattle affected with this syndrome. In this study, the

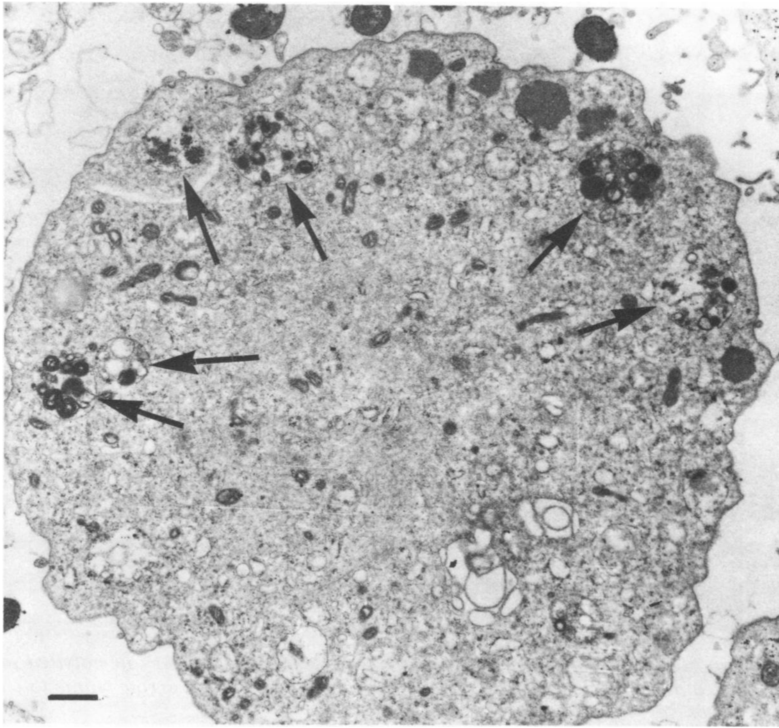

FIG. 5. *Electron photomicrograph of a PAM exposed to antiserum-treated P. hemolytica for 30 min. Numerous bacteria are in various stages of degradation inside phagosomes. The arrow points to bacteria undergoing degradation inside the phagosome. Several unphagocytized bacteria are also seen. ×5,860. Bar = 1 μm.*

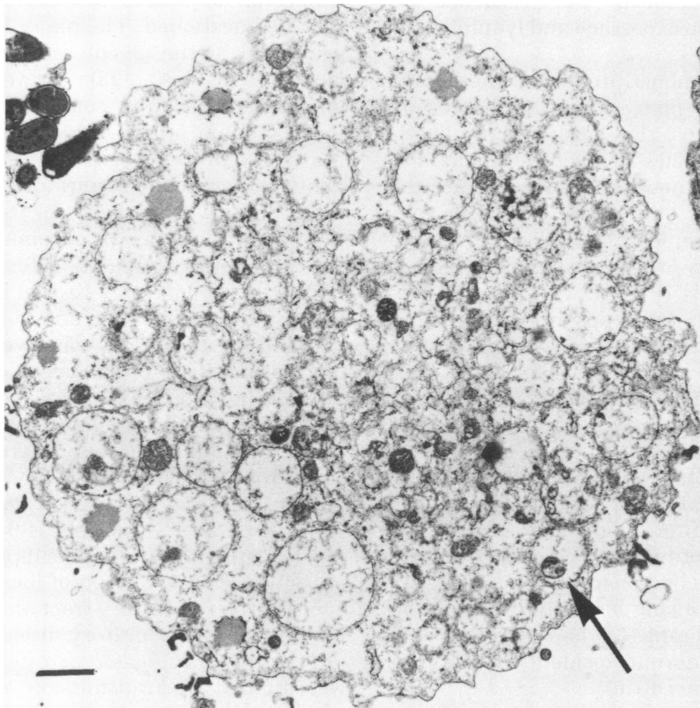

FIG. 6. *An electron photomicrograph of a PAM exposed to antiserum-treated P. hemolytica for 60 min. The PAM is filled with phagosomes containing bacteria which are almost completely degraded (arrow). The PAM also shows cytotoxic changes characterized by a complete loss of cellular organization in the cytoplasm. A few unphagocytized bacteria are also seen in the lower right-hand corner. ×6,919. Bar = 1 μm.*

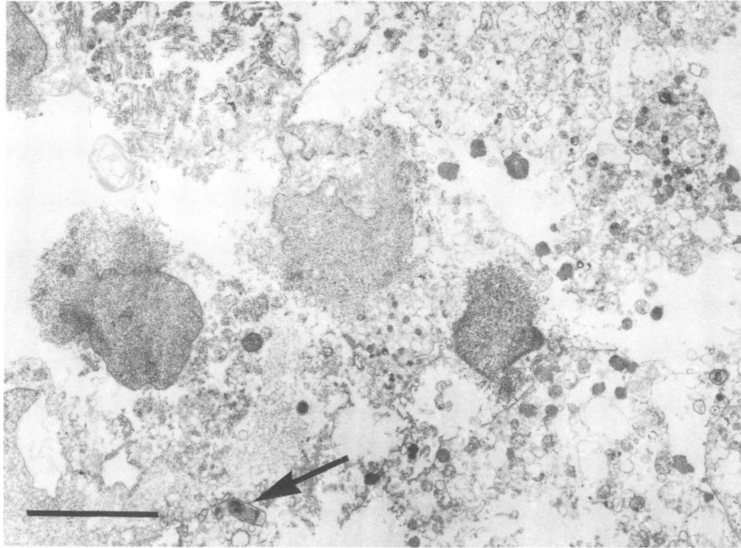

FIG. 7. *Electron photomicrograph of PAMs exposed to antiserum-treated P. hemolytica for 90 min. All PAMs appear to have undergone drastic cytotoxic changes and rupture. An arrow points to one unphagocytized bacterium. ×3,420. Bar = 5 μm.*

phagocytosis and killing of *P. hemolytica* by bovine PAMs were evaluated. The experiments performed involved the utilization of a virulent *P. hemolytica* organism. The virulence was maintained by mouse passage and lyophilization (4).

Our studies demonstrated that these organisms were poorly phagocytized in the absence of opsonin. Perhaps, the capsule found on the surface of this species of bacterium was antiphagocytic as has been demonstrated in other species of bacteria (19). However, in the presence of opsonins found in normal serum of adult cattle or antiserum, they were avidly phagocytized by the PAMs (Fig. 1). These studies also showed that heat-stable opsonins (antibodies) found in normal serum and antiserum were relatively more important than heat-labile opsonins (complement) in the phagocytosis of *P. hemolytica*. Furthermore, the findings that these bacteria were almost completely phagocytized (82.6% uptake) in the presence of the heat-stable opsonin found in normal serum suggests that serum from adult bovines contained antibodies (indirect bacterial agglutination titer of 64) against the components of *P. hemolytica*. This was not surprising because previous work by Hamdy and Trapp (7) had shown that *P. hemolytica* were normal residents in the nasal passages of healthy cattle.

A fourfold increase in agglutinating titer was noted with hyperimmune sera as compared to the normal adult bovine serum. In spite of this, no significant difference was apparent in opsonizing capability with phagocytosis as an indicator. The implications of this are speculative. The biological validity of using serum as a source of opsonin for phagocytes of the respiratory tract may be questioned. The major immunoglobulin generated in the alveoli is secretory immunoglobulin A (IgA) (23). However, significant amounts of IgG and complement components are also found in the alveoli. The presumption that these proteins originate as plasma components and enter the respiratory system by transudation provides a basis for using serum from peripheral blood as an opsonin source. Therefore, a possible explanation for the lack of significant difference may rest on the class or subclass of immunoglobulin stimulated by systemic vaccination. It is quite conceivable that the biological property of the specific antibody may only be in serological reactions and not in the opsonizing reactions. Alternatively, the lack of significant difference may merely indicate the saturation point of the assay system since the level of phagocytosis seen when hyperimmune serum was used as opsonin is 94 to 95%. These results and the questions support the need to investigate the induction of local sources of opsonin in the respiratory tract and what role they play in bovine respiratory disease.

The fate of *P. hemolytica* ingested by PAMs was studied by transmission electron microscopy, and the percentage of the organisms killed was determined by the viable plate count. Our electron microscopic observations confirmed that *P. hemolytica* entered the PMAs only by

phagocytosis since all bacteria were found in the cytoplasm inside a phagosome (Fig. 5). Furthermore, these studies also indicated that all *P. hemolytica* that were phagocytized by the PAMs were completely degraded 60 min after ingestion. This study also showed that when the ratio of bacteria to PAM was 20:1, approximately 10% of the bacteria were not phagocytized and that prolonged incubation of this mixture resulted in cytotoxic changes of the PAMs (Fig. 7). However, if the PAMs were exposed to bacteria at a ratio of 10:1 (bacteria/PAM) or less and incubated up to 180 min, all organisms were completely phagocytized and degraded, and no cytotoxic changes on the PAMs occurred. These findings indicated that the excess bacteria that were not phagocytized by the PAMs at this higher bacteria/PAM ratio (>10:1) released substances which were cytotoxic to PAMs. These results are consistent with the observations of Benson et al. (2), who found that live bacteria and culture supernatants, and not heat-killed *P. hemolytica*, were cytotoxic to bovine PAMs. More recently, Markham and Wilkie (16) confirmed the above findings and found that this phenomenon was not observed with *Yersinia entercolitica*.

The PAMs are the resident mononuclear phagocytes of the lung and function in the primary defense against bacteria and other particulate matter which reaches the lung (11). This may be very complex. *P. hemolytica* is part of the normal flora of the nasopharynx of cattle and exists for long periods of time without apparent harm to the host (7). Overt disease (pneumonia) may only occur after exposure to various compromising factors (environmental or viruses) which affect the bovine respiratory system (13). The question arises as to how *P. hemolytica* multiply in the nasopharynx and migrate to the lung, because they are not part of the normal lung burden. In experimental mice (22), various compromising factors, such as viruses, have been shown to have injurious effects on the mucociliary apparatus and impair phagocytic functions of the PAMs. In cattle it has been shown that the various compromising factors damage the pulmonary tissues and mucociliary cleansing mechanisms, thus permitting establishment of *P. hemolytica* to produce a bronchopneumonia (20). Therefore, the maintenance of large numbers of PAMs to handle *P. hemolytica* which enter the lung is very important to the host. Our in vitro results indicate that excess bacteria cause cytotoxicity and killing of PAMs. *P. hemolytica* is capable of multiplying so rapidly in the alveoli that the normal pulmonary defenses by PAMs may be overwhelmed by the toxic factor produced by excess bacteria. Since the PAMs are the primary elements in the lungs' cellular defense against inhaled microorganisms and other particles, a substance(s) produced by *P. hemolytica* which is cytotoxic to PAMs should be considered as a potential pathogenic mechanism of infectivity.

These findings may have practical implications in the control of pasteurella pneumonia in cattle. For example, it is quite conceivable that PAMs may be activated by lymphokines or immunomodulators such as levamisole (1) and that such activated PAMs could phagocytize more bacteria and thus prevent this cytotoxicity phenomenon. Another consideration in immunity to pneumonic pasteurellosis is the potential role of other protective mechanisms which may be present in the lungs of cattle. For example, the phagocytic role of lung neutrophils in the presence of local opsonizing antibody found in the alveolar secretions should not be ignored. It is quite possible both PAMs and neutrophils (as effector cells) and the opsonizing and bactericidal effects of alveolar secretions may act together in destroying *P. hemolytica* in immune cattle. Also, identification of the factor from *P. hemolytica* that is cytotoxic to PAMs may shed light on the pathogenesis of the disease. Any attempts at disease prevention should focus on the development of an immunoprophylactic agent that will generate a pulmonary defense mechanism involving PAMs, neutrophils, and bronchial secretions, which will work together in destroying the *P. hemolytica* long before they exert their cytocidal effect on PAMs.


## ACKNOWLEDGMENT

This study was supported by grant 63-031 from the U.S. Department of Agriculture.


## LITERATURE CITED


1. **Al-Ibrahim, M. S., R. S. Holzman, and H. S. Lawrence.** 1977. Concentrations of levamisole required for enhanced proliferation of human lymphocytes and phagocytosis by macrophages. J. Infect. Dis. **135**:517-523.
2. **Benson, M. L., R. G. Thomson, and V. E. O. Valli.** 1978. The bovine alveolar macrophage. II. In vitro studies with *Pasteurella hemolytica*. Can. J. Comp. Med. **42**:368-369.
3. **Carter, G. R.** 1956. Some remarks on shipping fever in Canada. Can. J. Comp. Med. **20**:289-293.
4. **Evans, H. B., and P. W. Wells.** 1979. A mouse model of *Pasteurella hemolytica* infection and its use in assessment of the efficacy of *P. hemolytica* vaccines. Res. Vet. Sci. **27**:213-217.
5. **Frank, G. H., and G. E. Wessman.** 1978. Rapid plate agglutination procedure for serotyping *Pasteurella hemolytica*. J. Clin. Microbiol. **7**:142-145.
6. **Friend, S. C. E., B. N. Wilkie, R. G. Thompson, and D. A. Barnum.** 1977. Bovine pneumonic pasteurellosis: experimental induction in vaccinated and non-vaccinated calves. Can. J. Comp. Med. **41**:77-83.
7. **Hamdy, A. H., and A. L. Trapp.** 1967. Investigations of

nasal microflora of feedlot calves before and after weaning. Am. J. Vet. Res. **28**:1019–1025.

8. **Ho, C. K., and L. A. Babiuk.** 1979. Long-term culture of canine peripheral blood monocytes in vitro. Can. J. Comp. Med. **43**:223–228.
9. **Holt, P. G.** 1979. Alveolar macrophages. I. A simple technique for the preparation of high numbers of viable alveolar macrophages from small laboratory animals. J. Immunol. Methods **27**:189–198.
10. **Jericho, K. W. F., S. E. Magwood, and P. H. G. Stockdale.** 1976. Prevention of experimental pneumonic pasteurellosis by exposure to infectious bovine rhinotracheitis virus. Can. Vet. J. **17**:194–195.
11. **Kaltreider, H. B.** 1976. Expression of immune mechanisms in the lung. Am. Rev. Respir. Dis. **113**:347–379.
12. **Kishimoto, R. A., B. J. Veltri, P. G. Canonico, F. G. Shirey, and J. S. Walker.** 1976. Electron microscopic study on the interaction between guinea pig peritoneal macrophages and *Coxiella burnetti*. Infect. Immun. **14**: 1087–1096.
13. **Lillie, L. E.** 1974. The bovine respiratory disease complex. Can. Vet. J. **15**:233–242.
14. **Lopez, A., R. G. Thomson, and V. E. O. Valli.** 1976. The pulmonary clearance of *Pasteurella hemolytica* in calves infected with bovine parainfluenza-3 virus. Can. J. Comp. Med. **40**:385–391.
15. **Maheswaran, S. K., and E. S. Thies.** 1979. Influence of encapsulation on phagocytosis of *Pasteurella multocida* by bovine neutrophils. Infect. Immun. **26**:76–81.
16. **Markham, R. J. F., and B. N. Wilkie.** 1980. Interaction between *Pasteurella hemolytica* and bovine alveolar macrophages. Cytotoxic effect on macrophages and impaired phagocytosis. Am. J. Vet. Res. **41**:18–22.
17. **Nathan, C. F., R. Asofsky, and W. D. Terry.** 1977. Characterization of the nonphagocytic adherent cell from the peritoneal cavity of normal and BCG-treated mice. J. Immunol. **118**:1612–1621.
18. **Smith, G. R.** 1961. The characteristics of two types of *Pasteurella hemolytica* associated with different pathological conditions in sheep. J. Pathol. Bacteriol. **81**: 431–440.
19. **Smith, H.** 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. **41**:475–500.
20. **Stockdale, P. H. G., E. V. Langford, and C. le Q. Darcel.** 1979. Experimental bovine pneumonic pasteurellosis. I. Prevention of the disease. Can. J. Comp. Med. **43**:262–271.
21. **Verhoef, J., P. K. Peterson, and P. G. Quie.** 1977. Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: a quantitative assay using ($^3$H) thymidine-labeled bacteria. J. Immunol. Methods **14**:303–311.
22. **Warshauer, D., E. Goldstein, T. Akers, W. Lippert, and M. Kim.** 1977. Effect of influenza viral infection on the ingestion and killing of bacteria by alveolar macrophages. Am. Rev. Respir. Dis. **115**:269–277.
23. **Wilkie, B. N., and R. J. F. Markham.** 1979. Sequential titration of bovine lung and serum antibodies after parenteral or pulmonary inoculation with *Pasteurella hemolytica*. Am. J. Vet. Res. **40**:1690–1693.