AN ABSTRACT OF THE THESIS OF

Eva Andrea Wallner-Pendleton for the degree of Master of Science in Veterinary Medicine presented on November 5, 1987.

Title: The Early Pathogenesis of Pasteurella Multocida Infection Studied by Immunohistochemical Techniques.

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Initial sites of localization and multiplication of Pasteurella multocida were examined in chickens intravenously inoculated with varying doses of bacteria. Sections of lung, liver, kidney, bone marrow and spleen were prepared for light microscopic examination. Tissue sections were stained with the hematoxylin and eosin stain, Gram's stain or the avidin-biotin-peroxidase complex immunoenzymatic technique. Of the three, immunohistochemistry proved to be far more sensitive in localizing bacteria within the various organs. As early as five hours postinoculation, bacteria were evident in low numbers in splenic and hepatic mononuclear phagocytes. Bacteria were not observed within the other organs or the blood. With increasing time, bacteria were observed in large numbers within these cells, causing necrosis of phagocytes, and liberating bacteria into the sinusoids. In birds succumbing to the infection, bacteria were seen in large numbers in all organs and in the blood, both intracellularly and extracellularly. These results are consistent with the hypothesis that P. multocida multiplies within hepatic and
splenic phagocytes in an early phase of infection. The possibility that *Pasteurella multocida* may be able to resist intracellular killing and may in fact multiply within the mononuclear phagocyte is discussed.
The Early Pathogenesis of Pasteurella Multocida Infection Studied by Immunohistochemical Techniques

by
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Typed by for Eva Andrea Wallner-Pendleton
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I wish to thank my husband, Ken, for continual support, faith, and love throughout these years, and baby George, for much happiness despite trying times.

I wish to dedicate this thesis to Dr. George Wallner, my father, who taught me the love of veterinary medicine.
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The Early Pathogenesis of *Pasteurella Multocida*

Infections Studied by Immunohistochemical Techniques

Chapter I

Literature Review
**Pasteurella multocida**

*Pasteurella multocida* is a bacterial pathogen responsible for many diseases in cattle, swine, wild and domestic birds, and rabbits. Infections in humans are occasionally observed secondary to animal bites (9,11,28,31,49). Diseases caused by this agent include acute septicemia, bronchopneumonia, rhinitis, and chronic localized infections (49). In birds, the acute and chronic forms of the disease have been collectively named "fowl cholera." The disease has been recognized since the 1700's. In 1800, Louis Pasteur was the first to isolate the organism and develop one of the first vaccines against the disease (47). Despite its long history, a satisfactory vaccine has yet to be developed, and more information is needed concerning the pathogenesis and immune mechanisms of this infection.

*Pasteurella multocida* has been classified as a Gram-negative, facultatively anaerobic, nonmotile, nonsporeforming, rod-shaped bacteria. Recently isolated organisms show bipolar staining properties with the Giemsa, Gram's or Wright's stains. *Pasteurella multocida* grows readily on sheep blood agar plates at 37°C as smooth, gray-white colonies with no hemolysis. The organism does not grow on MacConkey's agar. Isolates usually produce oxidase, catalase, and peroxidase and are indol-positive. No H₂S, gas, or urease are produced in appropriate media (47).

Encapsulated organisms will show characteristic iridescence under obliquely transmitted light. Nonencapsulated organisms show blue or gray colonies under the same light. In addition, colonies
may dissociate, that is, go from highly iridescent to blue or gray on subculture in artificial media. This may result in a decrease in virulence of that organism for its host species (22).

P. multocida isolates have also been grouped according to a variety of serologic and immunologic tests. Little and Lyon separated P. multocida isolates into three groups according to slide agglutination and passive protection tests in mice (30). Carter, using the indirect hemagglutination test, found four serotypes based on capsular polysaccharides (8). Namioka and Murata (37) defined somatic serotypes by the use of decapsulated organisms. Heddleston devised the gel diffusion precipitin test and distinguished 16 serotypes based on heat stable antigens (17).

Fowl cholera is a disease of major economic importance to the poultry industry. The disease can be particularly devastating in turkey and waterfowl populations where peracute to acute septicemias may result in the loss of much of the flock in a short period of time (28). In natural infections, the chicken appears to be more resistant with chronic infections more common (47). Transmission occurs through bird to bird contact, possibly by direct exposure through: chronic carrier birds, scratches or puncture wounds, contaminated food or drinking water (4,27,39,42). Wild birds, insects, and mammals are capable of carrying the organism and introducing the infection into a flock (17).

In acute fowl cholera, few signs may be observed initially, followed by anorexia, diarrhea, cyanosis, fever, and ultimately death. The mortality and morbidity often escalate exponentially
In chronic fowl cholera, morbidity and mortality tend to be lower. Depending on the site of bacterial localization, signs may include swelling of the wattles, torticollis, lameness, sinusitis, ophthalmia or respiratory signs from pneumonia (38). Gross necropsy lesions in acute fowl cholera are typical of septicemia and include pinpoint necrotic foci in the liver, petechial hemorrhages on serosal surfaces of the heart, lung, spleen, as well as other organs, and generalized venous congestion (28, 46). Acute foci of coagulation necrosis are seen in the liver and spleen. Heterophil infiltrates are present in most organs, especially the liver and spleen. Acute fibrinoid necrosis is present within arterioles in the spleen with accompanying necrosis of RE and lymphoid cells. Heterophil depletion has been described in the bone marrow with necrosis of hemopoietic cells. Bacteria are observed in high numbers in many organs, especially in blood vessels and hepatic sinusoids. Disseminated intravascular coagulation resulting in fibrin thrombi in the liver, lung, spleen and kidney has been reported (41).

Pathogenesis of Pasteurella multocida infections: Bacteria are present in the nasal and conjunctival secretions of infected birds (27, 42). The organism spreads via the aerosol route or by consumption of contaminated feed or drinking water (39). By some unknown mechanism, bacteria are thought to adhere to the mucosa of the upper respiratory tract then enter the blood stream resulting in septicemia (33). Virulent strains of the bacteria then multiply freely within infected tissues and blood in susceptible animals.
Large amounts of endotoxins are released in the process, resulting in death. This theory of pathogenesis is supported by the observations that birds are readily infected with live vaccine strains administered via the drinking water and endotracheal route (5,33). Birds appear to be refractory to infection when bacteria are administered directly into the lower gastrointestinal tract (27). The choanal slit swabbing method is also an effective means of experimentally reproducing the disease. However, it is possible that bacteria are inadvertently introduced directly into the bloodstream because of microtrauma produced on the choanal mucosa. Animals can also become infected experimentally via intramuscular, intraperitoneal, intravenous, and subcutaneous injection. Septicemia and death often ensue when virulent organisms are injected parentally into susceptible animals (11).

**Virulence factors associated with Pasteurella multocida infections in avians:** The phenomenal rate in which P. multocida multiplies in infected hosts, and its ability to evade host defenses is poorly understood. To date, significant exotoxins have not been reported in avian pasteurella infections. It has been postulated that the presence of a hyaluronic acid capsule might prevent phagocytosis and uptake of bacteria into phagocytic cells and allow the bacteria to multiply in the blood as if in liquid media (11,34). However, research by Pabs-Garnon and Soltys (40), and Tsuji and Matsumoto, (53) suggest that the organisms first localize in the liver and spleen and re-enter the blood in later stages of the disease. Recent experiments by Tsuji and Matsumoto
further suggested that encapsulation had little effect on bacterial uptake by phagocytic cells. However, they observed differences in organism survivability within phagocytes when comparing encapsulated and unencapsulated strains (53).

Besides the hyaluronic acid capsule and endotoxin, little is known about other virulence factors of this organism. Resistance plasmids have been studied by Hirsh et al. (24), which confer antibiotic resistance to the organism to a variety of antimicrobials. These R-plasmids were found to be nontransmissible and not widely dispersed in nature. Borisenkova (6) examined the production of hyaluronidase and neuraminidase in several high and low virulent strains of *Pasteurella multocida* isolated from hens, ducks, and turkeys, including an avirulent vaccine strain. In this study, a direct correlation was found between levels of bacterial hyaluronidase and virulence; however, the neuraminidase levels remained the same in all strains. Hyaluronidase would allow for rapid spread of bacteria in intercellular spaces, increasing invasiveness of the organism and rapid bacteremia. The neuraminidase might play a role in bacterial adherence at the site of initial infection. It was concluded by the author that hyaluronidase and neuraminidase may both play an important role in pathogenesis of cholera infection.

Iron is an essential nutrient for many parasitic microorganisms. Some bacteria produce complex iron-binding molecules, called siderophores, from which to obtain iron from the host. Siderophore production has been observed by Hu et al. (26) in
Pasteurella multocida organisms isolated from turkeys which died from fowl cholera. Since iron is important in bacterial macromolecular synthesis, it has been hypothesized that reduction of plasma iron may be an important part of the host's defense against bacterial infections, including fowl cholera (26).

Resistance factors of the host: The roles of humoral and cell-mediated immunity in Pasteurella multocida infection in poultry have not been well studied. Major emphasis of research has been placed on vaccine production and humoral immune response. Some, though not always, linear correlations are observed between antibody titers and protection. Antibody titers can be measured using several accepted serological tests including agglutination tests or the enzyme-linked immunosorbent assay (ELISA) (1,7,14,18,20).

Multivalent, inactivated vaccines have been shown to significantly protect immunized hosts when challenged with homologous strains of Pasteurella multocida, but field outbreaks continue to occur despite immunization (21,36). Live, attenuated, and naturally-occurring avirulent strains have been used parentally or in the drinking water (4,5,20,35). Some cross-protection has been observed with these vaccines against different serotypes. Unfortunately, significant mortality directly attributed to the use of live vaccines has been occasionally observed. Inactivated bacterins produced in vivo in tissues or embryos have been shown to induce some cross-protection in the same hosts. The mechanism for this cross-protective immunity is unknown (19,44). Several antigens have been isolated from Pasteurella multocida in avians and tested
for their immunogenicity in the hopes of producing subunit vaccines. Capsular polysaccharides have been isolated from turkeys by Maheswaran (33) and identified as being composed of primarily hyaluronic acid. A crude capsular antigen isolated through saline extraction by Kodama et al. (29), proved to be immunogenic in turkeys; however, the purified polysaccharide antigen was not. Polysaccharide-protein complex antigens have some immunogenicity which is lost following treatment with phenol. In addition, some protective immunity is stimulated by potassium thiocyanide extracted antigens. Many other antigens can be detected when pasteurella organisms are sonicly disrupted, but their identification and immunogenic potential have yet to be determined (1). It has been assumed that both killed and live vaccines protect the host through stimulation of the humoral immune system. Live vaccines may stimulate both local and systemic antibody production. It is not known whether these antibodies function as antitoxic, opsonic, complement activating, or perhaps serve some other immune function. Sensitivity to complement has been suggested by some to be an important nonspecific factor in host resistance to infection with fowl cholera. Snipes and Hirsh (51) demonstrated that P1059-1A, an avirulent fowl cholera strain, was more sensitive to a heat-labile substance in the serum than its virulent counterpart P1059-1. This heat-labile substance was most likely complement according to the authors. They concluded that the lipopolysaccharide configuration may be different between the two strains, resulting in differences in the activation potential of complement.
Cellular Immunity: The primary cellular response in *P. multocida* infections has been described as polymorphonuclear in nature (11,46). Little, if any, mononuclear cell component has been noted in the initial inflammatory cell response. The role of the heterophil in the pathogenesis and natural immunity against *Pasteurella multocida* infections in poultry is poorly understood. Studies by Snipes et al. (50) indicated *in vitro* the ability of mononuclear phagocytes to engulf and kill a virulent encapsulated *Pasteurella multocida* strain in turkeys and its avirulent variant. In those experiments, macrophages were able to phagocytose some bacteria (one to three percent), though not very well. Most phagocytized bacteria were destroyed. No differences were observed between the virulent and avirulent strain regarding phagocytosis. In another experiment by the same authors, an *in vivo* clearance was performed with the same two strains. The virulent encapsulated strain showed increased propensity for multiplication in the blood, liver, and spleen when compared with the avirulent strain. They postulated that the virulent strain may be able to multiply more effectively in blood, resisting humoral bactericidal defenses of the host (50).

Cell-mediated immunity was demonstrated *in vitro* against *Pasteurella multocida* by Maheswaran et al. (35), via the lymphocyte stimulation assay which is based on the principle that mitosis of sensitized lymphocytes occurs three to five days after contact with an antigen. This blastogenesis was shown to be antigen specific. A similar experiment by Dua and Maheswaran (14) measured both humoral
and cell-mediated immunity by the passive hemagglutination test and the lymphocyte stimulation assay. Both humoral and cell-mediated immunity was induced following vaccination with different strains of *P. multocida* although the immunity did not persist for equal lengths of time among animals vaccinated with different strains. Baba (2) demonstrated that cell-mediated immunity was transferable from immunized to normal chickens by the transfer of culture supernatent from previously sensitized spleen cells. Peritoneal macrophages from immunized hosts or macrophages sensitized with culture supernatant fluid from immune spleen cells did not allow intracellular proliferation of bacteria while normal macrophages allowed considerable intracellular proliferation. Baba et al. (3), in a previous experiment showed that surgical thymectomy had a more deleterious effect on the survival rate of chickens challenged with *Pasteurella multocida* than did hormonal or surgical bursectomy. However, in a subsequent experiment with turkeys, Schlink and Olson suggested that survival rate was negatively influenced by bursectomy in young turkeys as well (48). This effect was less important with increasing age of the birds, presumably because of the presence of secondary centers of antibody-producing cells of bursal origin scattered throughout the body. It appears likely that both cell-mediated and humoral immunity may play important roles in protecting poultry against *Pasteurella multocida* infection.
Immunohistochemistry

Immunohistochemistry is a subclass of histochemistry, which is the study of the chemical composition of tissues or cells without the disruption of morphology. Immunohistochemistry combines the principles of histochemistry and the immunologic specificity of the antigen-antibody reaction. Antibody can be labeled (i.e., conjugated) with enzymes or fluorescent markers to specifically locate antigens in histologically-prepared tissue sections. The location of the antigen-antibody complex may then be demonstrated with the addition of a substrate (enzyme histochemistry) or by the presence of fluorescence (immunofluorescent histochemistry). Both techniques are widely used in diagnostic pathology and research (55).

**Immunoperoxidase histochemistry:** Horseradish peroxidase has gained widespread use in enzyme histochemistry because of its relatively low endogenous level in most tissue samples. The indirect immunoperoxidase conjugated method is a classic example of this technique and will be used to illustrate this method.

First, a tissue section, which contains the antigen in question, is prepared histologically. Primary antibody prepared in species B and directed against the antigen is added to the tissue section. Secondary antibody, produced in species C and directed against species B immunoglobulin, is next added to the slide. This secondary antibody is also labeled with peroxidase enzyme. Finally, the addition of the substrate chromogen chemical produces a colored end product. In this example, the substrate is hydrogen peroxide.
and the chromogen is 3,3'-diaminobenzidine (usually referred to as DAB). In this reaction, oxygen is liberated, oxidizing the DAB and forming an insoluble brown end product at the site of antigen localization (12). See Figure 1.

**Avidin: Biotinylated Horseradish Peroxidase Complex Technique (ABC technique):** A modification of the immunoperoxidase procedure has been developed employing avidin and biotin. The technique is based on the fact that avidin has very high affinity for biotin. The two substances form an essentially irreversible complex. Avidin has four binding sites for biotin. Biotin can be easily conjugated with enzymes, hence the term biotinylation. The resulting complex of biotinylated enzyme and avidin has been termed "ABC."

The procedure involves incubating the antigen-containing tissue section with primary antiserum, prepared the same way as in the previously described immunoperoxidase technique. A secondary antibody, directed against the species in which the primary antibody was produced, is then added to the slide. This secondary antibody is labeled with biotin. The avidin biotinylated horseradish peroxidase is next added to the tissue section. Finally, the hydrogen peroxide-chromogen complex is added, resulting in a colored end product at the site of antigen localization in the tissue (25). See Figure 2.

**Advantages and Disadvantages to the ABC Technique:** The ABC technique is reported to be 40 times more sensitive than other immunoperoxidase techniques (25). Highly diluted amounts of primary antibody can be used, thus reducing the cost of production. There
is less background staining reported with this method. Routinely processed paraffin-embedded tissue sections, as well as tissue smears and frozen sections, can all be stained with this technique. These tissues may then be observed with an ordinary light microscope, as opposed to the fluorescent technique in which only frozen sections can be stained and fluorescent microscopy must be used. Once stained, the DAB slides can be stored for years without significant fading or deterioration. A wide variety of antigens may be detected with this technique including tumors, hormones, toxins, drugs, or infectious agents. The technique lends itself to ultrastructural examination as well. A number of commercially available kits have been developed containing standardized reagents including secondary antiserum produced in a variety of hosts (rabbit, goat, guinea pig, mouse, sheep, etc.). They also contain quenching solution (to block endogenous tissue peroxides), diluted normal serum (used as a blocking serum), and the ABC reagent. This greatly simplifies the technique and standardizes the test.

Disadvantages to this technique include some background staining occasionally observed. This may be due to high levels of endogenous peroxides or nonimmunologic binding of antisera to the tissue. This background staining is minimized by pretreating the tissue with 0.3% H₂O₂ in methanol, then by the addition of normal diluted serum to the tissue section. The normal serum is usually produced in the same species as the secondary antibody. Incubation times with the quenching solution or blocking serum can be varied
depending on the amount of background staining present. Non-specific staining can also be minimized by using the highest
dilution of primary serum that still stains well the antigen in
question. This dilution varies considerably and must be determined
for each antigen. The quality of the primary antiserum is also
important. Prompt fixation of the tissue is helpful in preserving
antigenic determinants and preventing diffusion of intracellular
antigen stores into extracellular spaces which may result in poor or
excessive staining (23).

The cost of the kits, and particularly the cost of producing
quality primary antiserum, can be high, especially if monoclonal
antibodies are used. Another disadvantage to the technique is the
suspected carcinogenicity of the chromogen substance. All necessary
precautions to prevent inhalation or skin contact should be used by
laboratory personnel (23).

As in all immunological tests, adequate controls are very
important in interpretation of results. A positive and a negative
control slide should be included. Ideally, they should both be
treated with positive antisera, and with negative preimmune sera to
test for specificity and the amount of background staining. The use
of absorption controls is advocated by some. The abolition of
positive staining after absorption of the antibody with the antigen
under test is considered by some to be the most sensitive test for
specificity. Other controls such as the use of DAB alone, or
omission of one or more reagents, or testing with primary antibody
produced against other antigens can also be tried (23).
Chapter II

In Vivo Multiplication of X-73 Strain Pasteurella multocida in Chickens
Inoculated Intravenously
Summary

Chickens were inoculated intravenously with X-73 strain *P. multocida* and portions of liver, spleen and blood were taken from euthanatized animals at regular intervals. These samples were examined for bacterial multiplication. The first four hours after inoculation, the bacteria were present in roughly equal numbers in the liver, spleen and blood. At eight hours, the liver and spleen contained 100 times and 10 times the original inoculum per gram of tissue respectively. No bacteria could be detected in the blood at this time. The number of organisms steadily increased between 12 and 18 hours postinoculation, peaking at $10^9$ number of organisms per gram of tissue.
Introduction

Fowl cholera is an acute or chronic septicemic disease affecting poultry and wild birds. In the acute form, organisms can be found widely disseminated in all organs and the blood at time of death. It is widely believed that the bacteria can freely multiply in the blood as if in artificial culture media, resulting in septicemia. However, research by Paps-Garnon and Soltys (40) in turkeys demonstrated that the bacteria first multiply in the spleen and liver, and reenter the blood during the latter half of infection. They concluded that Pasteurella multocida are probably released into the blood at this time, rather than directly multiplying intravascularly.

An in vivo clearance experiment by Snipes et al. (50) in turkeys inoculated intravenously with 1059-1 strain P. multocida demonstrated a similar, though much earlier, disappearance of organisms from the blood stream, with a steady increase of bacteria in the liver and spleen. They concluded that the disappearance and subsequent reappearance of bacteria was due to dilution of the organisms in the turkey vascular system rather than a specific removal by liver and splenic phagocytes.

The purpose of this experiment was to determine if multiplication of Pasteurella multocida in chickens with the X-73 strain occurs in a pattern similar to that observed in turkeys inoculated with other Pasteurella multocida strains.
Materials and Methods

Fourteen twelve-week-old specific pathogen-free male broiler chickens were inoculated with $1.9 \times 10^3$ colony-forming units of \textit{Pasteurella multocida} X-73 strain intravenously as determined by the standard plate dilution count method. Two birds from the group were bled, euthanatized via electrocution and examined at necropsy at the following intervals: one-half hour, four, eight, twelve, and eighteen and a half hours. The blood was collected into sterile heparinized vacutainer tubes. Samples of liver and spleen were collected separately into sterile plastic bags. The tissues were homogenized and serially diluted 10-fold with brain-heart infusion broth. Likewise, serial dilutions were made with the heparinized whole blood samples. Standard plate counts were performed to determine the colony-forming units per gram of tissue or milliliter of blood. Blood samples were not collected at 18½ hours as the birds were dead at this time.

Results

The results of the bacterial counts are summarized in Table 1.
Table 1. Concentrations of *P. multocida* in the liver, spleen and blood of chickens inoculated intravenously.

<table>
<thead>
<tr>
<th>No. of Birds</th>
<th>Hours PI</th>
<th>Liver$^a$</th>
<th>Spleen$^a$</th>
<th>Blood$^b$</th>
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<tr>
<td>2</td>
<td>½</td>
<td>0</td>
<td>4.5 $\times 10^2$</td>
<td>4.7 $\times 10^2$</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1.8 $\times 10^3$</td>
<td>4 $\times 10^2$</td>
<td>2 $\times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 $\times 10^3$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>6.6 $\times 10^5$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6 $\times 10^6$</td>
<td>2.3 $\times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1.0 $\times 10^8$</td>
<td>3 $\times 10^7$</td>
<td>1 $\times 10^7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1 $\times 10^6$</td>
<td>--</td>
<td>1 $\times 10^6$</td>
</tr>
<tr>
<td>2</td>
<td>18½</td>
<td>1.4 $\times 10^9$</td>
<td>1.8 $\times 10^9$</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.1 $\times 10^9$</td>
<td>2.4 $\times 10^9$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Reported per gram of tissue
$^b$Reported per milliliter of blood

Discussion

In this experiment, a steady increase in the numbers of bacteria per gram of tissue was observed with increasing time. Between four and eight hours, the organisms could not be detected in the blood, but were present in moderate numbers in the liver and spleen. This would suggest that the bacteria may have been cleared in these organs as has been hypothesized by others. In this study, a few birds were able to clear bacteria from the spleen between four and eight hours postinoculation while maintaining numbers in the liver. This may be due to more efficient killing in the spleen. Unfortunately, the small numbers of birds used in the experiment make interpretation of this data difficult. At 12 and 18 hours,
bacteria were present in high numbers in the liver, spleen and blood in all birds, suggestive of very rapid multiplication of the bacteria a few hours before death. This suggests that in vivo multiplication of *Pasteurella multocida* in chickens with X-73 strain occurs in a similar fashion as has been observed in turkeys inoculated intravenously with pathogenic strains.
Chapter III

The Early Pathogenesis of *Pasteurella multocida*

Infection Studied by Immunohistochemical Techniques
Summary

Initial sites of localization and multiplication of *Pasteurella multocida*, X-73 strain, were examined in chickens inoculated intravenously with varying doses of bacteria. Sections of lung, liver, kidney, bone marrow, and spleen were prepared for light microscopic examination. Tissue sections were stained with Gram's stain as well as the avidin-biotin peroxidase complex (ABC) technique. Immunohistochemical examination of tissues proved to be far more sensitive in demonstrating *P. multocida* in low numbers within tissues than the Gram's stain. As early as five hours post inoculation with $1 \times 10^3$ colony-forming units (CFU) of the organism, minute foci of inflammation were observed in the liver with hyperemia observed in all organs. The organism was found within isolated mononuclear phagocytes in the liver and spleen in low numbers (approximately five to ten bacteria per cell). Bacterial numbers increased tremendously with time within these cells in the liver and spleen. The organism was not observed extracellularly in the blood, lung, bone marrow, or kidney until later stages of infection. Bacteria were not observed within the heterophils of examined tissues. The results suggested possible intracellular multiplication of these encapsulated bacteria within splenic and hepatic mononuclear phagocytes.
Introduction

*Pasteurella multocida* infections are of considerable importance to the livestock and poultry industries. Infections have been noted in man, cattle, chickens, turkeys, wild birds, and rabbits (9,11,28,31,49). In poultry, the organism may produce an acute to peracute fatal septicemia due to rapid multiplication of this Gram-negative bacteria (11). Presumably, the cause of death is due to endotoxic shock (11). The extraordinary rate at which the bacteria multiply within infected hosts suggests potent virulence factors exist in this bacteria, but the nature of such factors remains largely unknown. Collins, using his mouse model, observed that the host response to infection with *P. multocida* was primarily polymorphonuclear and that, in the absence of specific opsonins, phagocytosis rates were very slow. His observations suggested that the bacteria multiplied freely in an essentially extracellular environment (11). Snipes *et al.* (50), when comparing a virulent encapsulated *P. multocida* strain with its avirulent variant, observed that virulence of the encapsulated organism was due to its greater capacity to multiply extracellularly in the blood stream. Their findings suggested that bactericidal and bacteriostatic factors in the plasma such as complement or transferrin may have a major role in determining survivability of infected fowl (50,51). Investigations with turkeys by Paps-Garnon and Soltys (40), however, indicated that the bacteria, following intravenous inoculation, multiplied first in the liver and spleen and only later in the blood. Experiments in turkeys conducted by Tsuji and Matsumoto
suggested that *P. multocida* was readily engulfed by phagocytic cells in the liver and spleen, regardless of the presence or absence of hyaluronic acid capsule (53). In their experiments, differences were observed in *P. multocida* survivability within the phagocytic cells. The purpose of this experiment was to obtain information on initial localization and multiplication sites of *P. multocida* within infected tissues through the use of immunohistochemistry and clarify questions concerning early pathogenesis of this infection.
Materials and Methods

Experiment 1 - Specific-pathogen-free eggs from Single Comb White Leghorn chickens were obtained from a commercial source and hatched. Sixteen, five-week-old chicks, raised in isolation facilities within battery units, were used. Twelve birds were inoculated intravenously in the brachial vein with 1.0 ml of \( P. \) \textit{multocida} in brain-heart infusion broth containing \( 1.0 \times 10^3 \) CFU of bacteria as determined by the standard plate dilution count technique. Four birds served as uninoculated controls and were housed separately. The inoculum was prepared by incubating previously lyophilized X-73 strain (type A1) of \( P. \) \textit{multocida} overnight on blood agar plates at 37°C. Ten colonies were randomly selected and streaked for confluent growth on brain heart infusion (BHI) plates. Bacteria were then harvested from these plates after five hours incubation at 37°C in BHI broth. Dilutions of the broth suspension served as inoculum. Three inoculated and one control chicken were euthanatized at the following intervals: 2.5, 5, 10, and 20 hours postinoculation (PI). Sections of liver, spleen, lung, kidney, and bone marrow were removed from each bird and fixed for 48 hours in 10% neutral buffered formalin. Isolation of \( P. \) \textit{multocida} from the livers of infected and control animals was attempted in each bird according to standard microbiological methods.

Experiment 2 - Sixteen, seven-week-old SPF Single Comb White Leghorn chickens, raised in isolation, were used. Twelve were inoculated with varying doses of X-73 strain via the brachial vein. Four birds served as uninoculated controls. All animals were
euthanatized at five hours PI. Of the twelve, three were inoculated with $1 \times 10^3$, three with $1 \times 10^5$, three with $1 \times 10^7$ and lastly, three with $1 \times 10^9$ CFU organisms. Tissue collection and fixation, as well as inoculum preparation, were done in the same manner as in Experiment 1.

**Preparation of Tissues for Histopathology:** Tissues fixed for approximately 48 hours in neutral buffered formalin were dehydrated in a graded series of ethanol, cleared with xylene, and processed in paraffin. Sections were cut 5 µm thick and attached to glass slides. Tissues were stained with routine hematoxylin and eosin stains. Duplicate slides were also stained with Gram's stain.

**Preparation of Sections for Immunohistochemistry:** A commercial kit (Vectastain®) was utilized for the immunoperoxidase histochemical procedures. Reagents in the kit included normal goat serum, biotinylated goat anti-rabbit IgG, avidin, and biotinylated horseradish peroxidase. All reagents were prepared according to manufacturer's instructions. Primary polyclonal antibody was obtained from an adult New Zealand White rabbit hyperimmunized with formalin-inactivated whole cell suspensions of X-73 strain given subcutaneously at 0-, 28-, 55-, and 75-days. The rabbit was exsanguinated on day 105 and its serum frozen at -70 °C until use. Two milliliters of inoculum emulsified in Freund's complete adjuvant were used on all except day 55 at which time 0.5 ml organism suspension was given. Normal rabbit serum was used as a negative control serum. Both sera were diluted 1:1,500 as it was predetermined that this dilution provided sections with minimal
nonspecific background staining and maximum contrast. All steps in the procedure were done in a moisture chamber kept at room temperature. Wash steps were done with fresh Tris-saline buffer, pH 7.6.

**ABC staining procedure:** Tissue sections were deparaffinized and rehydrated through a xylene and a graded alcohol series. The slides were then rinsed five minutes in distilled water, and a 0.6% H₂O₂ in methanol solution was applied to block endogenous tissue peroxides for 60 minutes. Sections were washed for twenty minutes in Tris-saline, then incubated with diluted 1.3% normal goat serum for 20 minutes. The slides were subsequently incubated for 60 minutes with the primary antibody. After a wash step in Tris-saline for ten minutes, sections were incubated for 30 minutes with .045% biotinylated goat anti-rabbit IgG. After a ten minute wash, the slides were incubated with the ABC reagent for 60 minutes. After a ten minute Tris saline wash, the sections were covered with the substrate-chromogen solution. The substrate consisted of a solution of 0.1% 3,3'-diaminobenzidine (DAB) mixed with equal parts of 0.02% H₂O₂. This solution was prepared a few minutes before each use. Tris buffer, 0.1M, pH 7.2 was used to dilute the DAB solution. Staining times varied from 2 to 5 minutes, depending on the rapidity of the brown color change. Slides were washed in tap water for five minutes and counterstained with Mayer's hematoxylin for one minute. The slides were immersed in saturated lithium carbonate solution for 30 seconds and rinsed for 30 seconds in tap water. The slides were dehydrated with 95% alcohol, absolute alcohol, and cleared in
xylene, two changes each. Mounting of the slides was done with Permount®.

The following controls were used to assess the specificity of the ABC technique and the primary antibody: a) a control slide containing normal chicken tissues stained with the primary antibody; and b) a control slide containing heavy numbers of *P. multocida* bacteria stained with negative rabbit sera.
1Becton Dickinson Vacutainer Systems, Rutherford, NJ 07070

2Difco, Detroit, MI.

3Spafas, Inc. Rural Route No. 1 Roanoke, IL 61561.

4A kind gift of Dr. K.R. Rhoades, National Disease Center, USDA, Ames, Iowa.

5Vector Laboratories Inc., 1429 Rollins Rd., Burlingame, CA 94010.

6Pharmacia Fine Chemical Co., Piscataway, NJ.

7Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, NJ.
Results

**Microbiology:** Pasteurella multocida organisms were readily reisolated from inoculated birds in both experiments from swabs taken from livers. Liver cultures of control birds remained negative for P. multocida.

**Gross and Microscopic Lesions:** The gross lesions were essentially identical in birds from both experiments. The severity of the changes varied, however, with the bacterial dose given and the time of euthanasia. A generalized cyanosis of skin and musculature was observed. A light green fecal and urate pasting was seen on the vent and tail feathers. Internally, the livers were enlarged, pale, and friable with multiple pinpoint yellow or red foci. Spleens were sometimes enlarged, though not consistently, and mottled red and white in color. Petechial hemorrhages were occasionally seen on serosal surfaces of internal organs. Watery, green intestinal contents with reddening of the intestinal mucosa was a common finding.

In Experiment 1, no visible changes were noted microscopically at 2.5 hours PI in examined tissues. As early as five hours PI, a generalized hyperemia was seen in all tissues. An influx of heterophils was observed also in the liver with heterophils along endothelial margins of hepatic blood vessels (Figures 10,15). Minute inflammatory foci were occasionally observed in the liver, consisting of equal numbers of heterophils and mononuclear phagocytes (Figure 6). Gram's stained sections of liver, spleen, kidney, lung, and bone marrow failed to demonstrate bacteria.
Examination of immunohistochemically-stained tissue sections showed a very small number of macrophages containing *P. multocida* in the liver and spleen (Figures 3,4,7,8). The organisms were usually within cells associated with an inflammatory focus and numbered about five to ten organisms per cell. (Figure 4). No bacteria were seen in ABC-stained sections of lung, bone marrow, or kidney at five hours PI. In birds inoculated with $1 \times 10^3$ CFU bacteria both 10 and 20 hours PI, mononuclear phagocytes in the liver and spleen appeared to contain larger numbers of organisms (often too numerous to count) (Figure 9). Necrosis of these cells was common, releasing bacteria into the sinusoids (Figure 5). Fibrinoid necrosis was observed in the spleen (Figure 14). Infiltration of these organs with heterophils and mononuclear cells became more pronounced (Figure 15). Low numbers of bacteria were evident in the blood at this time. In dead or moribund birds, bacterial dissemination was widespread in all organs (Figures 11,15). They were readily observed with H&E, Gram's and immunohistochemical stains both intra and extracellularly. Few bacteria were seen in the kidney within blood vessels (Figure 17). The lung and bone marrow showed large numbers of bacteria in the blood. Necrosis of blood cell precursors, as well as a depletion of heterophilic granulocytes was observed in the bone marrow (Figures 12,13). The severity of the congestion increased in all organs at death. Fibrin thrombi were observed within glomerular blood vessels in the kidney (Figure 16). An interstitial pneumonia and nephritis consisting of infiltrating mononuclear cells and heterophils were observed in tissues taken from
dead or moribund birds (Figure 18, 19). Occasional thrombi were present within blood vessels of the lung and spleen.

In Experiment 2, the microscopic lesions in birds dosed with $1 \times 10^3$ and $1 \times 10^5$ CFU bacteria and killed at five hours PI were essentially the same as Experiment 1, when similar bacterial doses were given and the birds killed at five hours PI. Birds inoculated with $1 \times 10^7$ and $1 \times 10^9$ CFU organisms and killed at five hours demonstrated changes similar to birds killed at 10 and 20 hours PI in Experiment 1. However, necrosis of cells was not as prominent in this group microscopically, perhaps because not as much time had elapsed between inoculation of bacteria and euthanasia.
Discussion

Immunohistochemistry using the avidin-biotin-peroxidase technique has shown to be a highly specific and sensitive test for the demonstration of antigens in tissue, particularly for the demonstration of microorganisms \(12,23,25,54\). In the present experiment with this technique, it was possible to demonstrate \(P.\ multocida\) within phagocytic cells during very early stages of infection when no bacteria were observed with conventional H&E and Gram-stained tissues. Cell types were recognizable in immunohistochemistry-prepared sections; whereas, they were often difficult or impossible to recognize in gram-stained tissues. Some background staining did occur, however, especially in sections containing large amounts of blood such as the bone marrow and lung tissue sections. This was probably due to large amounts of endogenous peroxides found in red blood cells. Control slides were very helpful in interpretation of some sections where this was a problem. Several techniques were employed to minimize background staining and artifacts \(12,23\).

The histopathologic changes noted in birds that died from the infection were similar to those previously described with some notable differences \(46\). The early lesions suggest that these organisms are readily phagocytized and may in fact have multiplied within splenic and hepatic mononuclear phagocytes. Little, if any, phagocytosis by heterophils was seen by the authors. It may be that the heterophil is less effective at phagocytizing \(Pasteurella multocida\) in chickens. It should be noted, however, that even under
high magnification, cell borders between inflammatory cells were not always distinct so that absence of phagocytosis by heterophils cannot be stated with absolute certainty. The numbers of bacteria within mononuclear cells appeared to increase with time, suggesting intracellular multiplication, because it is unlikely that large numbers of bacteria could gain entrance into a cell via phagocytosis alone. This data supports that the reticuloendothelial cells of the liver and spleen appear to be the main sites of initial localization and multiplication of *P. multocida* infection in the chicken and does not support that the organism freely multiplies in the blood as was previously presumed. Multiplication of the bacteria in the blood would result in bacteria being present in all organs equally. This was definitely not the case in this experiment. Although phagocytic cells are present in many organs, the organism appears to be picked up primarily in the liver and spleen. This type of localization and multiplication pattern is not uncommon with facultatively intracellular bacteria (10,15).

An interesting microscopic finding in this experiment was the significant mononuclear component of the inflammatory response seen in the lung, kidney, and spleen in birds which had succumbed to the disease or were necropsied late in the infection. Previous reports on the microscopic lesions of acute fowl cholera describe a mostly heterophilic response. A mixed inflammatory cell response may be characteristic of some avian bacterial infections including fowl cholera. The information obtained from this experiment suggests that the mononuclear phagocyte may play a key role in natural
immunity against this infection. Baba (2,3) demonstrated that cell-mediated immune protection exists in chickens against P. multocida.

The possibility exists that chronic carriers of fowl cholera may harbor organisms intracellularly. This might protect the bacteria from harmful serum factors such as antibodies or complement. To date, most research has focused on increasing humoral immunity in birds. However, the most effective vaccines that provide some cross protection are the live-organism vaccines and killed vaccines derived from organisms grown in vivo. Perhaps, these vaccines stimulate cell-mediated as well as humoral immunity. According to Baba (2), the activation of macrophages by previously primed T-cells may be an important resistance mechanism which increases the effectiveness of intracellular killing by these cells.

More research is needed to explore the mechanisms by which virulent P. multocida organisms avoid intracellular killing in susceptible host.
Figure 1. The indirect immunoperoxidase method.
Figure 2. Avidin: biotinylated horseradish peroxidase complex technique (ABC technique).
Figure 3. Chicken portal area and vein (V) in the liver from a bird injected with $1 \times 10^5$ CFU bacteria and killed five hours PI. Note a macrophage laden with bacterial antigen (arrow). ABC stain 640X.
Figure 4. Liver from a chicken injected with $1 \times 10^5$ CFU bacteria and killed five hours PI. Note *P. multocida* within vacuole (arrow) of mononuclear phagocyte. Bacteria-laden cell is within hepatic sinusoid (S). ABC stain 800X.
Figure 5. Liver of chicken showing degenerating mononuclear phagocyte within sinusoid (arrow). ABC stain approximately 640X.
Figure 6. Inflammatory foci in liver of a chicken injected with 1 x 10^6 CFU organisms and killed five hours PI. Note a macrophage containing *P. multocida* (arrow). Surrounding heterophils do not contain organisms (H). ABC stain 640X.
Figure 7. Splenic mononuclear phagocyte (arrow) containing bacteria. The bird was inoculated with $1 \times 10^3$ bacteria and necropsied five hours PI. ABC stain 1600X.
Figure 8. Splenic macrophage containing Pasteurella multocida bacteria (arrow). Chicken injected with $1 \times 10^3$ CFU organisms and killed five hours PI. ABC stain 800X.
Figure 9. Liver from chicken inoculated with $1 \times 10^3$ CFU bacteria and killed 10 hours PI. Note bacteria within sinusoid (arrow) both extracellular and within necrotic phagocyte. ABC stain 1600X.
Figure 10. Hepatic vein (V) from a chicken inoculated with $1 \times 10^5$ CFU bacteria and killed five hours PI. Bacteria absent from blood or heterophils (H). ABC stain 1280X.
Figure 11. Inflammatory focus in a chicken liver (AB) containing large numbers of organisms. The bird had been injected with $1 \times 10^3$ CFU bacteria and killed 20 hours PI. ABC stain 512X.
Figure 12. Bone marrow from a chicken inoculated with $1 \times 10^3$ CFU bacteria and dead at 20 hours PI. Note heterophilic granulocyte depletion and necrosis of blood cell precursors. H&E stain 400X.
Figure 13. Normal chicken bone marrow with abundant heterophilic granulocytes. H&E stain 640X.
Figure 14. Chicken spleen demonstrating fibrinoid necrosis (N). The bird was inoculated with $1 \times 10^3$ CFU bacteria and killed ten hours PI. H&E stain 400X.
Figure 15. Chicken hepatic vein (V) demonstrating accumulation of heterophils along endothelial margins (H). H&E stain 400X.
Figure 16. Chicken kidney demonstrating thrombi (TH) within glomerular tufts in uppermost glomerulus (G). H&E stain 400X.
Figure 17. Chicken kidney in bird injected with $1 \times 10^9$ CFU bacteria and killed five hours PI. Note large numbers of bacteria (B) within blood vessel (V). Gram's stain 640X.
Figure 18. Chicken lung demonstrating interstitial thickening and hyperemia. The bird was inoculated with $1 \times 10^3$ CFU and necropsied 20 hours PI. H&E stain 320X.
Figure 19. Normal chicken lung. H&E stain 320X.
Bibliography


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