Characterization, identification, and purification of the bacterial receptor expressed by turkey peripheral blood monocytes for serogroup A:3 of Pasteurella multocida

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Characterization, identification, and purification of the bacterial receptor expressed by turkey peripheral blood monocytes for serogroup A:3 of *Pasteurella multocida*

by

Ingrid Mireille Pruimboom

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Major Professors: Norman F. Cheville and Mark R. Ackermann

Iowa State University

Ames, Iowa

1998
Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

*Pasteurella multocida* causes fowl cholera in both domestic and wild birds. The high morbidity and mortality associated with this disease results in significant economic losses to the turkey industry. Transmission of the disease usually occurs following contamination of drinking water with mucous secretions from infected birds. Experimentally, a small inoculum of virulent organisms (30-100) is capable of causing nearly 100% mortality within 48 hours. The organism is thought to enter the bird after colonizing the mucous membranes of the pharynx or upper air passages, eventually gaining access to the blood stream where it rapidly reaches very high numbers, often exceeding $10^8$ ml^{-1} of blood (109). However, the precise mechanisms of pathogenesis are still unclear.

Serogroup A strains of *P. multocida* are the major cause of fowl cholera in poultry. Virulent strains are often resistant to phagocytosis by avian leukocytes and withstand complement-mediated lysis by normal turkey plasma (47). Survival of *P. multocida* outside the host and resistance to phagocytosis in the host are associated with the presence of a capsule. The capsule of serogroup A strains of *P. multocida* is composed of hyaluronic acid, an anionic mucopolysaccharide composed of repetitive disaccharidic units of N-acetyl-D-glucuronic acid and N-acetyl-D-glucosamine (114). In turkeys, the bacterial capsule inhibits opsonization or creates physical interference of receptor-ligand binding between phagocytes and opsonized bacteria (128). Whether or not these effects in turkeys are exclusively attributed to the hyaluronic acid has not been determined. Phagocytosis is an event which follows adhesion of host cells to
opsonized or non-opsonized bacteria. Although studies of *P. multocida* adhesion to HeLa cells indicated that capsular hyaluronic acid promotes bacterial binding, studies of *P. multocida* adhesion to avian air sac macrophages are lacking (30).

CD44, an 85-kDa transmembrane glycoprotein found on a wide variety of cell types, is one of several receptors capable of binding hyaluronic acid. Because CD44 is a hyaluronic acid receptor also associated with blood monocytes (23, 43), it is my hypothesis that CD44 is involved in adhesion of serogroup A strains of *P. multocida* to turkey peripheral blood monocytes. Other reports indicate that freshly isolated human peripheral blood monocytes do not bind soluble hyaluronic acid; however, increased mucopolysaccharide binding occurs *in vitro* after 8 to 16 hours of incubation (77) or after exposure to phorbol myristate acetate (79). Similarly, in septicemic cases of fowl cholera, minimal association occurs between peripheral blood monocytes and capsulated strains of *P. multocida* (129) (Figure 1.1). It has not been investigated whether or not mucopolysaccharide can bind freshly isolated turkey peripheral blood monocytes, or if cultures and phorbol esters affect this binding.

Hyaluronic acid is recognized as a major component of the extracellular matrix in animals. Interactions of blood monocytes with the extracellular matrix components have a central role in their tissue-specific migration, differentiation, and function. Hyaluronic acid proteins bind with high affinity to hyaluronic acid, and also bind fibronectin, collagen (I, II, and IV), and laminin (13, 142, 144). I hypothesized that by culturing turkey peripheral blood monocytes on an extracellular matrix composed of entactin, laminin, and collagen IV, expression of hyaluronic acid-binding proteins may be enhanced, and thereby bacterial adhesion may be increased. The studies presented in this dissertation were designed to: 1) investigate whether or not hyaluronic acid
promotes adhesion of serogroup A *P. multocida* to turkey air sac macrophages, 2) demonstrate that cultures and or chemical treatments up-regulate bacterial adhesion to turkey peripheral blood monocytes, 3) identify, characterize, and isolate the receptor involved in bacterial adhesion to turkey peripheral blood monocytes, 4) produce polyclonal antibodies against the receptor. Ultimately, the results of this dissertation could provide the material required for a better understanding of the pathogenesis of fowl cholera.

**Literature Review**

**Recognition of fowl cholera and discovery of its etiology**

Fowl cholera is a contagious disease of domestic and wild avian species that occurs worldwide. Because of its major contribution in the development of the discipline of bacteriology, some historical events will be reviewed in this chapter. Much of the following history of fowl cholera was originally described by Gray in 1913 (42).

In 1600, Androvandus reported for the first time a deadly epizootic of fowl cholera in poultry that occurred in Italy. During the second half of the 18th century (1770-1800), several epornitics among fowl occurred in Italy and France. In 1817, fowl cholera raged in the East Indies and a series of outbreaks occurred in France between 1825 and 1900. The considerable knowledge acquired during this period permitted Maillet to introduce the name of fowl cholera in 1835. Since that time, the disease has received many names including avian cholera, avian pasteurellosis, avian hemorrhagic septicemia, bird cholera, avian septicemia, and *Pasteurella avicida* infection.

In 1851, at the Alfort Veterinary School (France), Renault, Reynal and Delafond,
emphasized the importance of contagion in fowl cholera, and demonstrated the transmissibility of the disease to various species by inoculation. Benjamin (France), at the same period, suggested that fowl cholera could be spread by cohabitation and with this knowledge, formulated procedures for its prevention. In 1869, Moritz (France) demonstrated by light microscopy the presence of special granulations in the blood of inoculated birds. A few years after the first microscopic evidence (1877 and 1878), Perroncito (Italy) and Semmer (Russia) observed in tissues of affected birds an organism with rounded shape, occurring singly or in pairs. In 1879, at the Toulouse Veterinary School (France), Toussaint demonstrated that a single organism was the cause of the disease. In 1880, Louis Pasteur finally isolated and cultured the causative bacterial agent in chicken broth (114). The genus to which this bacteria belonged was later named after Louis Pasteur. In his classical experiments on vaccination, Louis Pasteur used an attenuated strain of the organism to immunize chickens. The history described that during the summer of 1879, some flasks containing fowl cholera cultures, were forgotten during several months and lacked laboratory care (14). When Pasteur returned from holidays, in the fall of 1879, two chickens were inoculated with these cultures. The chickens became ill, but unexpectedly recovered from the disease. Following this finding, Pasteur and his collaborators, Chamberland and Roux, inoculated the same chickens with new, virulent cultures. Surprisingly, the chickens died only four days after the inoculation and not more acutely. These results helped Louis Pasteur to develop the concept of vaccination. However, the history often describes inaccurately that the chickens did not died after the second inoculation.

In the United States, occurrence of the disease was first reported in poultry in 1867 (Anonymous, 1867) and was then studied by Salmon in 1880 (119). In 1900, the disease became
prevalent in Great Britain and was studied by McFadyean. In 1932, Devolt and Davis gave the first detailed description of fowl cholera in turkeys. In 1899, Salmon (120) and Gray (42) reported that wild birds were a possible source of infection for poultry. However, detailed reports on the disease in wild birds did not appear until 1936 with a report of *P. multocida* from wild ruffle gouse. The first reported epizootic in wild waterfowl occurred at the Lake Nakuru in Kenya during February of 1940 (115).

**Epidemiology**

*Geographical distribution, host range, and economical impact*

Fowl cholera affects both domestic and wild birds. Economically, the disease causes significant worldwide poultry losses, estimated to be 200 million U.S. dollars annually (114). Ecologically, it is a threat to the survival of several endangered avian species. Poultry, particularly chickens, turkeys, ducks, and geese are commonly affected and most epidemiologic studies and research programs focus on these hosts. Carpenter *et al.* performed a retrospective study of the epidemiology and financial impact of fowl cholera in California meat turkeys (16). They concluded that birds in fowl cholera outbreaks had a higher relative mortality rate (52% higher in toms and 26% higher in hens), and tripled medication costs than non-vaccinated birds. A study by the University of Georgia estimated that fowl cholera was the most prevalent disease in both commercial tom flocks (18.0%) and breeder hen flocks (14.7%) as compared to colibacillosis (15.9% in commercial tom flocks), and aspergillosis (8.5% in commercial tom and 4.9% in breeder hen flocks) (88). Outbreaks of fowl cholera have also occurred in Africa, North and South America, Asia, Europe, and Oceania (Anonymous, 1987).
Fowl cholera is also of major importance in wild birds, game birds and caged birds. Over 1000 wild geese died of fowl cholera overnight as reported by Vaught et al. (146). Another outbreak of fowl cholera in green pheasants (Phasianus colchicus) was reported in Japan (118). The first outbreak of fowl cholera in Muscovy ducks (Cairina moschata) was described in Okinawa Prefecture of Japan, causing 25% of the mortality among the birds. \textit{Pasteurella multocida subsp. multocida} were isolated in pure culture from these animals (90).

\textit{Transmission}

Dissemination of \textit{P. multocida} within a flock occurs primarily through indirect contact. Excretions from the mouth, nares, and conjunctiva of diseased birds contaminate the environment such as feed and water. In experimental studies, groups of susceptible, non-infected turkeys separated only by wire netting from infected turkeys became infected when water troughs were shared, but not when water was provided in separate troughs, suggesting the importance of contaminated water as a transmission factor rather than droplet aerosol (97). Feces do not likely represent a source of contamination since \textsuperscript{32}P labeled \textit{P. multocida} are inactivated in the proventriculus, leaving no viable organisms within feces (61). Experimental inoculation into the conjunctival sac of turkeys resulted more efficiently in the development of the acute disease than intranasal exposure, suggesting that conjunctival exposure may be an important site of transmission (7). Transmission \textit{in ovo} is unlikely. A study of more than 2000 fresh and embryonated eggs from chickens affected with chronic fowl cholera yielded no evidence of vertical transmission (114). Vehicles, contaminated feed bags, or any equipment may serve as mechanical carriers in introducing fowl cholera into susceptible flocks.
The possibility of insects as vector for fowl cholera transmission has been investigated. Several studies have demonstrated that flies, ticks, and mites experimentally or naturally contaminated by exposure to infected birds, could transmit the disease to susceptible flocks. These studies confirm that insect transmission can occur (64, 100, 125). However, under natural conditions, this is likely to be uncommon.

Reservoir of infection

Chronically infected birds may shed organisms for years and thereby are likely the principal source of infection for susceptible birds (46, 145). Virulent strains of *P. multocida* can indeed be isolated from the oropharynx of turkeys in flocks that had experienced outbreaks of fowl cholera, but not in healthy flocks (15, 101). Additionally, it had been demonstrated that *P. multocida* can rapidly increase its virulence by bird-to-bird transmission (83).

Wild birds are often infected and, through direct contact, may perpetuate the disease in other wild birds and cause outbreaks in poultry flocks. Numerous epidemiologic studies have been performed and underscore the importance of healthy carriers in wild fowl (8, 152). In one of these studies, serotype 1 strain of *P. multocida* was isolated from the pharynx of healthy wild snow geese and its virulence was demonstrated experimentally in white Pekin ducks (121).

Farm mammals may also become potential carriers of *P. multocida*; however, most of these organisms, with few exceptions, are avirulent for fowl. Tonsil isolates from cattle and sheep are not pathogenic for poultry, but porcine isolates are virulent in birds (62). Healthy carrier pigs can transmit fowl cholera to poultry as efficiently as diseased birds (63). Isolates from cats on farms with recent history of turkey pasteurellosis were also able to transmit the disease to poultry (24).
Heddleston et al. identified 27 cultures of *P. multocida* in the human upper respiratory tract but none of them were shown to be pathogenic for turkeys (50). Among procyonidae, raccoons are a possible reservoir of *P. multocida* and can transmit the disease through bites (44). Rodents such as mice and rats, are another possible reservoir. Between 1973 and 1984, 243 isolates of *P. multocida* were recovered from rodent specimens submitted for plague tests. The rodent serotypes were predominantly A:1 and A:3 which suggests an epizootiologic role for these animals in outbreaks of avian cholera that commonly involve these serotypes (104).

**Pasteurella multocida**

*Morphology*

Rimler et al. described many of the basic microbiological features of *P. multocida* (114). *Pasteurella multocida* is a nonmotile, nonsporogenous, coccobacillus or short rod which stains Gram-negative and occurs often singly or in pairs. In tissues and fresh cultures, the bacterium shows bipolar staining with Giemsa or Wright's stain due to the condensation of volutin granules at the organism extremities. The bacteria are 0.2-0.4 x 0.6-2.5 μm but become pleomorphic and filamentous after repeated subcultures.

Virulent strains of *P. multocida* are usually encapsulated. The capsule is composed of mucopolysaccharides and differs in size and chemical composition amongst strains. In serogroup A:3 strains of *P. multocida*, the most commonly isolated strains in turkey fowl cholera, the mucopolysaccharide is hyaluronic acid. With fresh isolates, multiple staining methods can be used in light microscopy to demonstrate the capsule: alcian blue, congo red, and crystal violet are used for direct staining; Jasmin and India ink are employed for indirect staining.
By transmission electron-microscopy, the bacteria presents the typical outer envelope (cell wall) and cytoplasmic membrane of Gram-negative bacteria. Internal structures such as nucleoid fibrils, mesosome, and ribosomosomes were also identified (102). In *P. multocida* isolated from infected tissues or septicemic blood, dense, polar, agranular vesicles with similar morphology to those observed in magnesium starved cells of *Escherichia coli* B were observed (11). The hyaluronic acid capsule is very hydrophilic, and dehydration procedures involved in transmission electron microscopy cause it to collapse. Alternative techniques to study the capsule by electron-microscopy include polycationic ferritin (66), and negative stains with ruthenium red or platinum (45).

**Growth requirements**

*Pasteurella multocida* is a facultative anaerobe bacterium that grows optimally at 37°C. The optimal pH ranges between 7.2 and 7.8. Isolation of the organism is usually performed on enriched agar media (casein hydrolysate, peptone) with 5% heat-inactivated serum (60°C for 30 minutes), blood, or peptic digest of blood. Blood or serum from equine, bovine, and ovine blood may inhibit the growth of the organism (114). In contrast, dextrose starch agar with 5% avian serum is considered an excellent medium for isolation and growth of *P. multocida*. Several selective media for isolation from contaminated material have been described by Das (26), Knight (71), and Morris (114). For isolation from nasal cavities of rabbits or pigs, specialized medium have been described by Garlinghouse (39), Smith (127). *Pasteurella multocida* also grows in chemically defined medium, such as the Wessman formula (149). Thiamine, nicotinamide and pantothenate are essential for growth in these media. Growth for large-scale vaccine production
is possible with media and enrichments, that are often chemically undefined. Finally, iron is also an essential element for *P. multocida* growth. Under iron limited conditions, the bacteria synthesizes a siderophore-like compound named Multocidin (57).

**Colony morphology and related properties**

Colony morphology is determined after 24 hours of culture in aerobic conditions, at 37°C, with constituents such as serum, blood, or dextrose starch agars. Colonies range from 1 to 3 mm in diameter and are usually smooth and mucoid. Rough colony formation is rare. The mucoid aspect is attributed to the capsular mucopolysaccharides (17) and may vary from slight to markedly moist and viscous (also referred as watery mucoid). Smooth colonies are more discrete, and are often composed of both capsulated and noncapsulated strains of *P. multocida*.

One of the most useful characteristics of *P. multocida* is the colony morphology observed on transparent media in obliquely transmitted light with a stereomicroscope (54). Except for watery mucoid colonies that have a gray appearance, fresh isolates will display a yellowish-green, bluish-green or mother of pearl-like iridescence. This iridescence is related to the presence of a capsule. Noncapsulated strains are blue, grayish-blue, or gray. Evidence supporting a correlation between virulence and colonial morphology was reported by Hughes (59). Hughes identified three patterns: 1) the iridescent type which is highly virulent and relates to acute cases of fowl cholera outbreaks; 2) the blue type which is of lower virulence and occurs in flocks in which fowl cholera is enzootic; 3) and a mildly virulent and iridescent type. Repeated subculturing induce the transformation of iridescent colonies to blue colonies, along with the loss of the bacterial capsule and virulence. This change is termed dissociation.
Serotyping and other methods of grouping strains

Serologic typing is based on the detection of capsular and somatic antigens. Passive hemagglutination tests (18) are used to detect the capsular group. Somatic serotyping can be established by tube agglutination tests (91) and gel diffusion precipitin methods (53). More recently, DNA fingerprinting and ribotyping methods have increasingly been employed in epidemiologic studies (152).

Five capsular serogroups (A, B, D, E, F) are currently recognized. Only A, B, D, and F are isolated from species of avian origin (107). Among them, serogroup A strains are recognized as the main cause of fowl cholera in poultry, and produce the most severe form of the disease (17). In turkeys, capsular serogroups D and F are mildly (108) and variably virulent respectively (113). Although serogroup B is highly virulent in turkeys, no pathogenicity was observed in chickens (21, 112).

The simplicity of the gel diffusion test makes it a very popular method for serology. The method uses antisera prepared in chickens and heat-stable antigens extracted from formalinized saline suspension of bacteria. The lipopolysaccharidic component of the antigen complex is the major determinant in the reaction. To date, 16 somatic serotypes of *P. multocida* have been identified. Most of them are from avian hosts (9, 51) and of the capsular type A strains of *P. multocida* (107). Variation in somatic phenotypes has also been demonstrated within the others groups. Somatic types 1, 3, and 4 within capsular group A are considered the most frequent cause of fowl cholera. *Pasteurella multocida* serotype A:3,4 were isolated from 76% of field cases of pasteurellosis in turkeys from the southeastern United States, despite the use of vaccines (55). Serotype 1 is predominant in Central and Pacific flyways outbreaks, and serotypes 3 and
4 prevail in the Mississippi and Atlantic flyways (10).

Pathogenesis

*Symptoms and lesions associated with the disease*

The lesions observed in turkeys affected with fowl cholera relate to differences in the course of the disease. Acute cases of fowl cholera are characterized by vascular disturbances and chronic cases tend to be associated with localized exudative lesions (109).

In turkeys, the most commonly observed symptoms of acute fowl cholera are depression, ruffled feathers, fever, anorexia, mucous discharge from the mouth, diarrhea, and increased respiratory rate. Fecal material associated with the diarrhea is generally watery and predominantly white in the early stages of the disease, but in later stages becomes mucoid and green. Near the time of death, birds become cyanotic. Cyanosis is most particularly evident in unfeathered areas of the head, such as comb and wattles. Occasionally, symptoms will only appear a few hours before death, so that the only evidence of a disease outbreak is the discovery of dead birds.

Macroscopic lesions include systemic hyperemia that predominantly occurs in veins of the abdominal viscera and small vessels of duodenal mucosa. Petechial and ecchymotic hemorrhages are systemic, and hemorrhages are more commonly found in subepicardial and subserosal areas, lung, abdominal fat, and intestinal mucosa. Pericardial fluid and ascites frequently occur. Disseminated intravascular coagulation and fibrinous thrombosis are additional findings reported in experimentally-induced cases of the disease. In these cases, the liver is swollen with multifocal areas of necrosis. Lungs of turkeys are more severely affected with heterophilic and histiocytic
pneumonia than those of chickens. Large amounts of viscid mucus can be observed in the pharynx, crop and intestine. The genital system of laying hens is commonly characterized by flaccid mature follicles with poorly defined thecal blood vessels and yolk rupture. Immature follicles and ovarian stroma are often hyperemic. Chronic fowl cholera may follow an acute stage of the disease or may result from infection with low virulence organisms. Chronically infected turkeys often are emaciated and lethargic. Signs of the disease are the result from localized infections and include swelling of wattles, sinuses, periorbital subcutaneous tissues, legs, wing joints, sternal bursa and footpads. This swelling is due to fibrinosuppurative exudate and focal necrosis. Exudative conjunctivitis, pharyngitis, and torticolis are also commonly observed. Tracheal rales and dyspnea may result from respiratory infections.

*Entry of Pasteurella multocida into the body*

**Oropharynx and Respiratory tract.** In natural field infections of turkeys, *P. multocida* likely enter tissues of birds through mucous membranes of the pharynx or upper air passages (1, 59, 109). In mammals, tonsils of the oropharynx are common sites of residence or portal of entry of microorganisms into the body, and, by acting as a first defense against foreign agents, contribute to the local and systemic immunity. In avian species, tonsils are lacking, but the hard palate is pierced by a median cleft that communicates with the nasal cavities and is delineated by well developed lymphoid tissue. Because of this anatomical communication between oral cavity and upper respiratory tract, it also becomes difficult to distinguish oropharynx from respiratory tract events in the pathogenesis of fowl cholera.

The knowledge of the avian palatine lymphoid tissue are limited. Therefore, the defense
mechanisms of mammalian lymphoid structures will be reviewed in this chapter and contrasted to what is known about avian palatine lymphoid tissue. Mammalian tonsils have subepithelial lymphofollicular aggregates extending into the mucosa and occasionally down to the submucosa. The lymphoid follicles are composed of typical germinal centers (B lymphocytes) surrounded by a rim of reactive B lymphocytes. A diffuse population of T lymphocytes infiltrate the areas in between the follicular aggregates. The overlying epithelium protrudes away toward the respiratory lumen (dome) and contains numerous lymphocytes and monocytes. In avian species, lymphofollicular organizations do not occur and lymphocytes diffusely infiltrate the mucosa. The epithelium of the avian palatine cleft is likely stratified squamous. Therefore, defense mechanisms such as secretory IgA, mucociliary transport, and nonspecific antimicrobial factors (ie, mucin, lysozyme, and lactoferrin) characteristic of the respiratory epithelium are probably lacking in birds (65).

In avian species, the mechanisms by which the antigens are translocated from the epithelium into the lymphoid tissue have not been studied. In mammals, however, antigenic uptake of horseradish peroxidase by the nasopharyngeal tonsils was demonstrated through histochemistry and electron microscopy (38). Horseradish peroxidase particles were present in the intercellular space and endocytic vesicles. More endocytic vesicles containing horseradish peroxidase were observed in nonciliated cells than in the adjacent ciliated cells suggesting an enhanced phagocytic ability of the nonciliated epithelium. One type of nonciliated epithelial cell adjoining lymphocytes contained more phagocytic vesicles than any of the other epithelial cells and likely transported the phagocytized material into the lymphoid cells. This close relationship between epithelial cells and underlying lymphocytes mimics that of M cells in gut and bronchus associated lymphoid tissues.
In these tissues, M-cells enhance trapping and uptake of antigenic substances in order to develop the mucosal immunity. Fujioshi et al. suggested that M-cells likely differentiated from the adjoining columnar ciliated or nonciliated cells under the influence of the surrounding lymphocytic and antigenic environment (38). It is also my hypothesis that M-cells are part of the avian palatine cleft epithelium.

Experimental inoculation of turkey palatine cleft with *P. multocida* is followed by colonization of the dorsal pharynx within 6 hours and invasion of internal organs (spleen, liver) of the bird after 6 to 12 hours (106), emphasizing the importance of the palatine lymphoid tissue as a possible route of invasion. In these cases, pneumonia and air sacculitis which are common lesions of fowl cholera, could develop from the hematogenous dissemination. However, since the authors isolated few organisms from the lower trachea in the same experiment, the possibility of pneumonia resulting from the respiratory route may not be ruled out. Air sacs may, in this perspective, become a portal of entry for *P. multocida* into the systemic circulation, probably via damaged air sac epithelium (31).

Within the respiratory tract of mammals and likely of avian species, efficient mechanisms protect against inhaled foreign particles. In the nasal cavities, the mucociliated epithelium traps particles that are 10 μm in diameter or more, and drives them towards the throat for swallowing. In the trachea and bronchi, the so-called mucociliary escalator combines the action of ciliated cells with secretory IgA and IgG, and the mucus secretions from goblet cells and subepithelial glands that also contain mucin, lysozyme, lactoferrin and other products. Particles that are trapped in the mucus, moved upwards to the throat by the cilia. Particles that are less than 5 μm escape the escalator and, in avian species, end up in the terminal airways represented by air capillaries and
air sacs (abdominal and thoracic). In these later areas, protection is achieved by cells of the immune system, mainly alveolar (in mammals) and air sac macrophages (in birds) as well as heterophils. However, in avian species, the number of resident macrophages in the lungs and air sacs is low (138). In the terminal airways, the survival of *P. multocida* would likely depend on antiphagocytic properties or resistance to intracellular killing. After intratracheal inoculation with strain P-1059I into young turkeys (84), the organisms multiplied *in situ* during the first 2 hours, and gradually spread downwards to the lower respiratory tract. By six hours post-inoculation, the bacteria invaded the circulatory system and multiplied in reticuloendothelial organs. In some birds, the organisms appeared to have reached the liver from the upper respiratory tract instantaneously.

**The skin.** In mammals and likely in avian species, the skin is a natural barrier to microorganisms and generally can only be penetrated when macroscopic or microscopic discontinuities occur. Biting arthropods such as mosquitos, fleas, and mites penetrate the skin during feeding, and can introduce *P. multocida* in avian hosts. As discussed earlier, although the possibility of such transmission exists, the incidence is low. Biting mammals such as cats, raccoons, and rodents such as rats, mice are another source of the disease. Frame et al. reported a case of cutaneous form of pasteurellosis in a turkey flock (36). The infection was predominantly ventral and lateral to the tail and microscopic lesions included hemorrhages, necrosis, and cellulitis of the subcutis. The lesions contained occasional Gram-negative colonies within the necrotic areas. The origin of the outbreak was not established. Once introduced into the flock, the author suggested that infection disseminated within the flock by bird to bird transmission. Organisms were thought to be transmitted into feather follicles when turkeys self-
preened, fought, or picked, at the blood engorged developing tail feathers of flockmates.

**Intestinal tract.** In general, the potential of infection through the intestinal tract is probably minimal due to the presence of mucus, acid, secretory IgA, enzymes, bile, and the flushing effects due to intestinal peristalsis. Most epithelial cells, whether epidermal, respiratory, or intestinal have a limited capacity of phagocytosis. In avian species, it was suggested that foreign particle uptake primarily occurs through the follicle-associated epithelium that overlies the gut associated lymphoid tissue within the terminal ileum (Peyer’s patches-like foci), the cecal tonsils, and within the Bursa of Fabricius (6). In experiments, contaminated drinking water has often been used to infect animals (111). However, little is known about the mechanisms used by *P. multocida* to invade the avian host in the field and experimentally.

**Conjunctiva.** Conjunctival inoculation has been used in experimental cases of fowl cholera. The conjunctiva is kept moist and healthy by the continuous flow of secretions from lacrimal and other ocular glands. Although tears contain lysozyme and other antimicrobial substances, their principal protective action is the mechanical washing away of foreign particles. Clearly, there is little chance for infection to initiate in the normal conjunctiva unless microorganisms have some special ability to attach to the conjunctival surface. Conjunctiva are most commonly infected through mechanical deposition rather than through airborne microorganisms.

The conjunctiva-associated lymphoid tissue is an anatomical and immunological entity described in turkeys, rabbits, and guinea pigs. In turkeys, the lymphoid tissue is localized on the lower-eyelid conjunctiva and fully develops by three weeks of age. The conjunctiva-associated lymphoid tissue resembles, in many aspects, the mucosa-associated lymphoid tissues of the
intestine. In the ocular conjunctiva, the Harderian gland is markedly infiltrated by heterophils and plasma cells within the intralobular connective tissue. The Harderian gland is probably another critical element of the immune system and secrete immunoglobulins (5).

*Events occurring immediately after the entry of Pasteurella multocida*

Adherence of bacteria to mucosal epithelial cells occurs through recognition of a cell surface receptor. In the respiratory tract, bacterial adhesion on the mucosal surface allows bacteria to withstand primary defense mechanisms such as sneezing, mucociliary clearance, and coughing. Adherence of some type of bacteria to host epithelial cells involves the recognition by bacterial surface molecules such as fimbriae or afimbrial adhesins (i.e. *Neisseria* opacity protein, *Bordetella pertussis* filamentous hemagglutinin, *Escherichia coli* AfaD and AfaE, *Streptococcus pneumoniae* M protein) to one or multiple eukaryotic cells surface receptors. Through eukaryotic receptors such as matrix glycoproteins (116), integral membrane glycoproteins (117), or glycolipids (69), bacterial adhesins engage protein-carbohydrate, protein-protein and occasionally carbohydrate-carbohydrate interactions (143). Some bacteria even rely on the host inflammatory reaction to up-regulate the expression of their eukaryotic cell surface receptor. Thrombin treatment or TNFα stimulation of the umbilical cord endothelium enhances the expression of the eukaryotic receptor for *Streptococcus sp.*. In addition, cell signal transduction often plays a major role in bacterial adherence. *Bordetella pertussis* filamentous hemagglutinin has a two arg-gly-asd repeat sequence (105), and interaction between the arg-gly-asd sequence and a monocyte integrin further up-regulates the binding activity of a second class of integrin (CR3) that recognizes another domain of the filamentous hemagglutinin (34) to strengthen the
interaction. Bacterial recognition is followed by invasion. Many bacteria, such as *Yersinia enterolitica* and *pseudotuberculosis*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella* invade epithelial cells. This characteristic becomes an important virulence factor for *Shigella* which must invade intestinal M cells in order to reach their receptor, the α5β1 integrin located at the basolateral surface of the enterocytes (93, 34).

The understanding of fowl cholera pathogenesis and the mechanisms responsible for protection against the disease are still poorly understood. It was suggested that *P. multocida* colonizes the oropharynx and the respiratory tract of birds before invasion into the circulation (1, 59, 82, 84, 95, 109). However, it is unclear where and how the organisms invade the respiratory tissue. In turkeys, acute cases of fowl cholera are characterized by debilitating pneumonia and airsacculitis. In peracute disease, however, spleen and liver lesions occur before the onset of respiratory symptoms indicating that active bacterial invasion components are operative in the pathogenesis mechanisms. Evidence suggests that the organisms first colonize the epithelium of the palatine clefts and the underlying lymphoid tissue, then reach the circulation leading to systemic spread (106). Subsequently, pneumonia results via hematogeneous route.

*Pasteurella multocida* adhesion to epithelial cells has received some interest (29, 30, 31, 67, 75). Studies of the HeLa cell model revealed that the capsular hyaluronic acid of serogroup A:3,4 strains of *P. multocida* promotes bacterial adhesion (30). Electron microscopy studies of cultured turkey kidney epithelial cells indicated that *P. multocida* associates with cilia, and that internalization occurs on thickened protrusions of the epithelial cell surface (75). Although the author demonstrated that internalized bacteria were not associated or enclosed in cytoplasmic organelles, the author did not examine whether or not intracellular bacteria were resistant to
killing. It is my hypothesis that, similarly, capsular hyaluronic acid promotes adhesion, but not internalization, of *P. multocida* to the palatine cleft epithelium.

The principal cellular receptor for hyaluronic acid is CD44 (2, 86). CD44 receptors are broadly distributed cell surface glycoproteins that are involved in multiple physiologic cellular functions including cell-cell adhesion (92, 124, 132), lymphocyte activation and homing (27, 49, 58, 92), and cell-extracellular substance interaction (2, 19, 86, 130, 136). The most widely expressed isoform is the 85-90 kDa glycoprotein which represents the standard CD44 molecule (CD44H), as it does not contain the products of differentially spliced exons (41, 122, 131). This isoform is principally found on hematopoietic cells, fibroblasts, epithelial cells, and some neoplastic cells (56, 103, 131). The hyaluronic acid receptor principally recognizes hyaluronic acid on adjacent cells or bound to the extracellular matrix proteins. Studies with epithelial cells, including epithelial cells of the respiratory tract, suggest that CD44 expression is tightly restricted to the lateral plasma membrane and therefore functions in cell-cell rather than cell-matrix interactions (43, 93, 123). In this interaction, the carbohydrate moieties of hyaluronic acid bind noncovalently to the transmembrane CD44 glycoprotein. It is my hypothesis that the repeated disaccharide units of the capsular hyaluronic acid of *P. multocida* interact with and bring together several lateral CD44 receptors of the epithelial cells of the avian oropharynx, thereby stimulating auto-phosphorylations of the cytoplasmic tail and signal transduction cascade (76, 98). Taken together, the intracellular signal might enhance expression of the cell-surface receptor and might inhibit internalization and or intracellular killing by the epithelial cells. Resistance to phagocytosis and resistance to intracellular killing are subjects that will be reviewed later in the discussion of subepithelial invasion. Capsulated *P. multocida* would then be confined in between epithelial cells.
due to the numerous intercellular junctions (desmosomes, tight junctions, gap junctions, adherens junctions) where bacterial multiplication could occur. The subsequent swelling pressure exerted by the hyaluronic acid of the bacterial capsule (due to the polyanionic nature of hyaluronate, as mutual repulsion between the negatively charged carboxyl groups of the glucuronate moieties causes each molecule to occupy a large domain when in solution) may expand the intercellular spaces and separate the epithelial cells of the avian oropharynx as previously described for embryogenesis and tumor metastases (137). Expansion of the intercellular spaces could ultimately help bacterial migration into the submucosa and precipitate the bacterial invasion and or allow bacteria to migrate from epithelial cells to epithelial cells as already described for *Listeria monocytogenes* (34). Depending on the host immunologic state, systemic invasion through the palatine cleft lymphoid tissue would therefore occur faster or after bacterial invasion of the lungs and air sacs (84, 106).

Alternatively, passage of *P. multocida* may occur through the specialized M cells which internalize and transport foreign particles. Receptor-mediated endocytosis by the M cells would be followed by presentation of the bacteria to the underlying lymphocytes and macrophages of the superficial mucosa. Within the M cells and mucosal phagocytes, the capsular hyaluronic acid could protect the organism against antigenic processing. Indeed, most encapsulated strains of *P. multocida* are resistant to phagocytosis and organisms that are internalized are often resistant to intracellular killing (47, 129, 139).

Many animal species exhibit age-related changes of mucosal epithelial cell surface glycoproteins. For example, pre-weaning and post-weaning diarrhea in swine are related to changes in the receptor for pili at the surface of the enterocytes. Sialic acid residues of rat
intestinal epithelium decrease with maturity and are replaced with fucose (3). Similarly, turkeys exhibit an age-related susceptibility to fowl cholera. In particular, young turkeys are less susceptible to fowl cholera than adult birds (109). It is unknown which factors are responsible for this occurrence, but age-related changes in epithelial cell receptors may be partially responsible (75). The avian respiratory epithelium is indeed extensively covered by neuraminic acid residues (sialic residues) on which neuraminidase treatment exposes other carbohydrate residues, such as mannose and galactose (20). Many *Pasteurella* isolates produce neuraminidase, and activity was correlated to virulence in one mice study (89). However, the effects of the bacterial enzymes on the avian epithelial cell surface glycoproteins have not been reported.

The hypothesis of CD44 as the cellular receptor for *P. multocida* is consistent with the above discussion of epithelial cells changes. Indeed, Sutherland *et al* demonstrated that *P. haemolytica* produces a neuraminidase that cleaves extensively O- and N-glycosylated adhesion molecules CD34, CD43, CD44, and CD45 but not other receptors lacking O-glycosylations (134). Also, it is well established that glycosylation of CD44 regulates (positively or negatively depending on the cell type and organ physiologic state) CD44-mediated cell adhesion to hyaluronic acid (4, 70). Because there is a positive correlation between the ability of tissue to bind a bacterial pathogen and the susceptibility of the host to that pathogen, both age-related cell glycosylation and bacterial enzymatic secretions may promote *P. multocida* adhesion to the epithelial cells of the respiratory tract.
**Subepithelial invasion**

After invading the epithelial layer, *P. multocida* contact the basement membrane. The basement membrane acts as a physical barrier which can inhibit invasion to a degree, but its functional integrity is soon damaged by enzymes, free radicals, complement and other inflammatory mediators. The invading microorganisms can then invade the subepithelial mucosa where bacteria are exposed to tissue matrix components and the lymphoid tissue of the palatine cleft mucosa. It is likely that *Pasteurella* produces enzymes such as neuraminidase which likely enhances its ability to move within the extracellular matrix (133, 150).

Within the extracellular matrix, the requirements for a successful cellular defense can be summarized as follows: 1) directed migration of resident and blood-borne phagocytes, 2) attachment to and ingestion of bacteria by leukocytes, 3) stimulation of oxidative burst of the leukocytes, 4) phagosome-lysosome fusion, 5) activation of the specific immune response, 6) response of phagocytes to cytokines, and 7) removal of bacterial and cellular debris. Some of these aspects will be reviewed as they relate to *P. multocida*.

Generally, damage and infection of epithelium and mucosa are rapidly followed by microcirculatory changes. Capillary, post-capillary vessels, and lymphatics are dilated, and gaps appear between endothelial cells. Vascular permeability increases, followed by leakage from the blood of a protein-rich fluid. Increased amount of immunoglobulins, complement components and other proteins are then present in tissues. Circulating leukocytes (especially heterophils and monocytes) adhere to endothelial cells, and this is followed by diapedesis and chemotaxis of leukocytes between endothelial cells toward the site of inflammation. Migration of blood and tissue leukocytes toward bacteria or bacterial products may be stimulated by the bacterial
components or may require the presence of serum for the generation of chemoattractants, especially C5a (22). With serogroup A strains of *P. multocida*, leukocyte chemotaxis was demonstrated by experimental inoculation of turkey air sacs with cell-free culture filtrates. The inoculation was rapidly followed by air sacculitis characterized by severe heterophilic infiltration of air sac membranes, large numbers of heterophils present within air sac lavage samples. These lesions were indistinguishable from those observed after experimental inoculation of the posterior thoracic air sacs with whole cells of *P. multocida* (31, 32). This experiment additionally suggested the presence of a toxin in the cell-free culture filtrate that could cause lesions to the air sac epithelium and subsequent leukocytes chemotactism to the inflammatory site. Air sacculitis can be produced by intra-air sac injection of lipopolysaccharides from *P. multocida* strain X-73 (R. A. Kunkle, personal communication.) It is plausible that the toxin suggested to cause the lesions observed in the air sacs experiments could have been bacterial lipopolysaccharide.

Binding of bacteria to phagocytes can occur in the absence of opsonins on the bacterial surface, but the presence of opsonins (e.g., immunoglobulin G, C3b, and other plasma proteins) facilitates internalization. Adhesion is followed by bacterial internalization into phagosomes and alterations of the plasma membrane lining the phagosome triggers an intracellular cascade that results in activation and mobilization of cytochrome oxidase to the inner surface of the phagosome and transportation of the lysosome to the phagosome (phagolysosome). Concomitant with this process, is the progressive decrease in the pH of the phagolysosome and a marked increase in both oxygen uptake and glucose utilization via the hexose-monophosphate pathway. The last two events provide both the energy and the reduced environment that are required for the stepwise reduction of oxygen to water and the production of numerous oxygen reactive
species. Taken together, these events create an intravacuolar environment that is extremely hostile to many bacteria, and ultimately, causes irreversible bacterial injury (25).

Survival of *P. multocida* within the host primarily depends on resistance to phagocytosis and this is reflected by the large number of bacteria present extracellularly into the blood of infected birds. The mechanisms by which encapsulated bacteria resist phagocytosis include decreased binding by serum opsonins (147), inaccessibility of ligands (immunoglobulin G, complement C3b) required for phagocyte binding (99, 151), and decreased hydrophobicity of the bacterial surface (145). Factors that enable Gram-negative bacteria to resist antibody-independent complement-mediated killing include lipopolysaccharides, outer membrane proteins, and polysaccharidic capsule. The finding that capsular hyaluronic acid of *P. multocida* 1059i (A:3) causes resistance to the bactericidal effects of the complement system in normal turkey serum (80) is supported by findings that decapsulation of 1059I with hyaluronidase induced sensitivity to complement activity. Since the amount of complement decreased in the presence of strain P1059I but the organism was not killed, it is likely that the capsule promotes serum-resistance by preventing a stable interaction between the membrane attack complex and the outer membrane. Further evidence is provided by Harmon *et al.* who compared two heavily encapsulated strains (as measured by polycationic ferritin labeling and electron microscopy) 86-1913 and CU of serotype A:3,4 of *P. multocida* to the avirulent vaccine strain M9, in phagocytosis and oxidative burst studies of dextran-activated turkey peritoneal macrophages (47). Strains 86-1913 and CU were more resistant to phagocytosis than the avirulent vaccine M9.

Production of the oxidative burst by turkey macrophages exposed to these three bacteria was compared in a chemiluminescence assay (47). Opsonization of *P. multocida* increased the
macrophage chemiluminescence index for macrophages exposed to all three bacteria. However, the chemiluminescence index for macrophages exposed to nonopsonized M9 was significantly higher than for macrophages exposed to isolates CU and 86-1913. To determine if differences in phagocytosis and chemiluminescence were due to differential cytotoxic activity of all three strains, culture supernatant was assessed. Supernatant did not, however, alter phagocyte viability or chemiluminescence. Furthermore, enzymatic removal of the capsule from isolate 86-1913 with hyaluronidase significantly increased phagocytosis by the turkey macrophages suggesting that capsule was at least partially responsible for resistance to phagocytosis. Unexpectedly, opsonization of capsulated strain 86-1913 only slightly increased phagocytosis, implying that the capsule could physically block phagocyte receptor access to antibody bound to the bacterial outer membrane. Since cytotoxicity was poor, the author suggested that capsule inhibited opsonization or compromised receptor-ligand binding between phagocytes and opsonized bacteria.

Avoidance of phagocytosis can also been produced by bacterial antiphagocytic proteins of strain 10591. A 50 kDa outer membrane protein blocked in vitro phagocytosis of Candida albicans by turkey peripheral blood monocytes (139). The factor was heat labile and digestible with trypsin, suggesting its protein nature. Removal of the capsule from the whole organism increased the antiphagocytic activity and trypsin treatment of unencapsulated organism abolished this activity, which implied that the compound was an outer membrane protein. Finally, antibodies specific to this 50 kDa compound neutralized the antiphagocytic activity of the isolated protein and turkeys given the antibody were protected against lethal challenge with P. multocida.

Phagocytosis is a two step process that includes the adhesion first, then the uptake of foreign particles. Some bacteria, such as Mycoplasma, readily attach to phagocytic cells but
manage to avoid subsequent internalization. By lacking the peptidoglycan outer layer, *Mycoplasma* inconspicuously adhere and proliferate on the leukocyte surface without being recognized. Adherence of serotype A *P. multocida* to HeLa cells is mediated by capsular hyaluronic acid (30). Internalization of the strain was not studied by the author. It is plausible that hyaluronic acid mediates adhesion of *P. multocida* but not internalization of the bacteria. Bacterial components or the mucopolysaccharide itself could subsequently protect against internalization, allowing the organisms to rapidly multiply within the extracellular matrix as it is observed in vivo. Although adhesion and internalization studies of *P. multocida* to epithelial cells and blood monocytes of various species have already been investigated, these studies have never been performed with turkey air sac macrophages.

Once bacteria are internalized, protective tactics are developed by some bacteria to escape the oxidative burst that occurs within the phagolysosome. Some bacteria fail to stimulate an oxidative burst when they are ingested, or elicit an oxidative burst that is weaker and short-lived. *Yersinia enterolitica* (78), and *Salmonella typhi* (72) are some examples of these bacteria. These effects can be mediated by lipopolysaccharide (73), and outer membrane proteins (78). Other bacteria such as *Nocardia asteroides* (33), *Listeria monocytogenes* (148) are resistant to oxygen free radical damages. With avian strains of *P. multocida*, organisms that are phagocytized are often resistant to intracellular killing (47, 129, 140). Suzuki et al (135) investigated the effects of hyaluronic acid on superoxide anion and hydrogen peroxide release after phorbol myristate acetate stimulation. The release of these oxygen intermediates was inhibited by high molecular weight hyaluronic acid in a dose dependent manner. Similarly, the capsular hyaluronic acid could promote resistance to intracellular killing by down-regulating the production of oxygen reactive
species.

**Spread via the blood and bacterial resistance to cellular defense mechanism**

Following epithelial invasion and mucosal migration, *P. multocida* may enter the blood circulation directly through the enlarged endothelial intercellular space or through the lymphatic circulation. Entrance may be also enhanced by interaction through hyaluronic acid binding adhesion molecules localized in the endothelial intercellular spaces (85). The blood is a rapid route for the spread of microbes in the body. Microorganisms that enter small blood vessels in the lungs are carried straight to the capillary beds in the systemic circulation and are transported within minutes to any part of the body.

Snipes *et al.* compared the virulence of capsulated and noncapsulated variants of the strain 1059 after intravenous exposure to 14-week-old turkeys (129). Both variants decreased in the blood 1 hour after inoculation. However, the concentration of the capsulated strain rapidly increased up to 100,000-fold the concentration of the non-capsulated strain after 1 hour. The same pattern was observed within liver and spleen, particularly 15 hours after inoculation. Histopathological examination indicated that differences between concentrations of the two strains were more likely related to an increased propensity for extracellular multiplication of the capsulated strain due to enhanced survival extracellularly rather than resistance to phagocytosis or resistance to intracellular killing. Noncapsulated organisms were likely to be more sensitive to the bactericidal effect of the normal turkey plasma. Since the bactericidal effect of the turkey plasma is destroyed by heat treatment or zymosan exposure, it is likely that the bactericidal effect resulted from complement activity and that the capsule provided resistance to the complement.
However, the capsule is not the sole criterion of virulence since capsulated isolates from birds affected with fowl cholera may not be virulent and intravenously inoculated *P. multocida* are rapidly cleared from the bloodstream regardless of the presence or the absence of a capsule (141).

Since phagocytosis follows adhesion of host cells to opsonized or non-opsonized bacteria, *P. multocida* may not be internalized by turkey blood monocytes because initial adhesion does not occur. Indeed, minimal association occurs between peripheral blood monocytes and serotype A strains of *P. multocida* during acute cases of fowl cholera (129). Subsequently, the bacteria are rapidly arrested in small capillaries and sinusoids where blood flow is slow. Leukocyte-bacterial interactions are particularly enhanced in the liver and the spleen where they encounter phagocytes of the reticuloendothelial system (Kupffer cells, splenic macrophages) that line the sinusoids, but few bacteria are internalized by either Kupffer cells or splenic macrophages. By avoiding phagocytosis, *P. multocida* can then multiply within the extracellular matrix.

Accumulation of capsulated strains of *P. multocida* within the liver may also be explained by the fact that hepatic endothelial cells express an hyaluronic acid receptor and become specific sites for hyaluronic acid degradation. Hyaluronic acid is mainly synthesized by mesenchymal cells and is released in the systemic bloodstream through the lymphatic system when tissue injury occurs. Ninety percent of the mucopolysaccharide is then removed from the blood primarily by the liver endothelial surface (28, 35, 37, 74, 126).

**Dissertation organization**

This dissertation is presented in an alternate format. The next three chapters are formatted as journal papers in which I was the senior author. The first manuscript was published in Avian
Diseases, the second has been accepted by Infection and Immunity, and the third manuscript will be submitted to the Journal of Avian Diseases. Following the third paper is a general conclusions chapter.

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Serogroup A strains of *Pasteurella multocida*, the major cause of fowl cholera, are resistant to phagocytosis in non-immunized birds. Adherence studies with a capsulated strain of *P. multocida* (serotype A:3) and turkey air sac macrophages in culture showed that the bacteria were capable of adhering in large numbers to the macrophages but were not internalized. A noncapsulated variant of the bacteria (serotype -.3) showed little or no adherence and was not internalized. These data indicated that the adhesive properties were due to the presence of a capsule on the bacteria. The role of capsular hyaluronic acid in adherence to macrophages was investigated. Depolymerization of the bacterial capsule with hyaluronidase increased phagocytosis by macrophage cultures, and addition of hyaluronic acid to the macrophages inhibited bacterial adherence. Additionally, exposure of macrophages to chondroitin sulfate B, an anionic polysaccharide similar to hyaluronic acid, did not affect the adhesive properties and resistance to phagocytosis of capsulated organisms. Treatment of macrophages with sodium metaperiodate
or trypsin suppressed bacterial binding. Collectively, these data indicate that *P. multocida* adhesion to air sac macrophages, but not internalization, is mediated by capsular hyaluronic acid and suggest that recognition of this bacterial polysaccharide is due to a specific glycoprotein receptor.

**INTRODUCTION**

*Pasteurella multocida* causes fowl cholera in both domestic and wild birds. The high morbidity and mortality associated with this disease results in significant economic losses to the poultry industry, especially the turkey industry. Transmission of the disease usually occurs following contamination of drinking water with mucous secretions from infected birds. Experimentally, a small inocula of virulent organisms (30-100) is capable of causing nearly 100% mortality within 48 hr. *P. multocida* usually enters tissues of birds through mucous membranes of the pharynx and upper air passages (8). Septicemia, pneumonia, and air sacculitis are common clinical features of turkeys that are affected by the disease.

Serogroup A strains of *P. multocida* are the major cause of fowl cholera in poultry. An ability to evade host phagocytic cells and resist complement-mediated lysis by normal turkey plasma are considered important virulence factors of pathogenic strains (5). Survival of *P. multocida* outside the host and resistance to phagocytosis in the host are associated with the presence of a capsule. With serogroup A strains of *P. multocida*, the capsule contains hyaluronic acid, an anionic polysaccharide that also occurs in host tissues (10). Hyaluronic acid is found in the capsules of other bacteria such as group A streptococci where it is recognized as a virulence factor (16). In turkeys, the capsule seems to inhibit opsonization or create physical interferences of receptor-
ligand binding between phagocytes and opsonized bacteria (11). Whether those effects in turkeys are due exclusively to the presence of hyaluronic acid is not yet clear. Although antiphagocytic properties are generally attributed to the capsule, a 50 kDa outer membrane protein capable of inhibiting phagocytosis has been described (13).

Phagocytosis is an event which follows adhesion of host cells to opsonized or non-opsonized bacteria. Although adhesion of *P. multocida* to respiratory epithelia has received some interest, studies on adhesion to tissue phagocytic cells are sparse. The adhesive properties of *P. multocida* to cultured HeLa cells and mammalian phagocytes have been examined (2, 3). Use of the HeLa cell model revealed that capsular hyaluronic acid could mediate the bacterial adhesion (2). Nevertheless, studies on *P. multocida* adhesion to avian phagocytes have not been described.

This study was designed to investigate whether: 1) capsulated *P. multocida* would adhere to turkey air sac macrophages, 2) adherence occurred by means of hyaluronic acid contained in its capsule, and 3) a receptor specific for hyaluronic acid occurred on the surface of macrophages.

**MATERIAL and METHODS**

**Animals.** Normal Beltsville small white turkeys, 8-12 weeks old, from the National Animal Disease Center were used.

**Bacteria.** Encapsulated (serotype A:3) and noncapsulated (serotype -.3) *Pasteurella multocida* strain P-1059 were used. The noncapsulated variant was derived by serial passage on dextrose starch agar and colonies that appeared blue in oblique-transmitted light were selected. The bacteria were grown overnight at 37 C on dextrose starch agar (DSA, Baltimore Biological laboratories, Cockeysville, MD). Bacterial cells were re-suspended in RPMI-1640 without
sodium bicarbonate and phenol red (mRPMI, Sigma Chemical Co., St Louis, MO). The suspensions were adjusted to a density equivalent to that of a number 2 MacFarland nephelometer standard (~2 x 10⁹ bacteria/ml) using a spectrophotometer (model 35, Perkin-Elmer, Oak Brook, IL).

**Phagocytic cell recruitment and collection.** A sterile suspension of Sephadex G-100 superfine (Pharmacia Fine Chemicals AB, Uppsala, Sweden), pre-swollen in 0.85% sterile saline, was the eliciting agent used to recruit phagocytic cells. For recruitment, left and right posterior thoracic air sacs were inoculated with the eliciting agent. The suspension was injected about 2 cm deep with syringe and needle at a site located dorsally in the last intercostal space. The injected volume was 1 ml of suspension/100 g of body weight. After 72 hr, turkeys were euthanatized (Sleepaway, Fort Dodge Laboratories, Inc., Fort Dodge, IA) and opened to expose the anterior and posterior thoracic air sacs. Care was taken to avoid cutting major blood vessels. Recruited cells were collected by flushing the air sacs with cold Hanks balanced salt solution (HBSS, Gibco Laboratories Inc., Grand Island, NY) that contained 0.5 U of heparin per ml; collection was done with a siliconized Pasteur pipet. The cells were centrifuged at 600 x g for 10 min at 22 C, and the pellet was resuspended in Minimum Essential Eagle medium (MEM, Sigma Chemical Co., St. Louis, MO) supplemented with 100 U/ml penicillin, 50 µg/ml streptomycin, 2 µg/ml fungizone, and 10% heat-inactivated fetal bovine serum (mMEM).

**Removal of the heterophils.** The cell suspension was layered on a density gradient medium (Accuprep [sp. G. 1.077], Accurate Chemical & Scientific Corporation, Westbury, NY) in 13 x 100 mm tubes according to the instructions provided by the manufacturer. Tubes were centrifuged at 600 x g for 15 min at 22 C. The cell layer above the gradient medium was
collected and diluted 1:3 with 0.85% sterile saline supplemented with 0.13% ethylenedinitrilo-tetra acetic acid (EDTA, Eastman Fine Chemicals, Rochester, NY) to reduce the density of the solution. The diluted layer was centrifuged at 500 x g for 10 min at 22 °C. The cell pellets containing the air sac macrophages (ASM) were resuspended in mMEM and cell counts were made using a cell counter (Nova Cell Track, Alicia Diagnostics, Oveido, FL). Four-well chamber slides (Lab Tek®, Nunc Inc., Naperville, IL) or 25 cm² tissue culture flasks (Corning Inc., Corning, NY) were seeded with 2 to 4x10⁵ macrophage/cm². After 2 hr of incubation at 37 C under 5% CO₂, the medium was changed and macrophage cultures were further incubated overnight before use in adhesion assays.

**Adhesion assays.** Overnight ASM cultures were rinsed 3 times with MEM plus 10% heat-inactivated fetal bovine serum and adjusted suspensions of *P. multocida* were added to give a final concentration of 10⁶ bacteria/10⁶ macrophages. After addition of bacteria, incubation was performed 2 hr at 37 C in an atmosphere containing 5% CO₂. For enumeration of bacteria adhering to ASM, the slides were rinsed 3 times with MEM, stained by Diff Quick (Baxter Healthcare Corporation, McGaw Park, IL) and examined with a light microscope. Adhesion was evaluated by observing 50-100 randomly chosen macrophages. Each experiment was repeated with three different birds.

A modification of the double fluorescence technique of Detilleux, et al. (1) was used to discriminate between adherent and internalized bacteria as follows: 1) chamber slides of ASM exposed to bacteria were washed (3 times) in PBS supplemented with 3% fetal bovine serum (mPBS); 2) residual ASM-associated bacteria were labeled for 1 hr at 4 C in the dark with a 1:3000 dilution of rabbit anti-Pasteurella serum; 3) the slides were washed 3 times with mPBS,
macrophages were fixed for 5 min in methanol at 4 C, dehydrated for 5 min in acetone at 4 C, and then air dried; 4) a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated, goat anti-rabbit antiserum (Kirkegarrde and Perry Laboratories, Rockville, MD) was applied for 1 hr at 4 C in the dark; 5) the slides were washed with mPBS (3 times) and treated for 3 min with propidium iodide (PI, Sigma Chemical Co., St. Louis, MO) solution (25 μg PI/ml PBS); and 6) slides were washed with mPBS, rinsed with distilled water, and immediately mounted (Vecta Mounting Medium, Vector Laboratories, Inc., Burlingame, CA) with coverslips. Fluorescence was observed with a BX50 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a reflected light fluorescent attachment and a filter cube (U-M51005) for propidium iodide and FITC. Because fluorescent bacteria could not be precisely enumerated, the adherence or the internalization were determined subjectively with 0, indicating no adherence or internalization and 1+, 2+, 3+ indicating slight (1 to 25 bacteria/macrophage), moderate (26 to 75 bacteria/macrophage, and heavy (≥76 bacteria/macrophage) adherence or internalization, respectively.

Antiserum. Anti-Pasteurella serum was made in rabbits with capsulated strain P-1059 as described by Rimler and Brogden (9). The antiserum was absorbed with turkey liver powder to remove any cross-reactive antibodies for turkey cells and tissues.

Decapsulation and adherence inhibition. To demonstrate the role of hyaluronic acid in binding, capsulated P. multocida were treated with 250 U of hyaluronidase (Sigma Chemical Co., St. Louis, MO) /1x10⁹ bacteria in mRPMI for 2 hr at 37 C before addition to macrophage cultures.

Adherence inhibition studies were performed by treating macrophage cultures in MEM for 2 hr at 37 C and 5% CO₂ with one of the following substances: hyaluronic acid (5 mg/ml, Sigma
Chemical Co.); trypsin (250 U/ml, Millipore Corporation, Freehold, NJ); chondroitin sulfate B
(5 mg/ml, Calbiochem, La Jolla, CA); and 4.278 mg/ml sodium metaperiodate. After each
treatment, \textit{P. multocida} were added to the treated macrophage cultures in MEM plus 10% fetal
bovine serum, and incubation was done for an additional 2 hr. The final concentration was $2 \times 10^8$
bacteria/1x10^6 macrophages.

Electron microscopy. To confirm adherence and internalization observed by the fluorescence
technique, the macrophages were transferred to sterile siliconized centrifuge tubes, using a rubber
policeman. The cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH
7.4) at 4°C. After fixation, the tubes were lightly centrifuged (for 5 min at 82 x g) to pellet the
cells. The glutaraldehyde was discarded, and the cells were stored in 0.1 M sodium cacodylate
buffer at 4°C. Macrophages were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate
buffer at 4°C, dehydrated in ethanol, infiltrated and embedded in epoxy resin, sectioned at 70 to
90 nm, and examined with a Philips 410 electron microscope.

Statistical analysis. Student's t-test was used to determine statistical probabilities.

RESULTS

Adhesion assays. Enumeration of bacteria associated with ASM was determined by light
microscopy and distinction between adherent and internalized bacteria was determined by
fluorescence microscopy. An example of capsulated \textit{P. multocida} adhered to ASM is shown in
Fig 2.1. Note that the bacteria are closely associated with macrophages and there are no bacteria
free in the intercellular spaces. With the double fluorescence method, primary antibodies do not
penetrate the plasma membrane of unfixed cells (1). Therefore adherent bacteria, but not
internalized bacteria, react to produce bright-green fluorescence with labeled secondary antibodies (Fig. 2.2). Since methanol-acetone fixation results in membrane permeability, both macrophage DNA (Fig. 2.2 and 2.3) and internalized bacteria fluoresce bright red (Fig. 2.3). Similar to light microscopic observations with the Diff Quik stain procedure, fluorescent microscopy showed bacteria were intimately associated with macrophages and there were no bacteria free in the intercellular spaces. The findings observed with fluorescence microscopy were confirmed by electron microscopy and an example of a macrophage with internalized bacteria is shown in Fig. 2.4.

**Influence of hyaluronic acid on bacterial adhesion.** Capsulated bacteria were adherent but not internalized by ASM (Table 2.1). Comparisons of adhesion between capsulated P-1059 and its noncapsulated variant indicated that the former had significant higher incidence of adherence (P< 0.05). These data suggested that the presence of a capsule promoted adherence. Depolymerization of the bacterial capsule with hyaluronidase resulted in a high degree of bacterial association with ASM (P<0.05). In contrast to non-treated bacteria, hyaluronidase-treated bacteria were internalized.

Pre-incubation of the ASM with chondroitin sulfate B, an anionic polysaccharide similar to hyaluronic acid, did not significantly affect adherence of capsulated bacteria (Table 2.2). Although exposure of ASM to a concentration of 5 mg/ml of hyaluronic acid diminished adherence of capsulated bacteria, adherence of hyaluronidase-treated *P. multocida* was not affected (data not shown).

Oxidation of the macrophage surface with sodium metaperiodate markedly reduced the bacterial adhesion. Moreover, proteolytic treatment of the macrophages with trypsin suppressed
bacterial binding (Table 2.2).

**DISCUSSION**

Pneumonia and airsaccultis are common findings in acute as well as chronic fowl cholera, and recent studies have suggested that damaged air sac epithelium may be a portal of entry for *P. multocida* into the systemic circulation (4). Respiratory tract macrophages are an important line of defense. However, numbers of resident macrophages in the respiratory tract of normal chickens and turkeys are very low compared with those in mammals of similar body weight (12). Although respiratory tract infection with *P. multocida* attract macrophages, non-opsonic phagocytosis by these cells has not been demonstrated. Adhesion to phagocytic cells and subsequent internalization are significant features in the pathogenesis, as well as the prevention, of many bacterial diseases. In the present study, with recruited air sac macrophages, capsulated *P. multocida* were adherent but not internalized. These findings are contrasted by those seen in preliminary studies in which bacteria neither adhered nor were internalized by peripheral blood monocytes (Pruimboom, unpublished). Capsule depolymerization with hyaluronidase increased adhesion and subsequent internalization of the bacteria by recruited macrophages. This observation together with the findings of the inhibition studies demonstrated the presence of a specific hyaluronic acid receptor. Apparently this hyaluronic acid receptor is absent on normal peripheral blood monocytes.

This report showed that hyaluronic acid is the capsular component that mediates adhesion to recruited air sac macrophages. Hyaluronic acid seems to prevent internalization as described for group A Streptococcus (16). It is clear from our studies, as well as others (5, 15), that enzymatic
depolymerization of the capsule enhances phagocytosis of *P. multocida*. This finding suggest that a receptor other than that for hyaluronic acid is necessary for adherence and non-opsonic internalization of capsule depolymerized bacteria.

Treatment with trypsin and oxidation with metaperiodate abolished or diminished hyaluronic acid-mediated adherence to the recruited macrophages. These findings suggest that the hyaluronic acid receptor is a glycoprotein. Further investigations will be required to confirm this observation.

The role of the macrophage hyaluronic acid receptor in fowl cholera is not clear. Following experimental intravenous infection, capsulated *Pasteurella multocida* are rapidly cleared from the blood and are found primarily in the liver and spleen (7,14,15). Because little or no non-opsonic phagocytosis occurs and the bacteria tend to concentrate and reproduce in these organs, it seems probable that a hyaluronic acid receptor on the resident macrophages may be a colonization factor. Identification of a hyaluronic acid receptor on resident macrophages of the liver and spleen and determination as to whether it is involved in clearance of *P. multocida* from circulating blood awaits further study.

**REFERENCES**


Table 2.1: Adherence and internalization of *P. multocida* by air sac macrophages.

<table>
<thead>
<tr>
<th></th>
<th>Adhesion of bacteria</th>
<th>Internalized bacteria</th>
<th>Number of bact. / macr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsulated P-1059</td>
<td>2+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1+</td>
<td>50.2 ± 6.2</td>
</tr>
<tr>
<td>Noncapsulated P-1059</td>
<td>1+</td>
<td>1+</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>Hyaluronidase treated P-1059</td>
<td>1+</td>
<td>3+</td>
<td>101.2 ± 16.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by fluorescent microscopy

<sup>b</sup> Determined by Diff Quick stain (mean of 3 experiments)

<sup>c</sup> 1+ = slight, 2+ = moderate, 3+ = heavy (see text)
### Table 2.2: Inhibition of adhesion of capsulated *P. multocida* to air sac macrophages.

<table>
<thead>
<tr>
<th>Macrophages treatment</th>
<th>Adhesion inhibitiona</th>
<th>Number of bact./macr. b</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No</td>
<td>50.2 ± 6.2</td>
<td>—</td>
</tr>
<tr>
<td>Hyaluronic acid (5 mg/ml)</td>
<td>Yes</td>
<td>3.8 ± 1.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Chondroitin sulfate B (5 mg/ml)</td>
<td>No</td>
<td>45.4 ± 1.5</td>
<td>Not Signif.</td>
</tr>
<tr>
<td>Metaperiodate (4.278 mg/ml)</td>
<td>Yes</td>
<td>6c</td>
<td>NDd</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Yes</td>
<td>1.1 ± 0.9</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

a Determined by Diff Quick stain

b Mean of 3 experiments (see text)

c Experiment done once

d Not determined
Figure 2.1: Example of Diff Quick stain of capsulated *P. multocida* adhering to recruited air sac macrophages. Bar: 10 mm = 5.27 μm.
Figure 2.2: Double fluorescence technique showing adherent capsulated $P.\ multocida$.

Adherent bacteria (3+, see text) appear green (arrow) and macrophage nuclei appear red. The cytoplasm of the phagocyte is lightly stained in red. Bar: 10 mm = 10.2 $\mu$m.
Figure 2.3: Double fluorescence technique showing internalization of *P. multocida* (3+; see text) after depolymerization of the capsule. Internalized bacteria (arrow) appear bright red.

Bar: 10 mm = 2.98 um.
Figure 2.4: Electron micrograph of turkey air sac macrophage with internalized bacteria.

Bar: 4.9 mm = 0.5 um.
CHAPTER 3: ENHANCED ADHESION OF PASTEURELLA MULTOCIDA TO CULTURED TURKEY PERIPHERAL BLOOD MONOCYTES

A manuscript submitted to the Journal Infection and Immunity.

Ingrid M. Pruimboom, Richard B. Rimler.

ABSTRACT

Capsular hyaluronic acid (HA) mediates adhesion of serogroup A strains of *P. multocida* to elicited turkey air sac macrophages (TASM). In contrast, freshly isolated turkey peripheral blood monocytes (TPBM) do not bind serogroup A strains. Following culture of TPBM for 6 days in chamber slides, adhesion of the bacteria to TPBM increased gradually. Incubation in chamber slides coated with Entactin-Collagen IV-Laminin attachment (ECL) matrix or exposure to phorbol myristate acetate (PMA) further enhanced the adhesion of *P. multocida* to TPBM. Addition of HA to TPBM culture, but not Arg-Gly-Asp peptide, inhibited bacterial adherence similarly to that previously reported for TASM. Exposure of TPBM to MAb directed against HA-binding cell surface proteoglycan (CD44) decreased binding of *P. multocida*. Collectively, these findings indicate that *P. multocida* adhesion to TPBM is mediated by capsular HA and can be up-regulated by culture on ECL matrix or PMA exposure. Additionally, the findings suggest that the capsular mucopolysaccharide of serogroup A strains of *P. multocida* recognizes an
ISOFORM OF CD44 EXPRESSED ON CULTURED TPBM.

INTRODUCTION

Pasteurella multocida causes fowl cholera, a widely distributed disease occurring in most poultry-producing countries of the world. Annual worldwide losses to the poultry industry were estimated at 200 million US dollars in 1986 (11). Serogroup A strains of P. multocida are the major cause of fowl cholera in turkeys. Survival of P. multocida outside the host and resistance to phagocytosis in non-immunized birds are associated with the presence of a capsule. With serogroup A strains of P. multocida, the capsule contains HA, an anionic polysaccharide composed of repetitive disaccharidic units of N-acetyl-D-glucuronic acid and N-acetyl-D-glucosamine. Invasion by P. multocida is believed to occur through the lymphoid tissues of the respiratory tract, and the bacterial capsule is suspected to play a major role in this event.

Previous studies from our laboratory demonstrated that serogroup A strains of P. multocida adhere to TASM but are not internalized. Although we showed that bacterial adhesion to TASM occurs through specific recognition of capsular hyaluronate by a cell surface glycoprotein (10), the host cell receptor was not identified. CD44, an 85-kDa transmembrane glycoprotein found on a variety of cell types, is one of several receptors capable of binding HA. Because CD44 is an HA receptor also associated with lung macrophages (3, 5), we suspected that it might be involved in adhesion of serogroup A strains of P. multocida to TASM. However, with avian species, the number of resident macrophages in the lungs and air sacs is low and recovery by lavage is poor (12). Consequently, our initial attempts to isolate CD44 from TASM failed due to the inability to acquire sufficient numbers of cells. This failure prompted a study to
determine whether freshly isolated TPBM could replace elicited TASM. We found that serogroup A strains of *P. multocida* do not adhere to freshly isolated TPBM. Similarly, others have reported that freshly isolated peripheral blood monocytes do not bind soluble HA but that *in vitro* culture for 8 to 16 hr (7), as well as exposure to phorbol myristate acetate (8), will increase HA binding. Hyaluronic acid is recognized as a major component of the extracellular matrix in animals. Interactions of blood monocytes with the extracellular matrix have a central role in their tissue-specific migration, differentiation, and function. These HA binding proteins, although binding with higher affinity to HA, also bind fibronectin, collagen (I, II, and IV), and laminin (1, 13, 14).

The goals of this study were to determine whether 6-day culture, exposure to PMA, or culture on ECL matrix of freshly isolated TPBM would increase adhesion of serogroup A *P. multocida*.

**MATERIAL AND METHODS**

**Animals.** Twelve to 24 week-old, male, Beltsville small white turkeys from the closed flock at the National Animal Disease Center were used. Females were not used due to high serum lipid concentration that impeded isolation of blood monocytes using centrifugation procedures.

**Monoclonal antibodies.** The following monoclonal antibodies purchased from Sigma (Sigma Chemical Co., St Louis, MO) were used: mouse anti-human CD44 monoclonal antibody (IgG1 isotype derived from hybridoma A3D8), FITC (fluorescein isothiocyanate) conjugated mouse anti-human CD44 monoclonal antibody (mouse IgG1 isotype derived from hybridoma A3D8), and the nonspecific mouse monoclonal immunoglobin G (IgG1).
**Bacteria.** Capsulated *P. multocida* strain P-1059 (serotype A:3) was grown overnight at 37°C on dextrose starch agar (DSA, Baltimore Biological Laboratories, Cockeysville, MD). The bacterial cells were resuspended in RPMI-1640 without sodium bicarbonate and phenol red (Sigma Chemical Co.). The bacterial suspension was adjusted to a density equivalent to that of a number 1 MacFarland nephelometer standard (1x10⁹ bacteria/ml) using a spectrophotometer (model 35, Perkin-Elmer, Oak Brook, IL).

**Turkey peripheral blood monocyte collection.** A two step-gradient procedure for isolation of monocytes was used as follows. Turkey peripheral blood was collected by venepuncture of the brachycephalic vein into EDTA treated-vacutainer tubes (Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ). Pooled blood from 3 birds was diluted 1:2 with RPMI 1640 containing L-glutamine but lacking sodium bicarbonate (Sigma Chemical Co.). When necessary, penicillin (100 U/ml), streptomycin (50 μg/ml), and fungizone (2 μg/ml) were added (mRPMI). For separation of white blood cells, 6 ml of diluted blood was layered onto 3 ml of density gradient medium (Accu-Prep®, sp. G. 1.077; Accurate Chemical & Scientific Corporation, Westbury, NY) in 13 x 100 mm tubes, and tubes were centrifuged at 800 x g for 15 min. at 22°C. The white blood cell layer above the gradient medium was collected and diluted 1:2 with mRPMI. Six ml of the diluted suspension was applied onto 3 ml of a second density gradient medium (1-Step Monocytes®, sp. G. 1.068; Accurate Chemical & Scientific Corporation) in 13 x 100 mm tubes. Tubes were centrifuged at 600 x g for 15 min. at 22°C. After centrifugation, the top gradient, including the interface, was removed to a level just above the cell pellet and diluted 1:3 with 0.85% NaCl containing 0.13% EDTA to reduce the density of the solution. The diluted gradient was centrifuged at 600 x g for 10 minutes at 22°C. The cell pellet containing the
monocytes was resuspended in mRPMI supplemented with 10% of heat-inactivated fetal bovine serum (ΔmRPMI), and cell counts were made using a cell counter (Nova Cell Track, Alicia Diagnostics, Oveido, FL). Cell viability was assessed using propidium iodide dye. Briefly, 25 μl of propidium iodide (0.5 mg/ml) was added to 100 μl of the monocyte suspension. This suspension was gently mixed and incubated for 5 min. at 22°C before spreading onto an hemacytometer. Counts were made using light and fluorescence microscopy; dead cells fluoresced orange. Cell viability above 85% was the minimum requirement for all experiments. Isolated monocytes were seeded (5 x 10^5 monocytes/cm²) in 4-well chamber slides (Lab Tek®, Nunc Inc., Naperville, IL).

**Adhesion assays.** Expression of *P. multocida* receptor by cultured TPBM was investigated daily for 6 days using adhesion assays. To perform assays, TPBM cultures were rinsed with warm (37°C) RPMI supplemented with heat-inactivated ΔRPMI and followed by re-incubation for 1 hour with warm ΔRPMI. Cultures were rinsed with warm ΔRPMI, and adjusted suspensions of *P. multocida* were added to a final concentration of 100 bacteria/monocyte. Culture slides were incubated for 1.5 hr at 37°C and 5% CO₂. Slides were rinsed 3 times with warm ΔRPMI to remove non adherent bacteria and stained with Diff Quick (Baxter Healthcare Corporation, McGaw Park, IL). Adhesion of *P. multocida* was evaluated with light microscopy by counting the total number of adherent bacteria on 200-400 randomly chosen monocytes. All experiments were repeated three times.

**Up regulation of bacterial adhesion.** To determine whether Entactin-Collagen IV-Laminin (ECL) attachment matrix would enhance bacterial adhesion, 15 μg/cm² of ECL cell attachment matrix (Promega, Madison, WI) was added directly onto chamber slides according to the
instructions provided by the manufacturer. Wells were seeded with $5 \times 10^4$ monocytes/cm$^2$ in AmRPMI and cultures were incubated for 6 days at $37^\circ$C in a 5% CO$_2$ atmosphere. For controls, uncoated chamber slides were treated similarly as described above.

To determine whether PMA (Sigma Chemical Co.) would increase bacterial adhesion, cultured TPBM were exposed to 15 ng PMA/ml AmRPMI for 12 min. at $37^\circ$C and 5% CO$_2$ on days 1 and 5 of incubation. Following exposure, supernatant was removed and replaced by warm AmRPMI. On day 6, 5 hr before performing adhesion assays, TPBM were re-exposed to PMA as before.

**Adherence inhibition studies.** Cell surface proteoglycans recognize specific amino-acid sequences or extracellular polysaccharides of the extracellular matrix. We previously showed that capsulated *P. multocida* adhesion to elicited TASM occurs through specific recognition of the capsular polysaccharide by a cell surface glycoprotein (10). Others have shown that adhesion molecules can also bind to a specific amino acid sequence of extracellular matrix components, such as the Arg-Gly-Asp peptide of laminin, fibronectin, and collagen (9). To determine whether the *P. multocida* receptor on cultured TPBM is the same as that previously described for TASM, 6-day old TPBM were incubated with 2.5 mg HA (Sigma Chemical Co.)/ml ARPMI and 1 mg Arg-Gly-Asp peptide (Sigma Chemical Co.)/ml ARPMI for 1.5 hr at $37^\circ$C and 5% CO$_2$ prior to adhesion assays. The culture fluid was discarded, the chamber slides rinsed with warm ARPMI, and the TPBM were used in adhesion assays.

To determine whether CD44 functions as receptor for *P. multocida* on cultured TPBM, 6-day old monocytes were exposed for 30 minutes to 20 μg of anti-CD44 MAb/5x$10^5$ TPBM in 1 ml of warm RPMI at $37^\circ$C and 5% CO$_2$ before adhesion assays. Antibodies were not removed.
during these assays. For control, isotype-matched non-specific mouse monoclonal IgG1 was used in the same conditions as above.

**Fluorescence microscopy.** Fluorescence microscopy was used to confirm CD44 expression by TPBM as follows: 1) 6-day old cultured TPBM were washed three times in warm RPMI; 2) TPBM were fixed for 5 minutes in methanol at 4°C, dehydrated for 5 minutes in acetone at 4°C, and air dried; 3) non specific binding sites on TPBM (4 x 10^6 cells) were blocked for 3 hr at 22°C with a 1:25 dilution of mouse isotype-matched control IgG1, in phosphate buffered saline (PBS, pH 8); 4) slides were washed three times with PBS; 5) TPBM were labeled with a 1:25 dilution of FITC-conjugated anti-human CD44 MAb in PBS for 3 hr at 22°C; 6) culture slides were washed three times with PBS and rinsed with distilled H2O; and 7) following washes, coverslips were immediately mounted with mounting medium (Vector Laboratories, Inc., Burlingame, Calif). To determine whether false positive reactions might arise due to monoclonal antibody Fc segment binding to monocyte Fc receptor, FITC-labeled anti-CD44 F(ab')2 was also tested as above, but starting at step 5. Fc portion of the anti-CD44 IgG was removed using the Immunopure® F(ab')2 Kit from Pierce (Rockford, Ill), according to the instructions provided by the manufacturer. Preparation fluorescence was observed with a BX50 microscope equipped with a reflected light fluorescent attachment and a U-MNIBA filter cube (Olympus Optical Co, Tokyo, Japan).

**Statistical analysis.** Student's t-test was used to determine statistical probabilities.
RESULTS

**Turkey peripheral blood monocyte collection.** Cells isolated by the 2-step gradient procedure consisted of monocytes as the only adherent cell population with small numbers of non-adherent lymphocytes. Heterophils and red blood cells were excluded from the monocyte fraction, and lymphocytes were removed by rinsing slides after adherence of monocytes.

**TPBM cultures and bacterial adhesion.** Due to meniscus formation by the media in the chamber slide wells, monocytes and bacteria tended to accumulate at the edges of the wells. Consequently, uniformly distributed monolayers were seen in the corners of wells but not at their centers. For each adhesion assay, the average number of bacteria per TPBM was estimated by counting 200-400 monocytes located along a diagonal line from well corner to well corner, using 63X magnification with oil. In each experimental trial, the blood from 3 birds was pooled. Non uniformity of monocytes throughout the well and pooling of the blood resulted in increased standard deviation of the observations. Nevertheless, adhesion of encapsulated P-1059 to cultured TPBM increased daily during the 6-day observation period to reach a maximum by day 6 \( P < 0.0001; \) (Fig. 1). However, when data from each experiments were considered individually, maximum of adhesion occured at day 4 in experiment 1 and day 6 in experiments 2 and 3. Adhesion was significantly enhanced \( P < 0.0001; \) (Fig. 1) when chamber slides were coated with ECL matrix. Synergism between increased time of incubation and growth on ECL matrix was observed in enhancing adhesion of *P. multocida* to monocytes \( P < 0.0001 \).

**Influence of PMA on bacterial adhesion.** Treatment of TPBM cultures with PMA, a diacylglycerol analogue, resulted in a significantly increased bacterial adherence to monocytes \( P < 0.0001; \) (Table 1). Light microscopic examination showed that treated monocytes
increased in size and were often multinucleated when compared to controls (Fig. 2.a, 2.b). Based
upon this observation, counts of bacteria/monocyte were determined by dividing the total number
of bacteria per monocyte by the number of nuclei within the observed cell. This condition was
applied for each of these experimental trials. Although bacterial adhesion was up-regulated,
bacteria were not internalized as determined by a double fluorescence technique (10).

**Adhesion inhibition studies.** Treatment of cultured TPBM with HA or anti-human CD44
MAbs inhibited adherence of the encapsulated bacteria ($P < 0.0001$). However, Arg-Gly-Asp
peptide treatment or non-specific isotype-matched mouse IgG$_1$ (control) did not (Table 1).

**Fluorescence studies.** Membrane and cytoplasm of 6 day-old cultured TPBM were
homogeneously and intensely stained by FITC-labeled anti-human CD44 MAb. Incubation with
isotype-matched control IgG$_1$,$\kappa$ did not suppress fluorescence. Although removal of the Fc
portion from the specific antibody did not abolish fluorescence, the intensity of the reaction was
reduced and photobleaching increased (Fig. 3).

**DISCUSSION**

The respiratory system of avian species markedly differs from that of mammals. After
entering the lung, air does not terminate in an alveolus. Air continues through the lung to
thoracic and peritoneal air sacs of the bird, thereby allowing gas exchanges at both inspiration and
expiration. Although air exchange efficiency is very high, the defense mechanisms against
airborne pathogens are suggested to be poorly developed due to the paucity of resident lung and
air sac macrophages (12).

In turkeys, pneumonia and airsacculitis are common features of acute as well as chronic cases
of fowl cholera. It is believed that damaged air sac epithelium is one portal of entry for *P. multocida* in naturally occurring cases of fowl cholera. Ficken *et al* (4) reported that experimental inoculation of air sacs with *P. multocida* is followed by an acute, marked heterophilic exudation with macrophage accumulation occurring to a lesser extent later in the inflammatory reaction. However, non-opsonic phagocytosis by these macrophages was not observed and adhesion of *P. multocida* to the macrophages was not described. Although we previously described *P. multocida* adherence to elicited air sac macrophages (10), we found that adherence did not occur with freshly isolated peripheral blood monocytes. Because elicited air sac macrophages most likely arise from peripheral blood monocytes, expression of HA receptor could be a hallmark of differentiation or activation of these phagocytes.

Bacterial adhesion was stimulated by culturing monocytes *in vitro* for 6 days. Others have shown and our own experience indicates that the presence of serum is required for monocyte activation. In this study, however, heat-inactivated serum did not induce opsonization-mediated adhesion of the bacteria. Adhesion was not blocked by exposure of monocytes to the Arg-Gly-Asp peptide, but was significantly decreased after treatment with HA. This evidence strongly suggests that adhesion of *P. multocida* to TASM as well as cultured TPBM is mediated by the mucopolysaccharide of the bacterial capsule.

Hyaluronic acid is a major component of the host extracellular matrix, as well as the principal capsular component of serogroup A strains of *P. multocida*. Macromolecules of the extracellular matrix are believed to be involved in the regulation of various cellular functions including cell adhesion, motility, growth, and differentiation *in vitro* (6). These functions are mediated by HA interacting with cell surface proteoglycans expressed on various cell types of which macrophages
are included. Although these proteoglycans bind with high affinity to HA, they can also bind to fibronectin, collagen (I, II, and IV), and laminin. We hypothesized that culturing TPBM on ECL extracellular matrix would enhance expression of HA-binding protein, by increasing bacterial adhesion. Our findings confirmed this hypothesis.

CD44H, the hematopoietic isoform of CD44, is a major protein expressed on human monocytes and lymphocytes. In the murine thymoma cell line BW5147, the cytoplasmic domain of CD44 interacts with ankyrin of the cytoskeleton, and this interaction is essential for HA binding. This event is mediated by protein kinase C (2). Phorbol myristate acetate is a diacylglycerol analogue, and diacylglycerol is known to trigger the activation and translocation of protein kinase C. We suspected that CD44 was the receptor for *P. multocida* on cultured TPBM and that triggering protein kinase C with PMA would enhance expression of monocytic CD44. In this study, we found that bacterial adhesion was enhanced after exposure to PMA and fluorescence microscopy clearly confirms the expression of CD44 by 6-day cultured TPBM. CD44 expression by freshly isolated TPBM was not investigated by fluorescence microscopy.

In conclusion, we have shown that *P. multocida* adhesion to TPBM is mediated by capsular HA and can be enhanced by culture on ECL matrix or PMA exposure. Recognition of the capsular mucopolysaccharide is due to a monocytic CD44 isoform present on cultured TPBM. This is the first report describing CD44 as a receptor for bacterial adherence. Recognition of CD44, a membrane glycoprotein involved in cell-matrix interactions, may be used by *P. multocida* to invade the host tissue. The isolation of this receptor from “activated” turkey blood monocytes awaits further study.
REFERENCES


Figure 3.1: Kinetics of *P. multocida* adhesion to cultured turkey peripheral blood monocytes. Values are the mean of 3 experiments.
Figure 3.2: Diff Quick stain of capsulated *P. multocida* adhering to cultured turkey blood monocytes. a: Day 1, control, Bar = 14.1 μm; b: Day 6, after exposure to phorbol myristate acetate, Bar = 18.9 μm.
Figure 3.3: FITC labeled anti-human CD44 Mab fluorescence after removal of the Fc portion
(arrow). Bar = 19.34 μm.
Table 3.1. Influence of various treatments (hyaluronic acid, phorbol myristate acetate, Arg-Gly-Asp peptide, anti-CD44) on *in vitro* adhesion of *P. multocida* to 6-day cultured turkey peripheral blood monocytes (TPBM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of <em>P. multocida</em> per TPBM</th>
<th><em>P</em> value</th>
<th>Effect of treatment</th>
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<tr>
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<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>None</td>
<td>21.06 – 0.81</td>
<td>24.19 – 1.69</td>
<td>21.74 – 1.53</td>
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<tr>
<td>Hyaluronic acid</td>
<td>1.35 – 0.09</td>
<td>0.78 – 0.07</td>
<td>1.42 – 0.16</td>
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<tr>
<td>None</td>
<td>12.62 – 0.95</td>
<td>11.03 – 0.60</td>
<td>8.79 – 0.65</td>
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<td>PMA</td>
<td>26.03 – 1.37</td>
<td>41.36 – 15.25</td>
<td>56.31 – 8.15</td>
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<tr>
<td>None</td>
<td>18.79 – 1.30</td>
<td>21.65 – 1.38</td>
<td>Not done</td>
</tr>
<tr>
<td>Arg-Gly-Asp peptide</td>
<td>20.69 – 1.16</td>
<td>20.56 – 1.13</td>
<td>Not done</td>
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<td>None</td>
<td>36.02 – 0.97</td>
<td>21.06 – 0.81</td>
<td>13.09 – 0.70</td>
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<td>Anti-CD44</td>
<td>7.30 – 2.69</td>
<td>3.77 – 0.24</td>
<td>13.09 – 4.49</td>
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</table>
CHAPTER 4: PASTEURELLA MULTOCIDA SEROTYPE A:3: IDENTIFICATION AND PARTIAL PURIFICATION OF THE BACTERIAL RECEPTOR EXPRESSED BY TURKEY PERIPHERAL BLOOD MONOCYTES

A manuscript to be submitted to the Journal of Avian Diseases

By Ingrid M. Pruimboom and Richard B. Rimler

SUMMARY

Serotype A:3 strains of P. multocida, the main serotype causing fowl cholera in turkeys, have an hyaluronic acid (HA) capsule. Previous studies have shown that the capsular polysaccharide promotes bacterial adhesion to cultured turkey peripheral blood monocytes. In this study, HA-Sepharose affinity chromatography was used to partially purify HA binding proteins (HABP) from cultured TPBM. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of HABP under reducing conditions, electroblots showed three major bands (81, 101, and 118 kDa) which reacted with a biotinylated HA probe. Both the 101 and 118 kDa proteins were eluted from preparative SDS-PAGE gels by reverse polarity electrophoresis and used to immunize rabbits. Preinoculation serum reacted with several TPBM antigens as well as the 101 and 118 kDa HABP. Immunization did not result in higher titers or increased specificity with the HABP.
INTRODUCTION

*Pasteurella multocida* causes fowl cholera, a disease affecting both domestic and wild birds throughout the world. Economically, the disease results in significant losses to the turkey industry, and ecologically, it is a threat to the survival of several endangered avian species. The pathogenic mechanisms used by *P. multocida* to invade and colonize the host are poorly understood. In natural infections of turkeys, *P. multocida* is believed to enter tissues of birds through mucous membranes of the pharynx and/or upper air passages. Colonization of the upper respiratory tract is rapidly followed by invasion into the circulation (19). Septicemia and pneumonia are therefore common signs of fowl cholera in poultry.

Serotype A:3 strains of *P. multocida* are the main cause of fowl cholera in turkeys (20). The capsule of these strains is composed of HA, a large anionic glycosaminoglycan which promotes adhesion to turkey air sac macrophages, but not internalization (16). The HA receptor causing adhesion of *P. multocida* was not found on freshly isolated turkey peripheral blood monocytes. However, cultures or phorbol myristate acetate treatment of the monocytes increased HA receptor expression as well as *P. multocida* adhesion.

In this study, our primary goal was to identify the HABP which may act as the HA receptor expressed by TPBM for serotype A:3 *P. multocida*. The second goal was to purify these HABP and to raise polyclonal antibodies against them. These reagents may be useful in further investigations of the invasion mechanisms of *P. multocida* during fowl cholera.
MATERIAL AND METHODS

Animals. Twelve to 24 week-old male, Beltsville small white turkeys from the closed flock at the National Animal Disease Center were used as a source of blood monocytes. Young adult male New Zealand white rabbits (Pasteurella-free Elite rabbits; HRP Inc., Denver, PA) were used for polyclonal antibody production.

Turkey peripheral blood monocyte collection. Peripheral blood monocytes were isolated using a slight modification of a previously described 2 step-gradient procedure (17) as follows. Blood (160 ml) was collected from the brachycephalic vein into EDTA-treated vacutainer tubes (Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ). Blood was diluted 1:2 with RPMI 1640 containing L-glutamine and antibiotics (100 U penicillin/ml, 50 μg streptomycin/ml, and 2 μg fungizone/ml) but lacking sodium bicarbonate (Sigma Chemical Co, St Louis, MO) (mRPMI). For separation of white blood cells, 13 ml of diluted blood was layered onto 7 ml of density gradient medium (Accu-Prep®, sp. G. 1.077; Accurate Chemical & Scientific Corporation, Westbury, NY) in 20x125mm screw-capped tubes. Tubes were centrifuged at 800 x g for 20 min. at 22°C. The white blood cell layer above the gradient medium was collected and diluted 1:2 with mRPMI. Thirteen ml of the diluted suspension was applied onto 7 ml of a second density gradient medium (1-Step Monocytes®, sp. G. 1.068; Accurate Chemical & Scientific Corporation) in 20x125 mm screw-capped tubes. Tubes were centrifuged at 600 x g for 15 min. at 22°C. After centrifugation, the top gradient, including the interface, was removed to a level just above the cell pellet and diluted 3:1 with 0.85% NaCl solution containing 0.13% ethylene diamine tetraacetic acid (EDTA) (Kodak, Rochester, NY). The diluted material was centrifuged at 600 x g at 22°C for 15 minutes. The monocyte-rich pellet was resuspended in
mRPMI supplemented with 10% heat-inactivated fetal bovine serum (AmRPMI), and cell counts were made with an automated cell counter (Nova Cell Track, Alicia Diagnostics, Oveido, FL). Isolated monocytes were seeded (5 x 10^5 monocytes/cm^2) in 150 cm^2 polystyrene tissue culture flasks (Corning Costar Corporation, Cambridge, MA) and incubated in AmRPMI for 6 days at 37°C in a 5% CO₂ atmosphere.

**Preparation of membrane proteins.** Membrane HABP were prepared from 6-day old cultured TPBM as follows. Cultured monocytes were washed once with a solution containing 0.85% NaCl and 0.13% EDTA. Flasks were held at 4°C for 10 minutes. The monocytes were harvested with the aid of a rubber policeman into a solution of 0.85% NaCl and 0.13% EDTA and collected by centrifugation at 600 x g for 15 minutes at 22°C. The monocytes were resuspended in 20 ml of the extraction buffer composed of 0.05M Tris buffer pH8, 1% 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS) (Sigma), 5 mM EDTA, 5 mM dithiothreitol (United States Biochemical Corp., Cleveland, OH), 0.5 μg/ml leupeptin (Sigma), 0.2 mM phenylmethylsulfonyl fluoride (Sigma) and 0.7 μg/ml pepstatin (Sigma) (pH 8.0). After gentle stirring on ice for 2 hours, the suspension was centrifuged for 30 minutes at 27,000 x g at room temperature. The detergent soluble phase (supernate) was removed and concentrated to 6 ml with a Centriprep 30 (Amicon, Beverly, MA) at 500 x g and 4°C for 25 minutes. Protein yield was estimated by spectrophotometric analysis at 260 and 280 nm.

**Hyaluronic acid affinity chromatography.** Hyaluronic acid was coupled to EAH-Sepharose® 4B (Pharmacia Biotech, Uppsala, Sweden) using the carbodiimide method accordingly to the instructions provided by the manufacturer. Briefly, 40 mg of low molecular weight HA from vitreous humor (#H-7630, Sigma) was dissolved in distilled H₂O to a
concentration of 5 mg/ml, and solution was adjusted to pH 4.50 with 1M NaOH. The HA solution was diluted 2:1 (v/v) with EAH-Sepharose. Solid 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) (Sigma) was added to the gel slurry to a final concentration of 0.1M. The suspension was gently rocked overnight at room temperature using a Vari-mix (Thermolyne; Dubuque, IA). The pH was maintained between 4.5 and 6 during the first hour using 1M NaOH. Following the reaction, the gel was thoroughly washed using three cycles of alternating pH, each cycle consisting of 0.1M acetate buffer pH 4.0 supplemented with 0.5M NaCl followed by a wash with 0.1M Tris HCl buffer pH 8 containing 0.5M NaCl. The washed gel was packed into 5 ml polypropylene columns (Pierce, Rockeford, Illinois) and thoroughly washed with 0.05M Tris HCl buffer (pH 8) containing 0.15M NaCl before loading the sample. Extracted membrane proteins were loaded onto the column and the column was incubated overnight at 4°C. The gel was then washed with 0.15M NaCl in 0.05M Tris HCl buffer (pH 8). HABP were eluted with 2M NaCl in 0.05M HCl buffer followed by 4M guanidine HCl in 0.05M Tris HCl buffer (pH 8). The eluted proteins were concentrated and equilibrated in 0.05M Tris HCl buffer (pH 8) using a Centriprep 30 concentrator (Amicon Inc.). Eluted HABP that were not immediately used for SDS-PAGE were stored at -70°C for a maximum of 2 weeks.

Preparation of the biotinylated hyaluronic acid. A slight modification of the method described by Pouyani et al. (18) was used to prepare the biotinylated HA probe.

High molecular weight Rooster comb HA (200 mg; #H-5388 Sigma) was dissolved in 50 ml of distilled H₂O by end-over-end tube rotation, overnight at 4°C. Adipic hydrazide (3.5g; Sigma) was added to this solution and pH was adjusted to 4.75 with 0.1N HCl. Solid EDC (382 mg) was slowly added to the solution and the pH of the reaction was adjusted to 4.75 by addition of
0.1N HCl. The reaction was allowed to proceed at pH 4.75 for 2 hours or until no further rise in pH was observed. The pH was then raised to 7.0 by addition of 1N NaOH. The solution was transferred to dialysis tubing (11-12 kDa M.W cut-off; Spectra/Por®, Spectrum Medical Industry Inc., Los Angeles, CA), exhaustively dialyzed against distilled water, and lyophilized. The lyophilized hydrazido-HA was dissolved overnight at 4°C in 0.1M NaHCO₃ (10 ml) by end-over-end tube rotation as above. Sulfo-NHS-Biotin (50 mg; Pierce, Rockford, IL) was added to the solution and stirred for 18 hours at room temperature. The solution was diluted 10-fold with distilled water, transferred to dialysis tubing (12-14 kDa cut-off), dialyzed exhaustively against water, and lyophilized.

**SDS-PAGE.** Electrophoretic analysis of HABP was first performed on 10% polyacrylamide (10% total monomer (T), 2.7% cross-linker (C)) slab gels (1.5x12x14 cm) under reducing conditions as described by Laemmli (7). 100 μl-size wells were loaded with 0.2 to 0.6 mg of column-eluted HABP, and run for 4 hours at 35 mA/gel. Protein bands were stained with Coomassie brilliant blue. For specific elution of HABP from preparative gels, 4.5 mg of HABP were electrophoresed in 10%T gels (1.5x12x14 cm) under reducing conditions by the blue SDS-PAGE method of Schagger et al. (22). Bands of interest were cut from the gels and proteins were electroeluted by reverse polarity as described by Abramovitz et al. (1). The electroeluates were concentrated at 4960 x g at 4°C for 35 minutes with a Centricon 30 (Amicon Inc.).

**Immunoblotting.** SDS-PAGE gels were equilibrated in transfer buffer (tris base 0.025M and glycine 0.192M, pH 8.2) for 5 minutes prior to transfer to supported nitrocellulose membranes (BA-S Optitran; Schleicher & Schuell Inc. Keen, NH) according to the method reported by Towbin et al. (24). Transfer was performed overnight at 15V and 15 °C, followed by 30 minutes
at 100 V at the same temperature. Unreacted sites of the nitrocellulose membranes were blocked by overnight incubation at 4°C in phosphate-buffered saline containing 1% fish gelatin (Hipure, Norland Products Inc. New Brunswick, NJ) and 0.05% tween 20 (Sigma, St Louis, MO). The membranes were rinsed (3 X 10 minutes) in Tris-buffered saline (0.05 M Tris, 0.1M NaCl, pH 7.5) supplemented with 0.05% tween 20 (TTBS), then incubated for 3 hours with 20 ug/ml of biotinylated HA at room temperature. The membranes were washed with TTBS (4 X 10 minutes), incubated with the Vectastain® ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes, and washed (3 X 10 minutes) in TTBS. Blots were developed with the DAB substrate kit for peroxidase (Vector Laboratories).

Antiserum specificity was examined by immunoblotting of HABP separated by SDS-PAGE. Blocked nitrocellulose sheets were incubated for 3 hours at 22°C with 1:100, 1:500, and 1:1000 dilutions of the rabbit serum or antiserum, with a 1:500 dilution of a mouse anti-human CD44 mAbs (Sigma; Clone A3D8). Membranes were washed with TTBS (4X10 minutes) and reincubated for 2 hours at 22°C with 1:1000 dilution of the secondary peroxidase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland) or secondary peroxidase labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories Inc.), respectively. Membranes were again washed (4X10 minutes) and reaction was detected with the DAB substrate kit for peroxidase.

**Preparation of antibodies to HABP.** Electroeluted HABP was emulsified in an equal volume of TiterMax® (CytRx, Norcross, GA) using a sonifier (Branson sonic power, Danbury, Ct). The emulsion (1 ml) was injected intramuscularly into the thigh region of a rabbit. Booster injections (1 ml) of the same preparation were given subcutaneously after 28 days. Animals were
bled before the first injection (preimmune serum) and antibody response was checked after 28 days. Heat-inactivated hyperimmune serum was adsorbed with freshly isolated TPBM for 12 hours at 4°C before use in immunoblotting analysis as described above.

**RESULTS**

**Characterization of HABP.** Using the two-step gradient protocol, monocytes were the only adherent cell population. Non-adherent lymphocytes were removed after 24 hours of incubation by gentle washing of the tissue culture flasks with warm mRPMI. The monocyte membrane fraction solubilized by detergent was estimated to contain 68.82 mg of protein prior to chromatography. After applying the detergent soluble material to the HA-Sepharose column, HABP were eluted with 2M NaCl in Tris HCl 0.05M pH 8. No further proteins were eluted when the HA-Sepharose column was washed with guanidine HCl. The peak fractions from the NaCl elution were pooled and after concentration with a Centripeg 30 (Amicon Inc.), protein yield was estimated to be 13.97% of the starting material (9.61 mg). Coomasie brilliant blue staining after SDS-PAGE indicated the partial purification and concentration of three bands of 81, 101, 118 kDa molecular weight respectively (Figure 1). The ability of these proteins to bind HA was demonstrated on electroblots using the biotinylated HA probe (Figure 2).

**Purification of HABP.** Using the reverse polarity elution technique of Abramovitz et al. (1), the 101 and 118 kDa HABP were isolated from blue SDS-PAGE gels. Their ability to bind to HA was confirmed by electroblotting and reaction with the biotinylated probe.

**Polyclonal antibody production and specificity.** The concentration of electroeluted proteins inoculated into the rabbits could not be determined due to the presence of Coomasie blue stain.
Both preimmune and hyperimmune serum reacted with the proteins in electroblots (Figure 3). Unexpectedly, the preimmune serum reacted more intensely with the HABP than the serum taken following immunization.

**DISCUSSION**

Adhesion of serotype A:3 *P. multocida* to cultured turkey monocytes is mediated by capsular HA (17). In this study, HA-Sepharose affinity chromatography was used to purify the bacterial receptor from cultured TPBM. Likely due to anionic-type of interactions with the HA-Sepharose, other proteins were also bound and eluted from the column. These proteins lacked HA binding activity and therefore a second purification step was required. Because of protease release and denaturation which may occur during elution, a second HA-affinity chromatography of the eluted material as recommended by LeBaron *et al.* (8) was not performed. Instead, preparative blue SDS-PAGE (1, 22) and electroelution were used to concentrate and purify the HABP.

The biotinylated HA probe is believed to detect HABP on electroblots with sensitivity and specificity (14). Its sensitivity is probably due to the long-spacer arm (adipic hydrazide linked to a 6-carbon hexanoic acid molecule) that facilitates access to the deep biotin binding pocket of the avidin conjugate of the detection kit. Also, the avidin binds to several biotin molecules, providing a powerful amplification system. Its specificity is probably higher than the one seen with the monoclonal antibodies used to detect HABP in conventional immunoblotting. The interaction of the biotinylated probe only occurs with biologically functionnal HABP whereas immunological reactivity can also be displayed by denatured proteins.
*Pasteurella multocida* poorly adhere to circulating blood monocytes, but cultures of blood monocytes on extracellular matrix or exposure to phorbol myristate acetate enhance bacterial binding (17) similar to that observed for human peripheral blood monocytes in HA adhesion assays (12, 13). Additionally, turkey monocytes exposed to anti-CD44 monoclonal antibodies have decreased bacterial binding thereby indicating that CD44 is the receptor for serotype A:3 *P. multocida* on TPBM. In this study, we identified three proteins with HA binding activity. These proteins were 81, 101, and 118 kDa in molecular size. These proteins are not expressed or poorly bind the biotinylated probe when using freshly isolated TPBM (Pruimboom, unpublished). Taken together, these findings indicate that the HABP detected on electroblots could represent multiple isoforms of CD44.

CD44 isoforms include the unspliced 85-kDa standard molecule (CD44S) expressed by freshly isolated human peripheral blood leukocytes and ten isoforms derived by alternative splicing (CD44V) of the gene. Freshly isolated human blood monocytes do not bind HA whereas alveolar macrophages express CD44 isoforms of 120kDa and above and are able to bind soluble HA (4). These changes in molecular weight result from posttranslational modifications such as O- and N- glycosylation and mRNA alternative splicing. These changes also have an important impact on the differentiation of circulating blood monocytes to tissue macrophages at inflammatory sites and the acquisition of new functions (4, 12, 13). *In vitro*, conversion of pre-existing CD44 molecules from a non-HA binding state to a form able to bind HA can be triggered (10, 11).

During this study, the 84-kDa HABP was intermittently detected by the biotinylated HA. This inconsistency may result from the methodology used to purify the receptor or the protein was
not activated to its HA-binding state as it would be expected for the CD44S isoform. The 101 and 118-kDa HABP were, therefore, the proteins of choice in attempts to produce specific antibody in rabbits. However, the attempt to produce polyclonal antibodies against these HABP failed. Preimmune serum and hyperimmune serum both recognized the 101 and 118 kDa proteins from the material eluted by chromatography and the TPBM membranes. Antiserum adsorption with freshly isolated TPBM did not remove the reaction with the preimmune serum. CD44 is expressed by a wide variety of cell-types and its functions are well conserved between species. It is likely that CD44 homology is high between rabbits and turkeys and a nonimmunized rabbit may recognize part of the molecule through antiidiotypes. Additionally, it is well established that tumor cells express high levels of CD44V₆ and subsequently become highly aggressive and metastatic (3). The recognition by the rabbit of turkey CD44 may represent part of a mechanism of regulation against tumor development. The production of polyclonal antibodies against the 81-kDa HABP is now under investigation.

REFERENCES


**Figure 4.1:** SDS-PAGE and brilliant blue coomasie stain. Lane 1 and 6, prestained molecular weight standard (241, 147, 99, 69, 57, 43, 29, 23, 18, 9 kDa); lane 2 and 5, molecular weight standard (220, 160, 120, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, 10 kDa); lane 3, TPBM membrane proteins; lane 4, HABP isolated by HA-Sepharose affinity chromatography.
**Figure 4.2:** Electroblot of TPBM membrane proteins. Nitrocellulose membrane was incubated with the biotinylated HA. Lane 1 and 4, prestained molecular weight standard (241, 147, 99, 69, 57, 43, 29, 23, 18, 9 kDa); lane 2, HABP isolated through HA-Sepharose affinity chromatography; lane 3, TPBM membrane proteins. Three proteins of 84, 101, and 118 kDa intensely reacted with the probe.
Figure 4.3: Electroblot of TPBM proteins. A, Preimmune serum; B, hyperimmune serum. Lane 1 and 4, prestained molecular weight standard (241, 147, 99, 69, 57, 43, 29, 23, 18, 9 kDa); lane 2, HABP eluted from HA-Sepharose affinity chromatography; lane 3, TPBM membrane proteins. Antiserum specificity was not confirmed since both preimmune serum and hyperimmune serum reacted with the injected HABP.
CHAPTER 5: GENERAL CONCLUSIONS

*Pasteurella multocida* causes fowl cholera in both domestic and wild birds. The high morbidity and mortality associated with this disease still results in significant economic losses to the turkey industry. In avian species, it is believed that *P. multocida* first colonizes the mucous membranes of the pharynx and upper air passages, and then causes pneumonia and septicemia. However, more studies remain to be done to understand the precise mechanisms of pathogenesis.

The studies presented in this dissertation were designed to: 1) investigate whether or not hyaluronic acid promotes adhesion of serogroup A *P. multocida* to turkey air sac macrophages, 2) demonstrate that cultures and or chemical treatments enhance bacterial adhesion to turkey peripheral blood monocytes, 3) identify, characterize, and isolate the receptor involved in bacterial adhesion to turkey peripheral blood monocytes, 4) produce polyclonal antibodies against the receptor. Ultimately, the findings in this dissertation could lead to a better understanding of the pathogenesis of fowl cholera.

Serogroup A strains of *Pasteurella multocida*, which are the major cause of fowl cholera in turkeys, are resistant to phagocytosis in nonimmunized birds. This resistance is believed to be associated with the presence of an hyaluronic acid capsule. We shown that in adherence studies with a capsulated strain of *P. multocida* (serotype A:3) and turkey air sac macrophage cultures, bacteria were capable of adhering in large numbers to the macrophages but were not internalized. However, a non-capsulated variant of the bacteria (serotype -:3) showed little adherence and/or internalization. Depolymerization of the bacterial capsule with hyaluronidase
increased phagocytosis by macrophage cultures, and addition of hyaluronic acid to the cultures, but not other polysaccharides, inhibited bacterial adherence. These findings demonstrated that *P. multocida* adhesion is mediated by the capsular hyaluronic acid. Furthermore, macrophages treatment with sodium metaperiodate or trypsin suppressed bacterial binding suggesting that recognition of the bacterial polysaccharide is due to a specific glycoprotein receptor.

In contrast to adhesion to turkey air sac macrophages, capsulated *P. multocida* did not bind to freshly isolated turkey peripheral blood monocytes, but I observed that culture in chamber slides for 6 days gradually increased adhesion. Incubation in chamber slides coated with entactin-collagen IV-laminin attachment matrix or exposure to phorbol myristate acetate further enhanced bacterial adhesion. Addition of hyaluronic acid to the monocyte culture, but not Arg-Gly-Asp peptide, inhibited *P. multocida* binding to air sac macrophages thereby demonstrating that only the capsular *multocida* promotes bacterial adhesion. Monocyte exposure to monoclonal antibodies directed against the major hyaluronic acid eukaryotic membrane protein (CD44) decreased binding of *P. multocida*.

Finally, hyaluronic acid-Sepharose affinity chromatography was used to partially purify hyaluronic acid binding proteins from cultured turkey peripheral blood monocytes. Material was eluted following sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and electroblots showed three major bands (81, 101, and 118 kDa) which reacted with a biotinylated hyaluronic acid probe. Both the 101 and 118 kDa proteins were eluted from the acrylamide gels by reverse polarity electrophoresis and used to hyperimmunize rabbits. However, preinoculation serum reacted with several TPBM antigens as well as the 101 and 118 kDa HABP, and immunization did not result in higher titers or increased specificity. Clonal
anergy may have resulted from the antigen injection. This concept is not fully understood, and its simplest form, may be stated as the inhibition of a B lymphocytes when the antigen is presented by the T lymphocytes without a co-stimulatory signal.

These findings indicate that serotype A:3 strains of *P. multocida* adhesion to air sac macrophages, but not internalization, is mediated by capsular hyaluronic acid. In contrast, capsulated bacteria do not adhere to freshly isolated blood monocytes but bacterial binding can be enhanced by culture on entactin-collagen IV-laminin attachment matrix or exposure to phorbol myristate acetate. Therefore, it is likely that recognition of the capsular polysaccharide is due to an isoform of the glycoprotein CD44. This receptor was partially purified from cultured blood monocytes but attempts to produce polyclonal antibodies against its higher molecular weight isoforms (101, and 118 kDa) failed. The production of polyclonal antibodies against the standard CD44 isoform of lower molecular weight (81-kDa) are still under investigation.
ACKNOWLEDGMENTS

I wish to thank Dominique for his support and understanding during this work. As an outstanding scientist, he constantly stimulated our quest for knowledge and as my fiancé, he made it all worthwhile.

To Drs. Norman F. Cheville and Mark R. Ackermann, my co-major professors, go my respect and gratitude. They introduced me to the fascinating field of veterinary pathology, and serve as scientific models of rigor and thinking that I will always acknowledge and remember. To Dr. Richard B. Rimler, my research advisor, goes all my appreciation and deference. He introduce me to the world of research and and provided me with constant support.

I am grateful to Dr. Harley W. Moon who gave me the opportunity to work at the National Animal Disease Center when he was still director. I am also thankful to Drs. Kim A. Brogden, Robert A. Kunkle, Steeve C. Olsen for their advice and stimulating influence as well as their friendship at the National Animal Disease Center.

I wish to thank the other members of my commitee; Drs. Janice E. Buss, John P. Kluge, Joseph S. Haynes, for their advice and time to review this dissertation.

For their excellent technical assistance and friendship, I wish to thank Gwen E. Nordholm, and Wendy A. Hambly. I also want to remember Judi Stasko for her help in electron microscopy. I also thank Gene Hedberg, Tom Glason, and Chuck Greiner for their outstanding work in the photography department.

For creating a stimulating, friendly, and innovative environment, I wish to thank all my colleagues and friends at the National Animal Disease Center as well as at Iowa State University.
I also want to remember Drs. Freddy Coignoul and Jacques Mainil, from Belgium who made this work possible.

I wish to thank my family and the Peters's family for the greatest support during this work, especially my mother and grandmother for their encouragement to seek higher education. And finally, I wish to remember my grandfather who deserves all the credit of this work.