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Pathogenesis and immunity involved in fowl cholera were studied with the turkey, one of the most susceptible natural hosts. The causative agent, Pasteurella multocida, was analyzed in terms of its virulence and immunogenic factors, using a highly virulent, encapsulated strain P-1059.

Protective immunity was induced by lipopolysaccharide (LPS)-protein complex antigens prepared by two different methods. The LPS prepared by Westphal's phenol-water method, in contrast, did not induce immunity. High titers of anti-LPS antibody were induced by LPS-protein complexes, but not by LPS. Immunogenicity of the LPS-protein complex was lost upon the treatment with periodate, but not with protease. The results suggest that immunoprotective determinants probably reside in the LPS moiety of the complex, and that the protein(s) may function as the carrier of LPS.

Virulence of the organism was closely associated with its capsule-producibility, since a non-encapsulated mutant derived from the strain P-1059 was significantly less virulent than the parent strain. The

capsule was removable by hyaluronidase, but the decapsulation did not cause the loss of virulence.

When the encapsulated or decapsulated form of organism, as well as the non-encapsulated mutant was intravenously inoculated into susceptible turkeys, the majority of bacteria were rapidly removed from the bloodstream, and trapped in the liver and spleen. The presence or absence of capsule did not influence the clearance of bacteria from the blood. Thus, the capsule appeared not to act as an anti-phagocytic factor to the phagocytes responsible for the intravascular clearance of bacteria. The non-encapsulated mutant was readily inactivated in the liver (but not in the spleen), while the encapsulated organisms multiplied freely in the liver and spleen. Hence, the capsule seemed to be important for the bacteria to resist the bactericidal activity of hepatic phagocytes.

Similar experiments with immune turkeys showed that specific immunity did not enhance the clearance of bacteria from the blood, but that immunity was essential for inactivation of the encapsulated bacteria entrapped in the liver. Immunity, however, did not facilitate the killing of bacteria in the spleen. Involvement of humoral and cellular defense factors in the bactericidal activity in the liver was investigated by the passive transfer of immune serum or by the treatment with macrophage activating agents. The results indicated that specific antibody has a primary role, and that activation of macrophages may also contribute to enhancing the bactericidal activity.

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Chapter I

Introduction

(Literature review)

Pasteurella multocida

Pasteurella multocida has been recognized as an important pathogen associated with many diseases in both mammalian and avian species.[1-3] Fowl cholera in poultry and hemorrhagic septicemia in cattle are the two most severe diseases caused solely by *P. multocida*. Other diseases are primarily respiratory infections. These include shipping fever in cattle, pneumonia in sheep and goats, atrophic rhinitis in swine, and rhinitis ("snuffles") in rabbits. In some of these respiratory diseases, however, it is not clear whether *P. multocida* is the primary cause or secondary invader. Infections in humans occur most frequently from animal bites and scratches (usually dogs and cats).[2]

Pasteurella multocida has been classified as a gram-negative, facultatively anaerobic, nonmotile, nonsporeforming, rod-shaped bacterium.[4] Organisms with bipolar staining are often observed in the specimens obtained from infected animals. The organism grows readily on sheep blood agar plates at 37°C, forming smooth, gray-white colonies with no hemolysis, but does not grow on MacConkey agar. Metabolically, the organism is catalase-positive, oxidase-positive, indole-positive,

and urease-negative. On triple sugar iron agar, it produces an acid slant, acid butt with no gas and no H_2S.

Pasteurella multocida, when isolated from the hosts, is usually encapsulated, but the organism often loses its capsule during the prolonged subculture in an artificial medium.[5,6] Colonial morphology observed under obliquely transmitted light is very useful to study encapsulation of P. multocida. The colonies of encapsulated organisms show characteristic iridescence (fluorescence), whereas non-encapsulated organisms form non-iridescent (blue) colonies.[1,6] However, these characteristics are not always clear-cut; there are many intermediate type colonies with variable iridescence. The capsule of P. multocida has been observed by various negative-staining techniques.[7-11]

Serological classification of P. multocida is complicated because of its antigenic complexity.[12] Various investigators have developed various serological methods,[13-16] but no internationally standardized classification system has yet been established. By convention, most workers use Carter's indirect hemagglutination method[15] for typing the capsular antigens and Heddleston's gel diffusion method[16] for typing the somatic antigens. Employing these methods, five capsular types (A, B, D, E, and F) and 16 somatic types (1 through 16) have currently been distinguished.[17,18] Capsular types B and E strains of P. multocida are the primary cause of hemorrhagic septicemia in cattle.[2,3] Most strains associated with acute fowl cholera belong to capsular type A, but their somatic antigens are heterogeneous.[19] The type A strains of P. multocida have been shown to produce a large amount of hyaluronic acid as a major component of their capsules.[20-22] This compound, however, is not the substance which is responsible for the capsular serotype-

specificity; hyaluronic acid is usually poorly immunogenic, and thus, serologically inactive.[22,23] The serotype A substance has yet to be defined. On the other hand, the antigenic substances responsible for Heddleston's somatic serotypes have been identified as lipopolysaccharides.[24]

Fowl cholera

Pasteurella multocida infection in avian species has been called fowl cholera.[1] The disease typically appears as acute fatal septicemia, accompanied with high morbidity and mortality, but chronic infections are occasionally observed in the field. The disease has world-wide distribution and affects a variety of avian species in both wild and domestic populations. In the United States, the problem caused by the disease is most serious in domestic turkeys; the turkey is much more susceptible than the chicken. Fowl cholera was ranked one of the top two infectious diseases causing significant economic loss to the turkey industry in 1985.[25]

Fowl cholera in turkeys occurs most often in late summer and fall.[1,26] Although turkeys are highly susceptible at all ages, the outbreaks have been observed most frequently at the ages between 7 and 13 weeks.[26] How the disease is introduced into a new flock is not known. The birds in the flock which have experienced the disease may become chronic carriers of P. multocida.[27,28] However, there is no evidence for the presence of apparently healthy carriers in a flock without a disease history.[28] Mammals and birds in wildlife have been suspected to be the reservoirs of P. multocida.[29] Once an outbreak occurs, the disease rapidly spreads within a flock, probably by direct

contact or via feed and drinking water that are contaminated with the excretions from the mouth, nose, and conjunctiva of affected birds.[1,30]

In acute fowl cholera, the birds usually die within a few days following infection. Clinical signs are observed only several hours before death, including: drowsiness, anorexia, fever, diarrhea, cyanosis, dyspnea, mucous discharge from the mouth, and sudden collapse. Gross lesions are characterized by vascular congestion, subserosal petechial hemorrhages, and pinpoint necrotic foci on the liver. Histopathological examinations[31,32] reveal typical lesions of septicemia, characterized by generalized passive hyperemia and massive dissemination of bacteria in the liver and spleen, as well as in the blood vascular system throughout the body. Accumulation of a large number of heterophils has been observed intravascularly in the liver and lung. Marked heterophil depletion has also been observed in the bone marrow with hemopoietic cell necrosis.

Chronic fowl cholera is usually characterized by localized infections. It may occur in a flock after an acute outbreak has diminished, or may result from the infection with P. multocida of low virulence.[1] Localized suppurative lesions are observed in various tissues: wattles, sinuses, conjunctiva, limb joints, foot pads, or sternal bursa. Torticollis sometimes occurs with neurological signs. Experimentally, chronic diseases (especially joint infections) are sometimes observed in vaccinated birds following challenge-exposure with a virulent strain of P. multocida.[33]

Pathogenesis of fowl cholera

Only limited information is available in the literature regarding

the pathogenesis of fowl cholera. The portal of entry for P. multocida is considered to be the pharynx and/or upper respiratory tract.[34-36] Maheswaran et al.[36] demonstrated that when only 5 x 10^3 organisms of virulent P. multocida were endotracheally inoculated into turkeys, the bacteria emerged in the blood and spleen as early as 6 hours after the inoculation. Thus, the organisms appear to invade the mucosal epithelium soon after colonization and enter the bloodstream. The mechanisms involved in this invasion step are largely unknown. Possibly, the organisms may be brought in by endocytosis activity of the epithelial cells, or they may directly penetrate through intercellular spaces. One of the most extraordinary characteristics of P. multocida is its rapid proliferation in the host tissues. Even when turkeys were inoculated with a very small number (30 to 100 organisms) of virulent P. multocida, nearly all birds develop acute septicemia and die within 48 hours.[33] At the time of death, the bacterial counts in the liver and spleen usually exceed 10^9 per gram of tissue. A large number of bacteria can be observed also in the blood vessels. The cause of death has been presumed to be disseminated intravascular coagulation induced by endotoxin released from a massive amount of bacteria.[37-39] How P. multocida causes such an acute septicemic disease is controversial. Collins,[40,41] based on his observations in a mouse model, claims that once the bacteria enter the bloodstream, they can freely multiply as in a liquid culture medium, resulting in fatal septicemia. However, the mechanism of pathogenesis in avian species may not be the same as that in mice. Pabs-Garnon and Soltys[42] demonstrated that virulent P. multocida, when inoculated intravenously into turkeys, initially localized in the liver and spleen. The bacteria

multiplied primarily in the two organs, and were abruptly released into the bloodstream at the terminal stage, resulting in septicemia. The implication of these observations is that the bacteria may be trapped by the reticulo-endothelial phagocytes in the liver and spleen, and multiply intracellularly in the phagocytes. Some histopathological evidence which supports this view has recently been reported.[43]

Virulence factors of Pasteurella multocida

Relatively little is known about the virulence factors of P. multocida. As described in the pathogenesis of fowl cholera, the virulence of this pathogen is attributable to its ability to invade and proliferate in the host tissues. This ability may reside in some specialized bacterial cell-surface components, since most avian strains of P. multocida appear not to produce any potent exotoxin in the culture medium.[40,44]

Virulent strains of P. multocida are capable of developing acute septicemia from the mucosal surface of the nasal cleft or trachea. Thus, the bacteria must have some special mechanisms by which they can attach to the mucosal surface and subsequently invade the mucosal tissue. The factors involved in these initial steps are largely unknown. The presence of fimbriae-like structures (or pili) have been identified with rabbit strains of P. multocida,[45] but such structures have not been found with the strains causing fowl cholera. Production of neuraminidase and hyaluronidase has been reported with some avian strains of P. multocida.[46] These enzymes may contribute to bacterial adherence to mucosa and to intercellular spreading.

The capsule of P. multocida is often regarded as one of the major virulence factors of this pathogen.[1,6,40,47] Highly virulent strains of P. multocida are usually encapsulated, whereas non-encapsulated strains tend to be less virulent. More convincing evidence is that the loss of ability to produce the capsule usually accompanies the loss of virulence.[6] However, the presence or absence of capsule does not necessarily correlate with the degree of virulence.[5,48,49] Many strains of P. multocida having large capsules are apparently non-pathogenic. Conversely, the presence of non-encapsulated P. multocida which shows relatively high virulence in mice has been recognized.[50] Hence, the capsule appears not to be the sole virulence factor of P. multocida. The capsules of type A strains of P. multocida primarily consist of hyaluronic acid.[21] This substance has been shown to act as an anti-phagocytic factor against the polymorphonuclear neutrophils.[51,52] Whether the capsular hyaluronic acid also acts as a resistance factor against mononuclear phagocytes is not clear.[53]

The endotoxin of P. multocida appears to play an important role in the pathogenesis of fowl cholera at the terminal stage. Substantial amounts of the endotoxin have been detected in the blood from turkeys that died of fowl cholera.[16,54] Toxic effects of free endotoxin prepared from an avian strain of P. multocida have been studied by Heddleston et al.[39] When chickens were intravenously inoculated with the free endotoxin, the birds showed several signs and lesions which were similar to acute fowl cholera. Relatively small amounts (0.1 to 2.0 mg) of the free endotoxin are lethal for mice, rabbits, chickens, and turkeys.[54] Whether the endotoxin (or lipopolysaccharide) is associated with the virulence of P. multocida is not known, due to the

lack of information concerning the chemical structure of the lipopolysaccharide.

Outer-membrane proteins are other possible factors which may be associated with the virulence of P. multocida, but their roles have not been well investigated. Recently, the presence of an outer membrane protein which exhibits anti-phagocytic activity against mononuclear phagocytes of turkeys has been reported.[55]

A potent protein toxin with dermonecrotic activity has been found in several strains of P. multocida, but these strains were isolated almost exclusively from pigs suffering from atrophic rhinitis or pneumonia.[56,57] Although the toxin has been shown to be lethal in a wide variety of animal species including turkeys,[58] it appears not to be related to fowl cholera for two reasons. First, most avian strains of P. multocida appear not to produce the toxin. Second, the intramuscular inoculation of a toxin-producing strain of P. multocida failed to produce any sign of fowl cholera in poults.[58]

Immunizing agents against fowl cholera

The first fowl cholera vaccine was developed by Pasteur in 1881.[1] Since that time, many investigators have attempted to develop effective immunizing agents for the control of this disease, but a satisfactory vaccine has yet to be developed.

Currently, two types of vaccines are commercially available. The first one, multivalent bacterin, consists of an oil adjuvant and inactivated whole bacterial cells of several somatic serotypes. An advantage of bacterin is its safety. Disadvantages are that it must be

parenterally inoculated into individual birds, and that it induces only serotype-specific immunity.[16] The quality of bacterin is controlled by U.S. Department of Agriculture to assure its potency. Nevertheless, occasional outbreaks occur in turkey flocks that are properly vaccinated. The reason for these "vaccine breaks" is not known. Several predisposing factors may play a role; including, other concomitant infectious diseases,[59,60] and some unknown environmental stress factors.

The second type of immunizing agent is live vaccine using a low-virulent strain of P. multocida, which is given to turkeys via drinking water.[61] Immunity induced by live vaccine is cross-protective against the challenge infections with heterologous somatic serotype strains of P. multocida. A serious disadvantage of this vaccine is that the vaccine strain of P. multocida, by itself, appears to cause significant mortality when it is administered to the birds under undefined stress or immunosuppressive conditions.[62,63]

A reasonable approach to develop an improved vaccine is to identify the antigenic components of P. multocida which provoke protective immunity against fowl cholera. Several immunogenic components have been isolated using various methods, but their identities are not clear. A soluble fraction obtained by extraction with 0.5 M potassium thiocyanate has been reported to induce immunity in chickens.[64] An antigen purified from an 2.5% NaCl extract by chromatographic methods has been found to be protective against fowl cholera in turkeys.[65,66] A lipopolysaccharide (LPS)-protein complex has been extracted with 0.3% formalin solution containing 0.85% NaCl and purified by differential centrifugation.[39,67] This substance has been

shown to provoke immunity against fowl cholera. However, the LPS prepared by Westphal's phenol-water method appears to be poorly immunogenic.[68] Baba reported that a ribosomal fraction prepared from an avian strain of P. multocida induced immunity in chickens.[69] Phillips et al., however, could not reproduce the results with highly purified ribosome preparations.[70] Subsequently, they showed that immunity was induced by the ribosome when it was cross-linked with a small amount of LPS.[71] Capsular polysaccharides purified from serotype B or E strains of P. multocida have been shown to induce immunity against hemorrhagic septicemia in cattle.[72] However, capsular substances of serotype A strains of P. multocida appear not to act as the protective antigen(s) against fowl cholera, because immunity can be induced by non-encapsulated as well as encapsulated forms of P. multocida organisms.[6] A cross-protective factor has been found in the cell lysate of in vivo-propagated P. multocida,[73] but its nature has yet to be defined. This factor may be related to the cross-protective immunity induced by live vaccine.

Host defense mechanisms

In contrast to the numerous studies on the immunizing agents, the host defense mechanisms involved in immunoprotection have not been studied thoroughly. Although hosts have various non-specific defense mechanisms consisting of both humoral and cellular factors, these are virtually incapable of controlling the growth of virulent P. multocida in the absence of specific immunity.

Specific antibody appears to have a primary importance for immunoprotection against fowl cholera, because immunity can be

transferred into susceptible birds by the administration of immune serum or the IgG antibody fraction.[74-76] However, it is not known whether the antibody acts as an opsonin, a complement activator, or possibly a neutralizing factor of an unidentified toxin. Antibody titers of vaccinated birds have been measured by several serological methods, such as agglutination test and enzyme-linked immunosorbent assay. Some workers[77,78] observed the correlation between antibody titers and protection rates against challenge infection, but others did not.[71,74]

There is no firm evidence for the role of complement working in association with antibody to directly lyse virulent P. multocida. However, several studies showed that fresh immune serum was bacteriostatic (or slightly bactericidal) against P. multocida.[79-81] The mechanisms of this bacteriostasis seem to be complicated. Not only the antibody and complement but also iron-binding proteins (mainly transferrin) seem to be essential for the bacteriostatic effect, because the effect has been shown to be abolished by the addition of iron compounds into the immune serum.[79,81] Importance of iron-binding proteins has also been demonstrated in vivo. In a study done by Bullen et al.,[82] mice were passively immunized with hyperimmune serum. The protection against P. multocida was abolished when mice were inoculated with various iron compounds. Hence, the host's mechanisms, by which iron compounds are sequestered from bacterial pathogens,[83] may also be involved in protective immunity against fowl cholera.

Cell-mediated immunity may also be important for immunity against fowl cholera. Baba reported that immunity was transferable from immune to normal chickens by the transfer of the immune spleen cells.[84] He further demonstrated that the peritoneal macrophages obtained from

immunized, but not unimmunized, birds were capable of inhibiting the intracellular multiplication of P. multocida. Macrophages sensitized with supernatant fluid of the immune spleen cell cultures also inhibited the intracellular growth of P. multocida.[84,85] Thus, the immune spleen cells appeared to produce some macrophage-activating factors.

Whether heterophils are also involved in controlling P. multocida has not been studied in avian species. However, several studies have been done in mice,[40,41,86,87] showing that the polymorphonuclear neutrophils were the primary phagocytes which responded to P. multocida infections. In the absence of immunity, however, these phagocytes were virtually unable to ingest virulent P. multocida. This inability was presumed to be due to the anti-phagocytic activity of capsular hyaluronic acid. When mice were intraperitoneally inoculated with immune serum-sensitized P. multocida, the bacteria were readily ingested by the phagocytic cells in the peritoneal cavity.[87] Therefore, the opsonization of the capsule by specific antibody appears to be important for the murine polymorphonuclear phagocytes to participate in immunoprotection. However, this finding may need to be confirmed in avian species, because some evidence indicates that the immune mechanism in mice differs from that in chickens. For example, in a study done by Heddleston and Rebers,[88] mice and chickens were immunized with an encapsulated or a non-encapsulated strain of P. multocida. Following challenge infection with virulent encapsulated P. multocida, the mice immunized with the encapsulated organism were protected, but those immunized with the non-encapsulated organism were not. In chickens, however, both encapsulated and non-encapsulated organisms

equally induced protective immunity. Similar findings have been reported by Yaw and Kakavas.[89] Opsonization of the capsule, therefore, may not be important for protection in chickens.

The studies reported in this dissertation were conducted to obtain further information concerning the pathogenesis and immune mechanisms involved in fowl cholera. The nature of the antigenic components of P. multocida which provoke protective immunity in turkeys was studied in chapters II and III. The relationship between the capsule and virulence of P. multocida was examined in chapter IV. The role of the capsule in the pathogenesis of fowl cholera was investigated in chapter V. The host immune defense mechanisms which prevent fatal septicemia were studied in the final chapter.

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Chapter II

Immunochemical Relationship of Three Antigens Purified from *Pasteurella multocida* Strain P-1059

Summary

Three antigens were prepared from a type 3 avian strain of Pasteurella multocida, and their chemical and immunological characteristics were studied. An antigen, designated 2.5S, was extracted with 2.5% NaCl solution and purified by chromatography. Lipopolysaccharide (LPS) was prepared by the phenol-water method, and another antigen, designated FS, was extracted with 0.3% formalin containing 0.85% NaCl and purified by differential centrifugation. The 2.5S and the FS antigens consisted of 40% protein and 15% carbohydrate, whereas LPS did not contain a substantial amount of protein. A major protein component with a molecular weight of 44,000 was detected in the 2.5S antigen as well as in the FS antigen. Of the three antigens, LPS had the highest activity in mouse lethality and Limulus lysate tests. Antigenic cross-reactions among the three antigens were demonstrated by immunodiffusion tests. The 2.5S antigen was indistinguishable from the FS antigen, as both antigens contained the LPS component in the amount of approximately 45%. Treatments with various reagents indicated that the 2.5S and the FS antigens contained at least two kinds of antigenic determinants. The first was a heat-stable protein sensitive to protease or phenol-water, and the second was a periodate-sensitive carbohydrate, which was a major antigenic determinant on the LPS antigen.

Introduction

Pasteurella multocida is a causative agent of many animal diseases, including fowl cholera; an acute septicemic disease of fowl causing high mortality and morbidity.[1] Protective immunity can be induced by a live low-virulent strain or an inactivated whole cell vaccine (bacterin). The live vaccine has advantages over the bacterin in its simplicity of application and induction of cross-protective immunity,[2] but its serious disadvantage is that the vaccination sometimes results in systemic infection.[3] Vaccination of poultry species with bacterins often resulted in ineffective immunity in the field, even though it demonstrated satisfactory results under controlled conditions.[1,4,5] A reasonable approach toward the development of an improved vaccine against fowl cholera would be to determine the immunogenic antigen(s) of P. multocida.

Antigenic substances responsible for immunity against fowl cholera have been studied by several investigators, and various antigens with protective immunogenicity have been obtained by various extraction methods.[6-11] Kodama et al.[10] compared immunogenic efficacy of subcellular fractions, which were prepared from a single strain of P. multocida by various extraction methods, including 0.3% formalin containing 0.85% NaCl,[6] 2.5% NaCl,[11] 0.5M KSCN[9] or 1M sodium salicylate.[10] They confirmed their protective immunogenicities in turkeys, but the identity of the protective antigens in these fractions could not be demonstrated well because crude materials were used in the experiment.

Three antigens of P. multocida are considered to be purifiable and reasonably well characterized. The first, a protective antigen

extracted with 2.5% NaCl solution, has been purified by chromatographic methods from avian strains,[11,12] and this antigen appears to be a complex substance with high molecular weight. A protein having a molecular weight of 43,000-44,000 has been detected as a predominant component in the antigens purified from several serotypes. Serotype-specific protection has been demonstrated in turkeys vaccinated with the antigens.[12] The second, an antigen extracted with formalin-saline, has been purified by differential centrifugation from encapsulated as well as non-encapsulated strains of P. multocida.[6,13] This substance is a lipopolysaccharide (LPS)-protein complex, referred to as "free endotoxin." The protective immunogenicity of this antigen has been demonstrated in mice, chickens and turkeys. The third antigen, lipopolysaccharide itself, has been extracted by Westphal's standard method, or by another method used for "rough"-type LPS, and purified by ultracentrifugation.[13,14] For protection in mice, LPS was a very poor immunogen, but in chickens it induced antibody production, and the transfer of the antibodies provided protective immunity in recipients.[7] Phillips and Rimler demonstrated that LPS induced active immunity in chickens when LPS was coupled with a suitable carrier such as ribosomal proteins.[15] Various investigators[6,11,13] have purified each of these three antigens from different serotypes of P. multocida; therefore, direct comparison has never been made among these three antigens.

The purpose of this study was to elucidate chemical and antigenic relationships among the three antigens: an antigen purified from a 2.5% saline extract; "free endotoxin;" and LPS, prepared from a single source. The relationships were analyzed by immunodiffusion tests, and

various physical and chemical treatments were used to examine the nature of antigenic determinants of the three antigens.

Materials and Methods

Bacteria--Pasteurella multocida, strain P-1059[a], belonging to Heddleston's serotype 3 was used. The growth medium and the culture procedure have been described.[11] The cells grown for 18 hours at 37°C were harvested from agar plates with a 2.5% NaCl solution (2.5% saline)[11] or 0.3% formalin containing 0.85% NaCl (formalin-saline).[6]

Preparation of three antigens--From the crude extract in 2.5% saline, an antigen (designated as 2.5S) was purified by Sephadex G-200 and DEAE-cellulose chromatography.[11] From the extract in formalin-saline, an antigen (designated FS) was purified by differential centrifugation according to the method described by Heddleston et al.[6] From the cell pellet obtained after the extraction with formalin-saline, Westphal-type lipopolysaccharide (LPS) was extracted and purified by the method of Rebers and Heddleston.[13] After repeated phenol-water extraction at 68°C for 15 minutes, the pooled water phase was dialyzed against distilled water to remove phenol, then dialyzed further in 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0) containing 10 mM $MgSO_4$ and 0.02% NaN_3 (Tris-Mg). The sample was treated with ribonuclease[b] and deoxyribonuclease[b] (each, 100 μg/ml) at 37°C for 24 hours, followed by dialysis against Tris-Mg buffer. A small amount of precipitate was removed by centrifugation at 12,000 x g for 20 minutes. Finally, LPS was pelleted by two cycles of centrifugation at 105,000 x g in Spinco 50.2 Ti rotor[c] for 3 hours at 4°C. The three antigen preparations were lyophilized after dialysis in distilled water. For reconstitution, the lyophilized powder was dissolved in distilled water and briefly sonicated[d].

Chemical and physical analysis--Total protein content of each antigen was estimated by the method of Lowry et al.,[16] using bovine serum albumin as the standard. Carbohydrate was determined by phenol-sulfuric acid method,[17] using glucose as the standard. The thiobarbiturate method[18] was used to estimate 2-keto-3-deoxyoctonate. Uronic acid content was measured by the carbazole reaction,[19] using glucuronic acid as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% polyacrylamide gel column by the system of Weber et al.[20] The protein samples used as markers for molecular weight estimation have been described.[11] After electrophoresis, the gels were stained with Coomassie brilliant blue R-250[e] for protein bands or with periodate-Schiff reagent[e] for carbohydrate bands.

Endotoxin assay--Swiss-Webster mice (9 weeks old) were inoculated intraperitoneally with serial 2-fold dilutions of each antigen (five mice/dilution) and observed for 6 days. Fifty percent lethal doses were calculated by the method of Reed and Muench. The Limulus amebocyte lysate test[f] was done according to the manual supplied with the test kit. Serial 2-fold dilutions of each antigen sample were prepared with pyrogen-free distilled water, and 0.2 ml of each dilution was tested. Endotoxin from Escherichia coli 0111:B4[g] was used as a reference, which gave the minimal concentration of 12.5 pg/ml for complete gelation.

Antisera and immunodiffusion test--Rabbit anti-2.5S sera were prepared by injecting Pasteurella-free rabbits[h] intramuscularly with the purified 2.5S antigen (100 μg protein/dose, 3 times at monthly intervals). The antigen was emulsified in equal volume with Freund's complete adjuvant[i] for the first injection, and with Freund's

incomplete adjuvant[1] for the second and third injections. Turkey anti-2.5S sera were made in adult Nicholas Large White turkeys with two subcutaneous injections of the 2.5S antigen (250 μg, dry weight/dose) emulsified in Freund's incomplete adjuvant at a 14-day interval. Anti-LPS sera was produced in adult chickens by intravenous injection with 100 μg of LPS (dry weight)/dose, and the injection was repeated 4 times at 7-day intervals. Ouchterlony gel diffusion tests were carried out with 1% agarose gel plates in 0.05 M sodium phosphate buffer (pH 7.2) containing 0.85% NaCl for testing rabbit antisera, or 8% NaCl for testing avian sera.

Treatments of the three antigens--Each purified antigen (1 mg/ml) was treated with protease, periodate, phenol-water, or hyaluronidase, or by heating. i) Protease-treatment: protease E (4.4 units/mg)[e], a broad-spectrum proteolytic enzyme from Streptomyces griseus, was added at a final concentration of 0.1 mg/ml to each antigen in 0.01 M sodium barbiturate buffer (pH 7.8) containing 0.85% NaCl and 0.02% NaN_3. After incubation at 37°C for 24 hours, the solution was heated at 56°C for 30 minutes to stop the reaction. ii) Periodate-treatment: each antigen was treated with 10 mM of sodium meta-periodate[e] in 0.05 M sodium acetate buffer (pH 5.0) for 24 hours at 4°C, followed by the addition of 1% glycerol to stop the reaction. iii) Phenol-water extraction: each antigen in distilled water was extracted with an equal volume of 90% phenol (v/v) at 68°C for 15 minutes, cooled on ice, and then centrifuged to remove the water phase. Subsequently, the phenol and interphase were extracted twice with equal volumes of distilled water. The water phases were pooled and dialyzed against distilled water, followed by concentration to the original volume. iv) Hyaluronidase-treatment:

hyaluronidase from bovine testes (980 units/mg)[e] was added at the final concentration of 0.1 mg/ml to each antigen in 0.1 M sodium acetate buffer, pH 5.0 containing 0.15 M NaCl, and incubated at 37°C for 24 hours. v) Heating: aliquots of each antigen in distilled water were sealed in glass ampules and heated in boiling water for 10, 60, or 120 minutes.

[a]Obtained from Dr. K. R. Rhoades, National Animal Disease Center, SARS, USDA, Ames, Iowa.

[b]Specific activities were 2812 units/mg for RNase and 3599 units/mg for DNase; Worthington Biochemical Co., Freehold, NJ.

[c]Beckman Instruments, Inc., Palo Alto, Calif.

[d]Disontigrator; Ultrasonic Industries, Clearwater, Fla.

[e]Sigma Chemical Co., St. Louis, Mo.

[f]M.A. Bioproducts, Waltersville, Md.

[g]Supplied with the test kit.

[h]ORRC:NZW obtained from the Rabbit Research Center, Oregon State University, Corvallis, Ore.

[i]Difco Laboratories, Detroit, Mich.

Results

Chemical analysis and toxicity--The three antigens, 2.5S, FS, and LPS, were prepared from a single source: 30 g of packed wet cells. Approximately 25 mg of 2.5S, 38 mg of FS, and 80 mg of LPS antigens were obtained. A chemical composition of the 2.5S antigen and that of the FS antigen were very similar (Table II.1); both contained protein and carbohydrate in approximate proportions of 40% and 15%, respectively. All three antigens contained 2-keto-3-deoxyoctonate. Based on the amount of this sugar, the 2.5S and the FS antigens were estimated to contain the LPS-component in proportions of 45% and 42%, respectively. Only small amounts of uronic acid were detected in the three antigens. In SDS-PAGE, both the 2.5S and the FS antigens had a major protein band with a molecular weight of 44 K and a broad carbohydrate band (Fig II.1). The LPS antigen contained a large amount of carbohydrate; carbohydrate staining after SDS-PAGE revealed a band with slower mobility than those of the 2.5S and the FS antigens. A small amount of protein (5.0%) was detected in the LPS sample by Lowry's method, but no protein band was observed on the SDS-PAGE gel, even when the amount of LPS applied was twice as much as the other two antigens. This finding suggests that a predominant protein component with a specific molecular weight may not exist in the LPS preparation.

Endotoxic activities of the three antigens were estimated by mouse lethality and the Limulus lysate test. In both tests, higher activities were demonstrated with LPS than with the 2.5S or the FS antigen, and the difference was more prominent in the Limulus lysate test than in the mouse test (Table II.1).

Cross reactions in the immunodiffusion--Antigenic cross reactions among the three purified antigens were examined by the immunodiffusion test. Antiserum made in a rabbit yielded precipitin patterns that were different from those produced by a turkey antiserum, despite the fact that the two animal species were inoculated with a single lot of the purified 2.5S antigen (Fig II.2). The three antigens formed a single precipitin line against the rabbit anti-2.5S serum, with LPS forming a faint line (Fig II.2A). No precipitin line was formed when the 2.5S, FS, or LPS antigens were added to the antiserum, indicating that the three antigens were antigenically identical against the rabbit anti-2.5S serum. With the turkey anti-2.5S serum, the 2.5S antigen formed at least three precipitin lines (Fig II.2B): two dense lines close to the antigen well, which did not separate well from each other, and another faint line close to the center well, fusing with the precipitin line of LPS. An identical precipitin pattern was obtained with the FS antigen. When the turkey antiserum was mixed with LPS, only single precipitin lines were observed with the 2.5S and FS antigens. No precipitin line was formed between the antigens and the serum mixed with the 2.5S or the FS antigen. These results indicate that the 2.5S and FS antigens contained an LPS-associated component and other antigenic components not related to LPS.

Analyses of antigenic determinants after various treatments--To further characterize antigenic determinants of the three antigens, immunodiffusion tests were carried out with the antigens treated with protease, periodate, phenol-water, or hyaluronidase, or by heating at 100°C. Typical results are shown in Fig II.3, where untreated (well 1) and treated antigens (well 2) were allowed to react against both

untreated antiserum (well a) and the antiserum previously mixed with the treated antigen (well b). The untreated 2.5S and FS antigens formed precipitin lines against both wells (a and b), whereas the protease-treated antigens formed a precipitin line against well a but not against well b (Fig II.3A), indicating that some antigenic determinants were lost by the protease-treatment. In contrast, neither untreated nor treated LPS antigens formed a precipitin line against well b, indicating that no change in antigenicity occurred by the protease-treatment. An analysis by SDS-PAGE revealed that protease digested the major 44K protein in the 2.5S and the FS antigens (Fig II.1). After the protease-treatment, the 2.5S antigen was passed through a Sepharose 6B column to remove small peptides and the enzyme. The antigen recovered at void volume of the column still contained 28% protein, indicating that only 25% of total protein was removed by the treatment (data not shown).

The treatment of the 2.5S and the FS antigens with periodate resulted in partially altered antigenicity; the effects were similar to those resulting from the protease-treatment (Fig II.3B). With the LPS antigen, however, the periodate-treatment nearly abolished its antigenicity, as indicated by the formation of a faint line between wells a and 2. The major antigenic epitopes on the LPS antigen appeared to be carbohydrate in nature because of the sensitivity to this reagent.

Both protease and periodate treatments partially altered the 2.5S and the FS antigens. To investigate the nature of their alterations, another immunodiffusion analysis was done (Fig II.4). Partial identity was observed between the precipitin lines of the untreated 2.5S antigen and those of the protease-treated antigen. Lines of partial identity were also formed with the untreated and periodate-treated samples. A

faint line closer to the serum well was missing in the periodate-treated antigen. The faint line, judging from the other results presented, corresponded to the line of LPS. Lines of nonidentity were seen between the two wells of treated antigens. Therefore, protease and periodate apparently altered the antigen in a different manner.

The treatment with phenol-water apparently denatured the protein components in the 2.5S and FS antigens, leaving only LPS-related antigenic component (Fig II.5). The three antigens treated with hyaluronidase, or heated at 100°C for 10, 60 or 120 minutes, did not cause any change in their precipitin lines.

TABLE II.1--Chemical composition and endotoxic activity of the three antigens from P. multocida strain P-1059

	Antigens		
	2.5S	FS	LPS
Protein (%, w/w)	37.7	43.0	5.0
Carbohydrate (%, w/w)	14.8	15.8	31.0
2-keto-3-deoxyoctonate (%, w/w)	0.564	0.531	1.25
Uronic acid (%, w/w)	1.0	1.6	3.8
Carbohydrate/protein	0.39	0.37	6.3
2-keto-3-deoxyoctonate/ carbohydrate	0.038	0.034	0.040
LD_{50} in mice (μg)	561	561	353
LLT * (pg/ml)	125	31	7.8

* The amount of antigen causing complete gelation in Limulus lysate test (LLT).

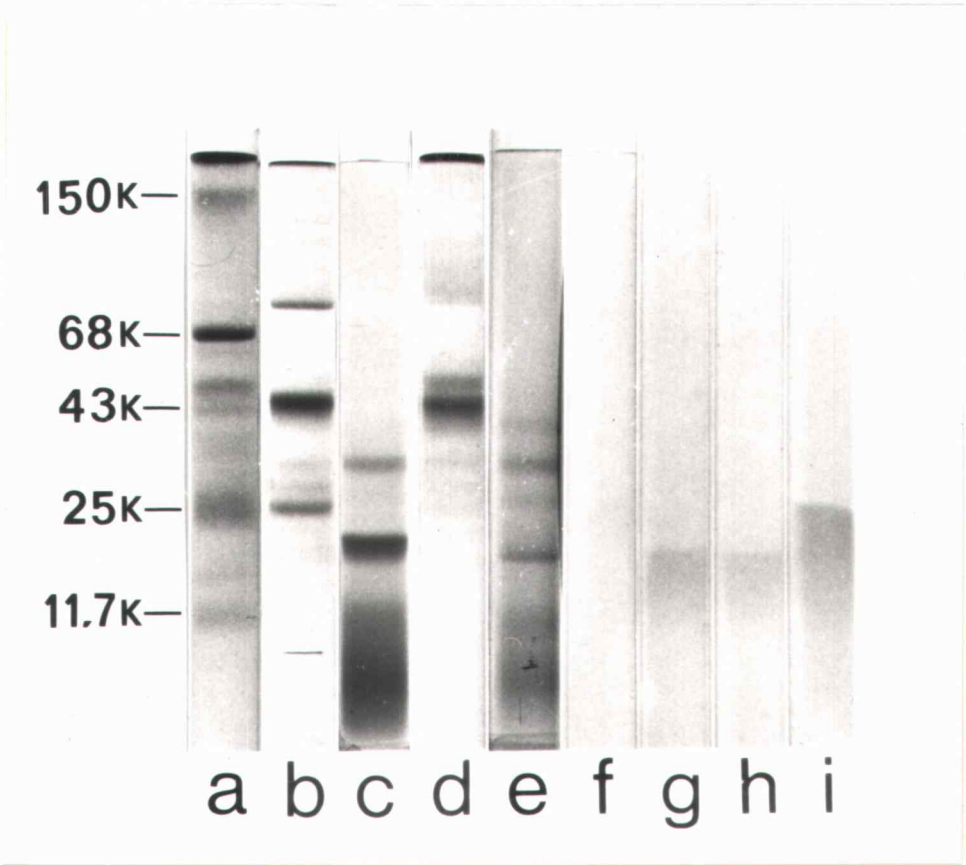

Fig II.1--SDS-PAGE of antigens purified from P-1059 strain. Columns; a, markers; b and g, 2.5S antigen (25 μg by dry weight); d and h, FS antigen (25 μg); f and i, LPS antigen (50 μg); c, protease-treated 2.5S; e, protease-treated FS. Columns a through f were stained with Coomassie blue for proteins, and columns g, h, and i were stained with periodate-Schiff reagent for carbohydrate.

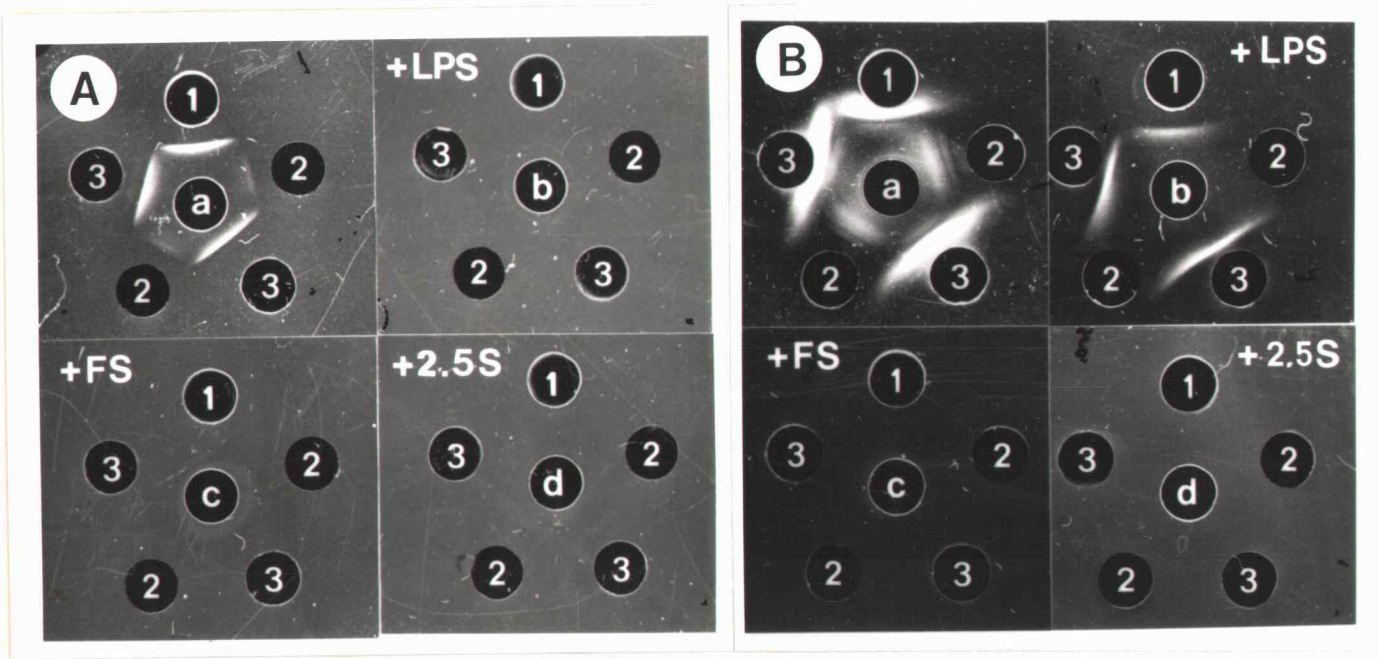

Fig II.2--Gel diffusion precipitin patterns of the three antigens tested against rabbit anti-2.5S serum (A) or turkey anti-2.5S serum (B). Wells; 1, FS antigen; 2, LPS; 3, 2.5S antigen (each 2 mg/ml). The center wells contained rabbit or turkey anti-2.5S serum only in "a," and the serum mixed with LPS antigen (1 mg/ml) in "b," with FS antigen (1 mg/ml) in "c," or with 2.5S antigen (1 mg/ml) in "d."

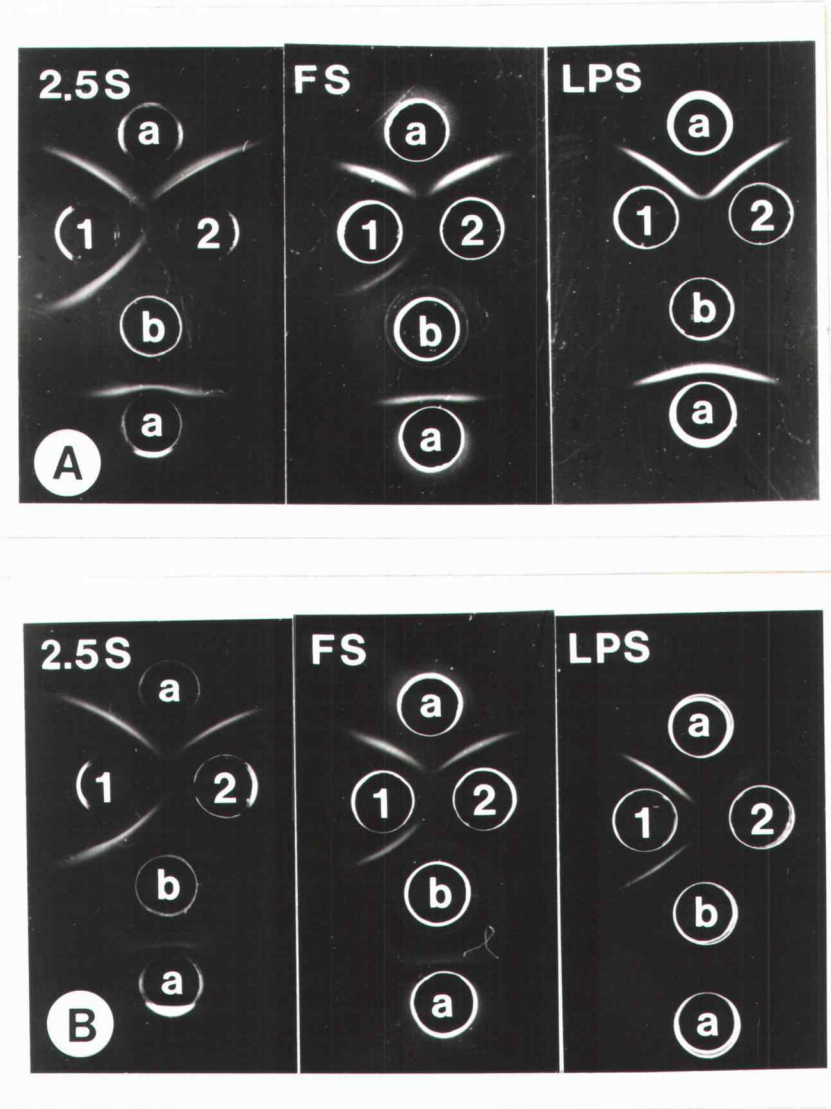

Fig II.3--The results of gel diffusion tests after protease (A) or periodate (B) treatment of 2.5S, FS, and LPS antigens. Untreated antigens (well 1) were compared with the treated antigens (well 2). Wells a and b contained anti-2.5S serum only and the serum mixed with the treated antigen, respectively. For LPS antigen only, chicken anti-LPS serum was used instead of turkey anti-2.5S serum. The precipitin lines between wells "b" and lower "a" indicate that an excess amount of antigen was added to the antiserum.

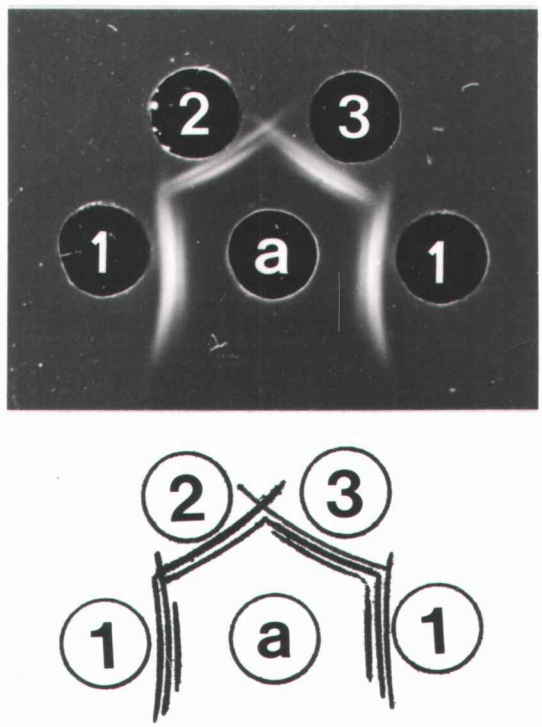

Fig II.4--Antigenic relationships among untreated 2.5S antigen (1), the antigen after the periodate-treatment (2), and after the protease-treatment (3) analyzed by the gel diffusion test with turkey anti-2.5S serum (center well, a). Precipitin lines are illustrated below.

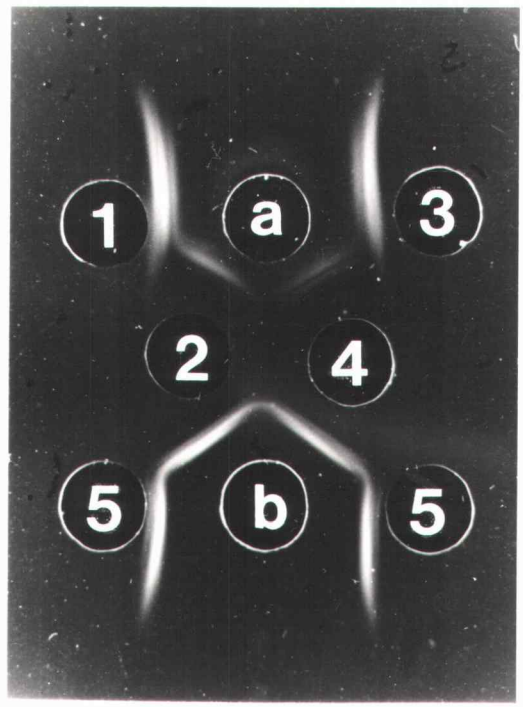

Fig II.5--The result of the gel diffusion test with the antigens treated with phenol-water (PW). Wells; 1, 2.5S antigen; 2, 2.5S antigen treated with PW; 3, FS antigen; 4, FS antigen treated with PW; 5, LPS antigen. Turkey anti-2.5S and chicken anti-LPS serum were added in wells "a" and "b," respectively.

Discussion

Gross chemical analysis showed that the 2.5S and the FS antigens were similar in terms of carbohydrate and protein content. The LPS antigen, on the other hand, had a substantially higher carbohydrate/protein ratio than the two other antigens (Table 1). The finding with the FS and the LPS antigens, in general, confirmed the findings of others.[7,13] The protein content of the FS antigen was considerably higher in the present study than that reported by Rebers and Heddleston.[13] However, the amount of protein in the FS antigen varied from 26 to 52%, depending on preparation lot.[21] The difference of culture medium may also have influenced the protein content. The 2-keto-3-deoxyoctonate content of the LPS antigen was close to that reported by Rimler et al.[14]

The pattern of protein and carbohydrate bands observed in SDS-PAGE also showed the similarity of the 2.5S and the FS antigen (Fig II.1). A protein band at 25K daltons seen with the 2.5S antigen was absent with the FS antigen. In the FS antigen, on the other hand, there were extra protein bands at 48K and at the top of the gel. These minor differences may have been attributable to the effect of formalin used for the extraction of the FS antigen. A protein band at 82K in the 2.5S antigen was not observed in an earlier study.[11] It probably was a dimer of 44K protein, because treatment of the 2.5S antigen with 2-mercaptoethanol resulted in disappearance of the band and a concomitant increase in the stain density of 44K band (data not shown).

The mouse lethality test demonstrated comparable levels of toxicity among the three antigens (Table II.1). The LD_{50} doses obtained

with the FS and LPS antigens were somewhat higher than those reported in other studies.[13,21] In the Limulus lysate test, LPS had the highest activity, followed by FS and 2.5S, in that order. The three antigens had greater differences in the Limulus lysate test than in the mouse lethal test, perhaps because of difference in sensitivity of the two tests, but physical aggregation of antigen molecules also may have influenced the tests. The lyophilized LPS antigen was less soluble than the other two antigens. To avoid aggregation, the reconstituted preparations were sonicated until gross aggregates disappeared. The interfering effect of aggregation, however, may not have completely been eliminated by this procedure.

In immunodiffusion analyses, some differences were found between the two anti-2.5S sera: one made in rabbits and the other made in turkeys (Fig II.2). The rabbit antisera could not differentiate LPS from the other two antigens. The 2.5S and the FS antigens consistently formed a single precipitin line against the rabbit sera, whereas multiple lines were obtained with the turkey sera. Presumably, the turkey antisera recognized a wider variety of antigenic epitopes on those two antigens than did the rabbit antisera. There are several previous reports concerning the difference in the antibody response and its specificity against the Pasteurella antigens between mammalian and avian species. Heddleston et al.[6] reported that free endotoxin from strain P-1059 formed a single precipitin line against rabbit antiserum, but that it formed multiple precipitin lines against chicken antiserum. Other studies showed that Westphal-type LPS from strain X-73 was poorly immunogenic in mice and rabbits, but that it produced antibodies in chickens by intravenous administration.[7,13] Because of that superior

antibody response, chickens have been recommended as the animal to produce serotype-specific antisera for Heddleston's somatic antigen typing.[22]

The multiple precipitin lines formed between the 2.5S antigen and the homologous turkey antisera may indicate the antigenic heterogeneity of the antigen preparation. The results, however, were not consistent with the conclusion of our earlier study,[11] wherein the purified antigen appeared to be antigenically homogeneous. The conclusion was based on the formation of a single precipitin line in immunodiffusion tests between the purified antigen and antisera raised against the crude saline extract, and between the crude extract and antisera raised against the purified antigen. In these analyses, however, rabbit antisera had been used. Subsequently, the purified antigen has been extensively used in our laboratory for vaccination of turkeys. With some sera from those immunized turkeys, a sufficient amount of the antigen produced multiple lines in immunodiffusion tests (Fig II.2B). Our close observation showed that a single sharp precipitin line was formed within 24 hours, followed by its gradual dissociation into two, or as many as three, separate lines at 48 to 72 hours of incubation. To isolate those antigenic components corresponding to each one of the precipitin lines, further purification has been attempted in vain by gel filtration and ion-exchange chromatography under several different conditions. The antigenic heterogeneity may have been induced under the high ionic strength environment. The precipitin reaction with turkey antisera was carried out in 8% NaCl. Inasmuch as the antigen was presumed to be a large protein-carbohydrate complex, with apparent molecular weight exceeding 2×10^6 daltons,[11] such high concentration

of salt may have partially dissociated the antigen complex, yielding a few distinct antigenic populations during the precipitin reaction.

The 2.5S antigen contained a substantial amount of LPS. The 2.5% saline extraction method, originally described by Maheswaran et al.,[23] was used to obtain "capsular polysaccharides." Our earlier study showed that the extraction caused some shrinkage of capsular structure.[11] Hence, it was assumed that the antigen primarily originated from capsular constituents, rather than from somatic components. The present study, however, showed firm evidence for the presence of LPS in the saline-extracted antigen: detection of a substantial amount of 2-keto-3-deoxyoctonate, a sugar unique to LPS of gram-negative bacteria; antigenic cross-reaction between the 2.5S and the LPS antigens; and positive reaction in Limulus lysate test. The 2-keto-3-deoxyoctonate/carbohydrate ratio of the 2.5S antigen was close to that of LPS, suggesting that the carbohydrate moiety of the 2.5S antigen may be identical to that of LPS. Regarding capsular materials, hyaluronic acid has been claimed as a major component of type A capsule.[23,24] The amount of uronic acid found in the 2.5S antigen, however, was very low, and the hyaluronidase-treatment of the antigen did not cause any change in its antigenicity. Therefore, the antigen contained an insignificant amount of hyaluronic acid. Possible presence of the other serotype-specific acidic polysaccharides was not investigated in the present study, but, judging from 2-keto-3-deoxyoctonate/carbohydrate ratio, it seemed unlikely that a large amount of such material was present in the 2.5S antigen. For further study, the 2.5S antigen from encapsulated organisms should be compared with that from non-encapsulated mutants.

At least two kinds of antigenic determinants were found in the 2.5S antigen. One was protein and another was carbohydrate in nature. To study the protein determinants, the antigen was treated with protease or phenol. Both agents similarly altered the antigenicity, as evidenced by changes in immunoprecipitin lines (Fig II.3A and 5). Phenol altered the protein to a greater extent than did protease. Neither treatment altered a determinant cross-reacting with LPS. A major protein component of 2.5S, having a molecular weight of 44K, was digested into smaller polypeptides by protease (Fig II.1). However, only 25% of the total protein in the 2.5S antigen was lost by protease-treatment following gel filtration. Conceivably, the fragmented polypeptides may have still associated with LPS-component by noncovalent force such as hydrogen bonds and hydrophobic interaction. Heating at 100°C for as long as 120 minutes did not affect the antigenicity of the 2.5S antigen, indicating that the protein as well as the carbohydrate determinants were heat stable. Carbohydrate determinants in the 2.5S antigen were examined by periodate-treatment, because the reagent has been shown to cleave vicinal hydroxyl groups in sugar without altering the structure of polypeptide chains.[25] The treatment resulted in loss of the antigenic determinant(s) relating to LPS.

The 2.5S and the FS antigens had similar physicochemical and antigenic characteristics. Both antigens have been shown to induce protective immunity in turkeys against fowl cholera,[10,11,21] and these two antigens can be categorized as a high-molecular-weight LPS-protein complex. The association between LPS and protein components is apparently noncovalent, judging from the results of SDS-PAGE (Fig 1), but the separation of each component was difficult, unless the complex

had been dissociated by detergents such as SDS and sodium deoxycholate. The dissociation, however, resulted in substantial loss of protective immunogenicity.[11] In the present study, the two treatments, with protease and periodate, specifically affected different antigenic components; primarily proteins and LPS, respectively (Fig II.4). Therefore, these treatments would be useful to investigate which component is responsible for protective capacity.

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Chapter III

Evaluation of Relationship among Three Purified Antigens from *Pasteurella multocida* Strain P-1059 and of Their Protective Capacities in Turkeys

Summary

Three antigens were prepared from Pasteurella multocida strain P-1059 and their immunogenicity and antigenic relationships were investigated. The three antigens were a soluble antigen purified from a 2.5% NaCl extract (2.5S), a similar antigen purified from an extract in 0.3% formalin solution containing 0.85% NaCl (FS), and Westphal-type lipopolysaccharide (LPS). The antigens were treated with various chemicals and enzymes to study their antigenic and immunogenic determinants. Antigenic analyses with ELISA inhibition tests indicated that 2.5S and FS were similar LPS-protein complex antigens. The 2.5S and FS antigens induced protective immunity in turkeys with high titers of antibody against LPS antigen. Although LPS was a component of 2.5S and FS, LPS itself was poorly immunogenic in turkeys. The antigenicity of protein components in 2.5S was deteriorated by protease-treatment, which, however, did not significantly diminish the protective immunogenicity. Treatment of 2.5S with sodium periodate, altering its carbohydrate moieties, decreased its immunogenicity. The immunogenicity of 2.5S was also abolished by phenol-water-treatment, owing to dissociation of the LPS-protein complex. These findings suggest that a certain form of LPS-protein complex is essential for the induction of immunity against the P. multocida infection in turkeys.

Introduction

Pasteurella multocida is associated with various diseases in mammalian and avian species.[1] Strains belonging to capsular type B or E cause hemorrhagic septicemia in cattle characterized by an acute systemic disease with high morbidity and mortality. A similar disease in birds is known as fowl cholera. Most avian isolates of P. multocida belong to capsular type A, but their somatic antigens are heterogeneous, as 16 serotypes have currently been distinguished.[2] Immunity against fowl cholera can be provoked by live vaccines using low-virulent strains or by killed whole bacterial cells (bacterins). Immunity induced by a live vaccine is cross-protective against the infections of heterologous serotype strains.[3,4] The organisms grown in vivo seem to produce a cross-protective factor, but its nature has not yet been defined.[5] In contrast, bacterins generally induce serotype-specific immunity,[6] and several protective substances have been isolated from the organisms.

Capsular polysaccharides of strain types B and E are protective against hemorrhagic septicemia.[7] However, capsular substances may not be important for the protection against fowl cholera, because immunity can be induced by non-encapsulated as well as encapsulated strains of avian P. multocida.[8] Soluble antigens extracted by potassium thiocyanate (KSCN) from type A strains have been found to be immunogenic in mice and chickens.[9,10] Baba[11] reported that a ribosomal fraction obtained from an avian strain of P. multocida induced immunity in chickens. Phillips et al.,[12] however, could not reproduce the results with highly purified ribosome preparations. Subsequently, they showed that immunity in chickens was induced by the ribosomes cross-linked

with a small amount of lipopolysaccharide (LPS) from P. multocida strain X-73.[13,14]

Lipopolysaccharides of P. multocida are regarded to be responsible for the antigenic specificity of somatic serotypes[2] and for the immunogenic specificity of bacterins.[6] However, LPS antigen purified by the phenol-water method is poorly immunogenic,[15,16] and in this regard, it behaves like a hapten. On the surface of gram-negative bacteria, LPS molecules form a native endotoxin complex in association with outer-membrane proteins.[17] An LPS-protein complex of P. multocida (free endotoxin) has been extracted with a cold formalinized saline solution and purified by differential centrifugation. This substance, designated FS, has been found to be immunoprotective in mice, rabbits, chickens, and turkeys.[16,18]

Another LPS-protein complex has been extracted by use of a hot 2.5% NaCl solution. The substance, designated 2.5S, has been purified by chromatographic methods and shown to be immunogenic in turkeys.[19] A direct comparison between 2.5S and FS antigen indicated that the two antigens were antigenically and physicochemically similar and that both antigens contained approximately 40% protein and 45% LPS.[20] Although associations between LPS and proteins are apparently noncovalent, the separation of each component is difficult without losing their native immunogenicity. Hence, it has not been determined whether a protein or LPS component is primarily responsible for the immunogenicity of the LPS-protein complex.

In the present study, three antigens, 2.5S, FS, and LPS, were prepared from P-1059 strain of P. multocida, and their antigenic relationship was quantitatively studied by an inhibition test with

ELISA. The antigens were treated with various chemicals and enzymes to characterize the nature of their antigenic and immunogenic determinants.

Materials and Methods

Bacterial strain and antigen preparations--Encapsulated P. multocida strain P-1059, serotype A:3, was used. The growth medium and culture conditions have been described.[19] The bacterial cells were extracted by one of the three procedures using hot 2.5% NaCl solution,[19] 0.3% formalin solution containing 0.85% NaCl[21] or phenol-water.[16] Each extract was purified as described elsewhere.[20] The three purified antigens were designated 2.5S, FS, and LPS, respectively.

Chemical and enzymatic treatments--The three antigens were treated with protease, periodate, phenol-water, or hyaluronidase, or by heating according to the methods described elsewhere.[20]

Conjugate preparation for ELISA--Turkey IgG was purified from pooled normal turkey sera by the sodium sulfate precipitation, followed by Sephadex G-200 and DEAE-cellulose chromatography.[22] Purity of the IgG preparation was verified by immunoelectrophoresis with an antiserum against turkey whole serum and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sheep were subcutaneously (s.c.) inoculated twice with 2 mg of the purified IgG emulsified in Freund's incomplete adjuvant.[a] The anti-turkey IgG was purified by an affinity column containing Sepharose 4B coupled with purified turkey IgG.[23] The purified antibody in 1 mg was mixed with 1 mg of alkaline phosphatase[b] (1,200 units/mg) in 1 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.02% NaN_3 (phosphate buffered saline; PBS). A single-step coupling reaction was carried out by the addition of 100 µl of 2% glutaraldehyde at 25°C for 1 hour.[24] Following dialysis against PBS, the reaction mixture was diluted into 10 ml with PBS containing 1 mM $MgSO_4$ and 1% bovine serum albumin[b] (BSA). The conjugate was

dispensed in 100 µl aliquots and stored at -70°C. For the conjugate titration, EIA microtitration plates[c] were coated with the purified turkey IgG (1 µg/ml in 0.1 M $NaHCO_3$, 100 µl/well) by incubating at 4°C for 16 hours. To prevent non-specific binding, the plates were incubated with 0.5% BSA-PBS at 25°C for 1 hour and washed 4 times with 0.05% Tween 20 in PBS (T-PBS). Serial 2-fold dilutions of the conjugate in T-PBS containing 0.1% BSA and 1 mM $MgSO_4$ (BSA-T-PBS) were added to the plates (200 µl/well), and incubated at 37°C for 60 minutes. The plates were washed 4 times with T-PBS, and a substrate solution consisting of 2.5 mM p-nitrophenyl phosphate[b] in 10% diethanolamine buffer, pH 9.8, with 1 mM $MgSO_4$ was added in 200 µl/well. After incubation at 37°C for 60 minutes, absorbance of each well at 405 nm was measured by an ELISA reader.[d] The conjugate showed an absorbance value of 1.0 at 1:2,200 dilution. The titer remained essentially unchanged for at least 6 months.

Standard antisera for ELISA inhibition test--Preparation of turkey anti-2.5S sera are described elsewhere.[20] A standard serum that had the highest ELISA antibody titer was selected. Anti-LPS sera were produced in 10 adult turkeys by intravenous (i.v.) injection of LPS in 0.85% NaCl (100 µg/dose, repeated 7 times at 7-day intervals). High titers of antibody were induced in only two of the ten turkeys. Antiserum with a higher titer was chosen as a standard. For titration of the standard antisera, microtitration plate wells were coated with 2.5S or LPS antigen in 0.01 M $NaHCO_3$ (3 µg/ml, 100 µl/well) until the solution completely evaporated. The plates were stored at 4°C. For testing, the plates were treated with 0.5% BSA-PBS and washed as described earlier. Serial 2-fold dilutions of each standard serum in BSA-T-PBS

were added to the plates and incubated at 37°C for 60 minutes. The plates were washed, and the conjugate (1:1,100 dilution) was added (200 µl/well). The standard anti-2.5S serum titrated on the 2.5S-coated plate gave an absorbance value of 0.5 at 1:5,200 dilution. The standard anti-LPS serum tested in 2.5S-coated and in LPS-coated plates gave an absorbance value of 0.5 at 1:2,000 and 1:3,200 dilutions, respectively.

ELISA inhibition test--Various antigens for the inhibition were diluted into 100, 30, 10, 3, 1, 0.3, 0.1, and 0.03 µg/ml with PBS, and each dilution was mixed with an equal volume of standard antiserum at a constant dilution. The antiserum dilutions were: anti-2.5S, 1:2,600 for the test on 2.5S-coated plate; anti-LPS, 1:1,000 for the test on LPS-coated plate; anti-LPS, 1:1,600 for the test on 2.5S-coated plate. The mixture of antiserum and an inhibitor was incubated at 25°C overnight, and 200 µl aliquots were tested in duplicate. The following ELISA procedures were the same as described for titration of the standard antisera, except that 1:550 dilution of the conjugate was used. Wells without antigen coating were used as blanks, and the wells containing antiserum alone as controls. Percent inhibition (X) was calculated from the equation:

$$X = \left(1 - \frac{\text{mean absorbance value of wells with inhibitor}}{\text{mean absorbance value of control wells}}\right) \times 100$$

A 50% inhibition dose of an inhibitor was obtained from a linear regression line fitted to the plots of inhibitor amount/well in $\log_{10}$ versus logit, $\log_{10} \frac{X}{100-X}$, of inhibition value.

Protection tests in turkeys--In the first experiment, each antigen solution was mixed with an equal volume of Freund's incomplete adjuvant, and a dose of 250 µg was injected twice s.c. into 10-week-old

Nicholas Large White turkeys at a 4-week interval. The turkeys were challenge-exposed intramuscularly (i.m.) with 360 colony forming units (CFU) of P. multocida strain P-1059 at 4 weeks after the second injection. Mortality was recorded daily, and survivors were euthanatized for necropsy at day 14 after exposure. Isolation of P. multocida was attempted from liver swab specimens obtained at necropsy. The organisms isolated were treated with hyaluronidase, and identified by an agglutination test with an anti-P-1059 serum.[25] Data on mortality and survival time were examined by Fisher's exact test and Mantel's survival statistics,[26] respectively.

In the second experiment, untreated or treated 2.5S antigens were emulsified with Freund's incomplete adjuvant and injected twice s.c. with a 14-day interval. The LPS antigen in PBS, 100 µg/dose, was inoculated 4 times i.v. at weekly intervals. The first trial was initiated with 9-week-old turkeys, and they were challenge-exposed i.m. with 350 CFU of P-1059 strain at 13 weeks of age. The second trial was initiated with 6-week-old turkeys, and they were challenge-exposed i.m. at 10 weeks of age with 530 CFU of strain P-1059.

ELISA--Turkey sera obtained at the challenge infection were examined by a conventional ELISA to measure the antibody titers against 2.5S or LPS antigen. The 2.5S (0.5 µg/ml in 0.01 M $NaHCO_3$) or LPS antigen (2 µg/ml in PBS) was added to microtitration plates, 200 µl/well, and allowed to dry out. The plates were treated with 0.5% BSA-PBS and washed as described earlier. Serial 3-fold dilutions of serum samples in 200 µl of BSA-T-PBS were added to 2.5S- or LPS-coated plates. The subsequent procedures were identical to those described in the titration of standard sera for ELISA inhibition test, except that 1:4,000 dilution

of the conjugate was used. Wells without serum were used as blanks. For a positive control, anti-2.5S serum was placed in 4 wells/plate, and the mean absorbance value was used as a reference value of each plate. The dilution of the control antiserum was 1:90 in 2.5S-coated plates or 1:300 in LPS-coated plates. Absorbance values of tested sera were processed by a programmable calculator,[e] and antibody titer was obtained as the reciprocal of the serum dilution having 30% of the reference absorbance value.[27]

[a]Difco Laboratories, Detroit, Mich.

[b]Sigma Chemical Co., St. Louis, Mo.

[c]Linbro EIA microtitration plate, Flow Lab., McLean, Va.

[d]Titertek Multiskan MC, Flow Lab., McLean, Va.

[e]HP-97 calculator, Hewlett-Packard, Corvallis, Ore. The program listing is available on request.

Results

ELISA inhibition tests with the three antigens--An inhibitory principle was applied to ELISA to analyze the relationship of the three antigens. Increasing amounts of each antigen were mixed with a constant amount of a standard anti-2.5S serum. The mixture was incubated overnight, and the antibodies free to react were measured by ELISA with 2.5S-coated plate. Decrease in the absorbance value was expressed by percent inhibition (Fig III.1A). In this system, the 2.5S and FS antigens produced identical inhibition curves, indicating antigenic similarity between the two antigens. Both antigens caused nearly complete inhibition at a dose of $10^{3.5}$ ng/well. The 50% inhibition doses of 2.5S and FS were estimated at $10^{1.18 \pm 0.04}$ ng ($10^{\text{mean} \pm \text{SE}}$) and $10^{1.26 \pm 0.01}$ ng, respectively. In contrast, LPS antigen caused only partial inhibition in the same system. The inhibition was not more than 40%, even when the dose was increased as much as 10 μg/well. The three antigens also were examined in a different assay system, where the antigens had been incubated with a standard anti-LPS serum and then assayed on an LPS-coated plate (Fig III.1B). The highest inhibition was obtained with the LPS antigen (50% inhibition dose, $10^{2.04 \pm 0.04}$ ng). The 2.5S and FS antigens also caused substantial inhibition, but their 50% inhibition doses were as much as $10^{3.32 \pm 0.10}$ ng and $10^{3.37 \pm 0.10}$ ng, respectively. These values indicated that their inhibitory activities were approximately 20 times less than that of LPS antigen.

ELISA inhibition tests with treated antigens--The effect of various treatments on the antigenicity of 2.5S was investigated in the same inhibition system as depicted in Fig III.1A. Protease and phenol-water treatments decreased the inhibition activity of 2.5S to 1/25 and

1/10 of the original, respectively, based on the 50% inhibition doses (Fig III.2A). An irregular sigmoidal inhibition curve was obtained with the protease-treated antigen. When 2.5S antigen was treated with periodate, a peculiar enhancing effect was observed at lower doses, but the inhibition with higher doses reached a plateau of approximately 70% inhibition. When FS antigen was treated with these reagents, the changes were similar to those of 2.5S (Fig III.2A). Effects of various treatments on LPS antigen were investigated in the system consisting of anti-LPS serum and LPS-coated plate (Fig III.2B). The periodate-treatment greatly affected the antigenicity of LPS, and its inhibition potency decreased to about 40%. Protease and phenol-water treatments also decreased the inhibition potency: 1/9 and 1/12 decrease, respectively, on the 50% inhibition basis. Treatment with hyaluronidase or heating at 100°C for 120 minutes did not cause any significant change in the inhibition curves of the three antigens.

Antigenic determinants in 2.5S antigen cross-reacting with LPS was further investigated in a heterologous system of ELISA inhibition test, in which the anti-LPS serum was mixed with the untreated or treated 2.5S antigen and tested on 2.5S-coated plates (Fig III.3). The inhibitory activity of 2.5S was not significantly altered by protease- or phenol-water-treatment, but it was virtually abolished by treatment with periodate. Similar results were obtained when untreated or treated 2.5S antigens were tested in a system consisting of anti-2.5S serum and LPS-coated plates (data not shown). These results indicated that the cross-reactive determinants were associated with periodate-sensitive carbohydrates.

Immunogenicity of the three antigens--The 2.5S, FS, and LPS antigens were injected s.c. into turkeys to examine their protective potency. Significant protection ($P<0.05$) was observed with 2.5S and FS antigens (Table III.1). The two antigens induced high titers of antibody against LPS antigen. In contrast, LPS antigen did not induce significant titers and no protection was observed. Pasteurella multocida was isolated from all of the dead birds and from none of the protected birds.

Immunogenicity of the treated antigens--The effect of various treatments on the protective immunogenicity of 2.5S antigen was tested in two trials (Table III.2). The protease-treatment of 2.5S did not affect its immunogenicity in either trial. On the other hand, the periodate-treated 2.5S failed to induce significant protection. The effect of periodate was more evident in the second trial than in the first. The periodate-treated antigen induced high titers of antibody against 2.5S antigen, but little response was observed against LPS antigen. Immunogenicity of 2.5S antigen was abolished by phenol-water treatment. The LPS antigen injected i.v. also was non-immunogenic in turkeys. Surviving birds had high anti-LPS titers (3.49 ± 0.75 in $\log_{10}$) at the challenge exposure. None of the turkeys having anti-LPS titers below 2.0 was protected. Most of the birds with anti-LPS titers above 2.0 were protected from death within 5 days after challenge-exposure. However, there was no correlation between anti-LPS titers and mortality occurred after the sixth day.

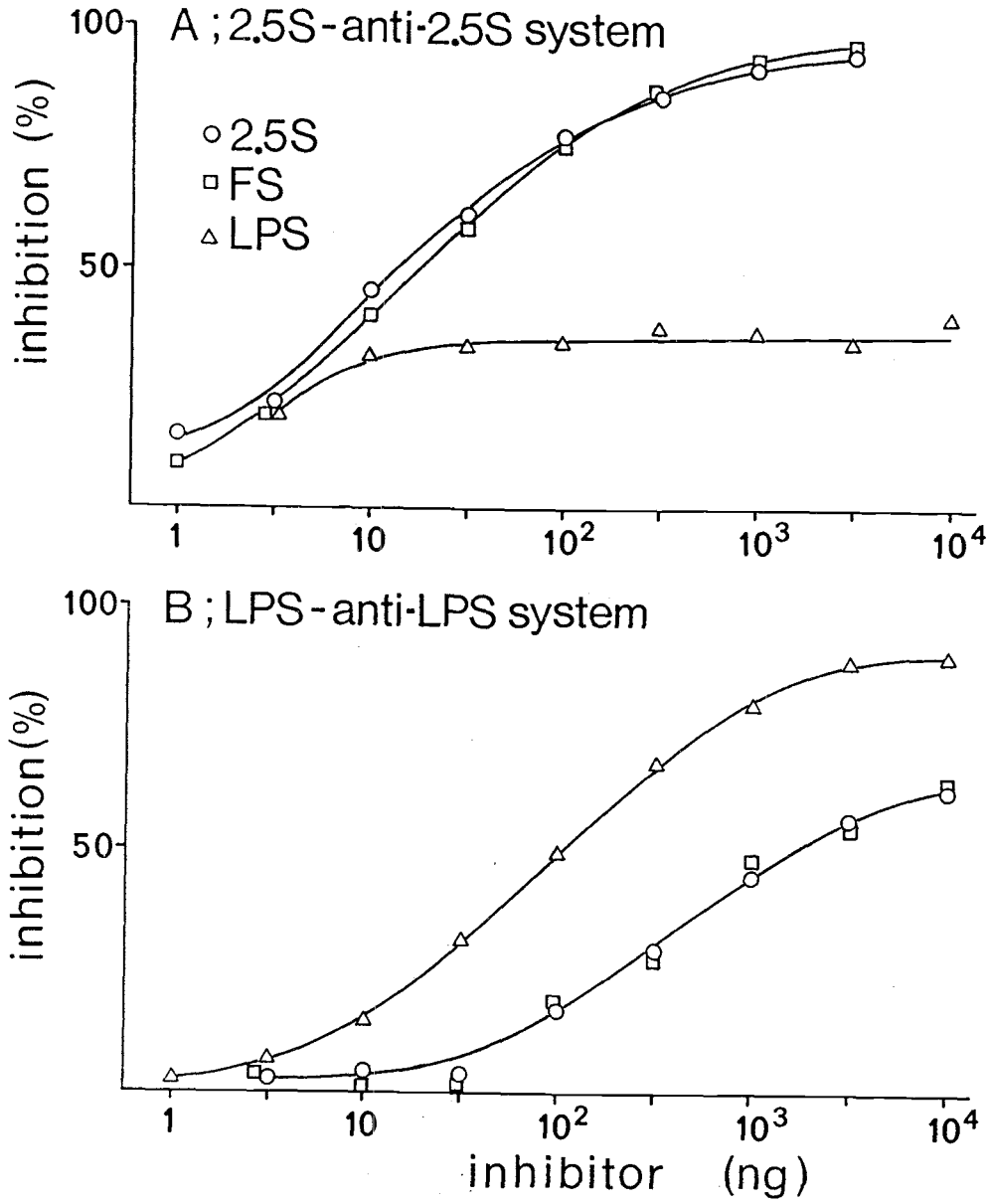

Fig III.1--Extent of cross-reaction among the three antigens examined by an ELISA inhibition test. Increasing amounts of each antigen were mixed with the standard anti-2.5S serum (A) or anti-LPS serum (B), and tested in 2.5S-coated or LPS-coated plates, respectively.

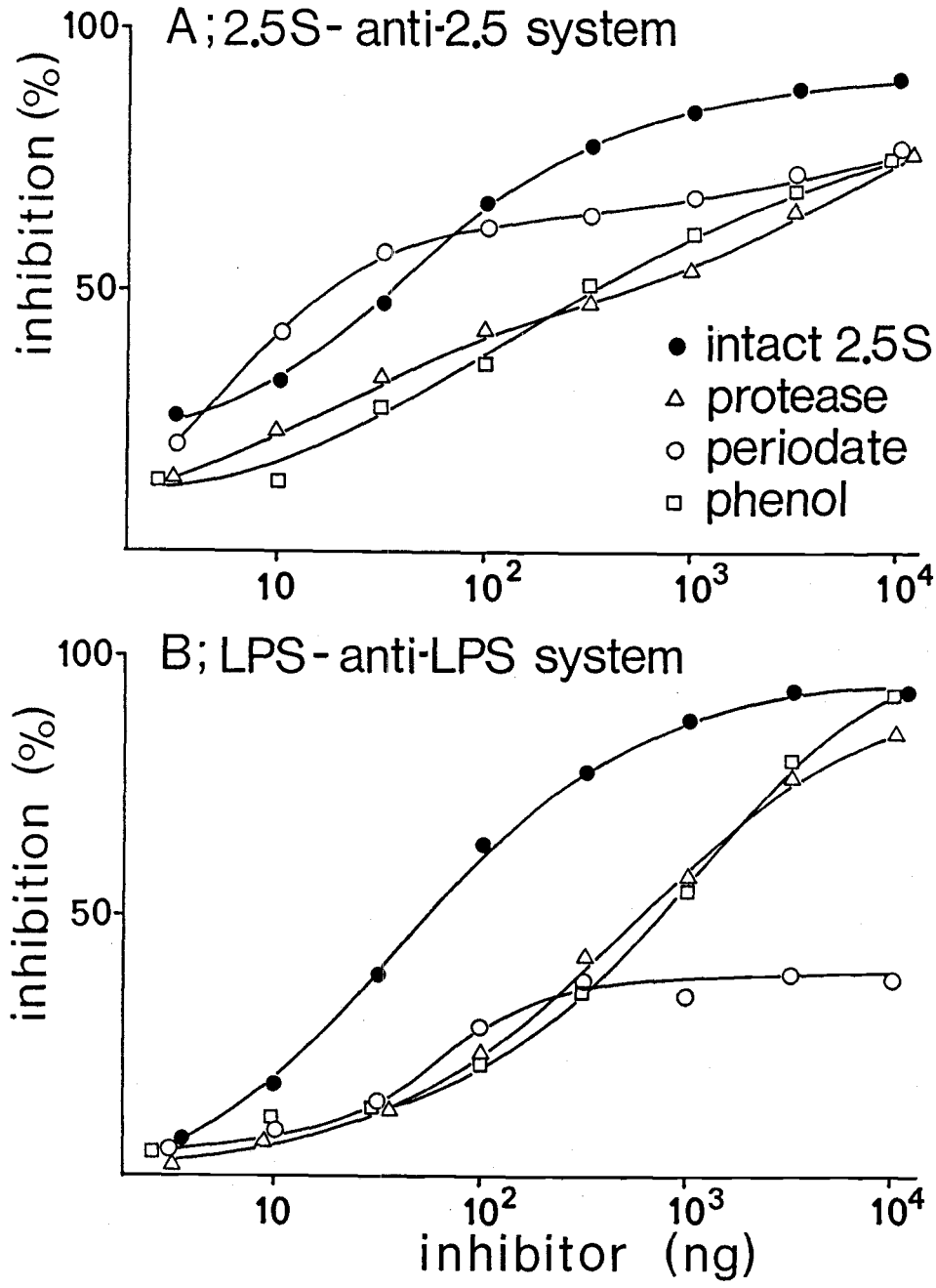

Fig III.2--Inhibition tests in ELISA with the antigens treated with various reagents. In A, increasing amounts of untreated, protease-, periodate-, or phenol-water-treated 2.5S antigen were incubated with the standard anti-2.5S serum, followed by the addition of the mixture to 2.5S-coated plates. In B, untreated LPS or treated LPS in various amounts were incubated with anti-LPS serum, and the mixture was added to LPS-coated plates.

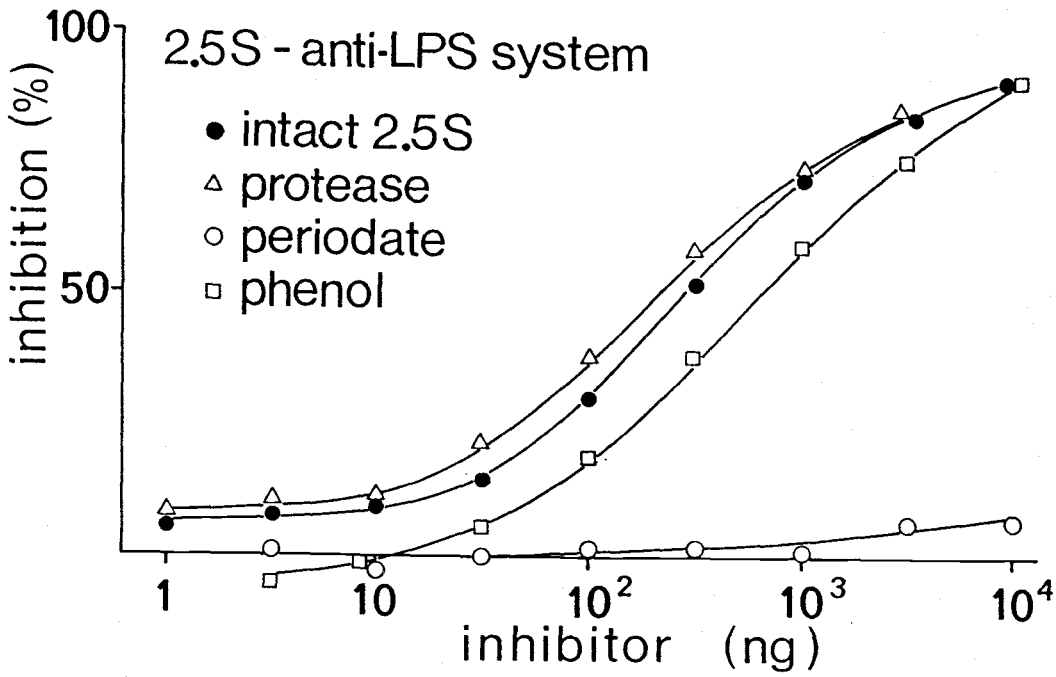

Fig III.3--Effects of several treatments on the antigenicity of 2.5S antigen analyzed by a heterologous system of ELISA inhibition test, which consisted of 2.5S antigen and turkey anti-LPS serum.

TABLE III.1--Protective potency of the three P. multocida antigens in turkeys

Antigen*	Mortality (dead/total)	MST (days)	Isolation[ϕ] (positive/total)	Antibody titer[ǂ] against 2.5S	Antibody titer[ǂ] against LPS
2.5S	2/5[a,b§]	>12.2	2/5	2.84 ± 0.26	3.00 ± 0.27
FS	0/5[a]	>14.0	0/5	3.23 ± 0.24	3.50 ± 0.41
LPS	5/5[b,c]	2.0	5/5	1.09 ± 0.31	1.29 ± 0.25
None	7/7[c]	1.9	7/7	1.00 ± 0.57	1.33 ± 0.15

* All antigens (250 μg/dose) were injected s.c. twice into turkeys when they were 10 weeks old and 14 weeks old, and the birds were challenge-exposed i.m. with 360 CFU of P. multocida strain P-1059 at 18 weeks of age. MST = Mean survival time. [ϕ]Isolation of P. multocida from liver. [ǂ] Antibody titer (mean ± standard deviation in $\log_{10}$) against 2.5S or LPS antigen measured by ELISA, with serum samples obtained at time of challenge exposure. [§] Groups without a common letter were significantly different ($P<0.05$).

TABLE III.2--Protective potency of untreated or treated 2.5S and LPS antigen in turkeys

Antigen*	Treatment	Mortality (dead/total) within 5 days	Mortality (dead/total) within 14 days	MST (days)	Isolation$^{\phi}$ (positive/total)	Antibody titer‡ (mean ± SD in $\log_{10}$) against 2.5S	Antibody titer‡ (mean ± SD in $\log_{10}$) against LPS
Trial 1							
2.5S	None	0/8[a§]	4/8[a]	>12.9[a,b]	4/8	3.64 ± 0.37	3.65 ± 0.41
2.5S	Protease	0/9[a]	2/9[a]	>12.6[a]	2/9	3.81 ± 0.59	3.86 ± 0.53
2.5S	Periodate	3/9[a]	7/9[a,b]	> 8.6[b]	8/9	3.06 ± 0.29	1.49 ± 0.35
2.5S	Phenol-water	8/8[b]	8/8[b]	1.6[c]	8/8	0.78 ± 0.13	0.74 ± 0.20
LPS, i.v.[Π]	None	9/9[b]	9/9[b]	1.7[c]	9/9	0.83 ± 0.17	0.79 ± 0.23
Control[¶]	—	9/9[b]	9/9[b]	1.7[c]	9/9	0.84 ± 0.19	0.92 ± 0.23
Trial 2							
2.5S	None	1/16[a]	9/16[a]	>12.0[a]	9/16	3.27 ± 0.32	3.31 ± 0.48
2.5S	Protease	2/16[a]	11/16[a]	>10.2[a]	13/16	3.33 ± 0.40	3.49 ± 0.39
2.5S	Periodate	14/16[b]	16/16[b]	3.1[b]	16/16	2.62 ± 0.53	1.03 ± 0.27
Control	—	16/16[b]	16/16[b]	1.7[c]	16/16	0.97 ± 0.20	0.83 ± 0.21

* All antigens except for the group "LPS, i.v." were injected s.c. twice, 250 μg/dose, with a 14-day interval. MST = Mean survival time; one side P values were calculated with Mantel's survival statistics.[26] $^{\phi}$ Isolation of P. multocida from liver.

‡ Serum antibody titers at time of challenge exposure, as measured by ELISA. § Groups within each column without a common letter were significantly different ($P<0.05$).

$^{\Pi}$ LPS (100 μg/dose) was administered i.v. four times at weekly intervals. ¶ Incomplete Freund's adjuvant only was injected twice.

Discussion

Relationship of the three antigens was investigated by an immunodiffusion test in another study.[20] Although their antigenic cross-reaction was demonstrated by the test, further details have not been explored, due to the lack of a suitable quantitative method. In the present study, we developed the ELISA inhibition test, which provided a useful tool to analyze the extent of antigenic cross-reactivity as well as to study the nature of antigenic determinants of those antigens. The assay was found to be sensitive enough to quantitate as little as 10 ng of antigen.

Major antigenic determinants on the LPS antigen appeared to be periodate-sensitive sugars. After the periodate-treatment, however, the antigen still retained approximately 40% inhibition (Fig III.2B). This residual activity might have been attributable to a small amount of contaminating protein in the LPS preparation, because the inhibition decreased after protease- or phenol-water-treatment. In addition, two things should be considered. First, periodate does not cause entire degradation of carbohydrate chains, but oxidizes only vicinal hydroxyl groups into aldehyde groups within a sugar. Such altered antigenic determinants still may have cross-reacted to the antibodies. Second, probably not all antigenic determinants are susceptible to the reagent, because some sugars may not have the vicinal hydroxyl groups.

Periodate-sensitive determinants were responsible for the cross-reaction between 2.5S and LPS antigen. It was clearly demonstrated with a heterologous system of ELISA inhibition test, wherein only the common components between the two antigens were selectively measured (Fig III.3). The test showed that the 2.5S antigen lost all inhibition

activity by the periodate-treatment, whereas the protease- or phenol-water-treatment did not significantly alter the activity. The effect of periodate, however, could not be demonstrated well in a homologous inhibition system (Fig III.1A). On the contrary, the protease and phenol-water treatments significantly decreased the inhibition activity of 2.5S antigen. The contribution to the total antigenicity appeared to be greater for the protein components than for the carbohydrates in 2.5S.

The protective potency of 2.5S was not affected by protease-treatment, despite the apparent changes in antigenicity (Table III.2 and Fig III.2A). Similar results have been obtained with free endotoxin (FS) antigen of strain P-1059 by Ganfield et al.[18] In our earlier study, the major 44K protein component was isolated from 2.5S antigen after SDS-PAGE, but it did not induce immunity.[19] Thus, the protein moiety of those LPS-protein complex antigens may not be responsible for the protective immunity in turkeys. Mukkur and Pyliotis, however, obtained contrasting results with a KSCN extract from type A:3 strain of P. multocida isolated from cattle.[9] Their experiments with mice showed that the protective substance(s) in the extract was destroyed by several proteolytic enzymes or by heating at 56°C for 45 minutes, but not by the periodate-treatment. The extract contained a substantial amount of LPS and outer membrane-like materials. Another KSCN extract of strain P-1059 has been shown to be immunogenic in chickens.[10] Although the results in mice clearly indicated that the immunity was associated with some proteinaceous substances in the extract, such an experiment has not been carried out in birds. The protective substance(s) for mice may not be the same as that for avian species.[28,29]

The LPS-associated component in 2.5S antigen appears to be important for the protective immunity, because the periodate-treatment significantly deteriorated the protective potency of 2.5S. The periodate-treated antigen induced a substantial titer against untreated 2.5S but not against LPS (Table III.2). All protected birds had high anti-LPS titers, whereas birds with low titers were not protected. These results, however, did not necessarily indicate that the fate of birds after challenge exposure were predictable by measuring their anti-LPS titers. In fact, some birds died in spite of the high anti-LPS response, although their survival time was significantly extended. Direct evidence for the immunoprotective role of LPS antigen could not be demonstrated because the LPS antigen itself was found to be poorly immunogenic in turkeys. No antibody was produced with the LPS after either s.c. or i.v. injection. It should be noted that the anti-LPS sera used for ELISA inhibition tests were made in turkeys with some difficulty. The haptenic nature of LPS antigen has been noticed by Rebers et al.[15] They reported that Westphal-type LPS from strain X-73 was not immunogenic in mice and rabbits. In chickens, however, the LPS behaved as a complete antigen, and optimal antibody production was obtained when a proper amount of LPS was administered i.v. at weekly intervals. Protective ability of the anti-LPS sera has been demonstrated by passive immunization in 7-day-old chickens. Although the chicken seems to be a better responder than the turkey, the extent of anti-LPS responsiveness was different between White Leghorn and New Hampshire Red chickens.[30] Furthermore, the immunogenicity of LPS antigens varied, depending on their serotypes.[30]

The LPS antigen probably requires some carrier protein to be immunogenic. The 2.5S antigen produced high titers of antibodies against LPS, but the immunogenicity was totally abolished by phenol-water treatment (Table III.2). Inasmuch as the treatment did not affect the LPS-crossreactive antigenicity of 2.5S, the treatment probably caused dissociation of the LPS-protein complex, releasing the LPS component as a free hapten. Rebers and Heddleston[16] also reported that the free endotoxin of strain X-73, as well as the whole cells, lost the immunogenicity upon treatment with phenol. More recently, Phillips and Rimler[13,14] clearly demonstrated that the immunogenicity of LPS from strain X-73 could be greatly enhanced by being cross-linked with ribosomal proteins of Brucella abortus or Aspergillus fumigatus. Such a LPS-ribosome complex induced protective immunity in chickens, and the immunity could be passively transferred into 7-day-old turkeys with the chicken antisera.[14]

Anti-LPS antibodies appear to have a crucial role in immunoprotection against fowl cholera, but the mechanism is not clear. The anti-LPS antibodies probably do not opsonize P. multocida very efficiently, because the organisms of highly virulent P. multocida strains are usually encapsulated. The thickness of morphological capsule is as large as the diameter of cell body. Some recent studies have indicated that LPS of P. multocida has only a short length of carbohydrate chain, similar to that of rough-type LPS of Enterobacteriaceae.[31,32] If this is the case, the LPS molecules probably do not project from the outermost surface of encapsulated organisms as the target of opsonins. It is not known, however, whether anti-LPS antibodies as well as complement freely permeate the capsular

structure, causing bacteriolysis and/or opsonization.

Toxicity of P. multocida endotoxin has been studied in chickens, wherein the i.v. inoculation of 0.16 mg of free endotoxin resulted in a 50% death rate in addition to signs and lesions similar to those of acute fowl cholera.[21] A substantial amount of endotoxin has been detected in the serum of turkeys at the terminal stage of infection.[6] The anti-LPS antibodies may neutralize the endotoxin; however, such an effect would not explain how the immune host controls the bacterial multiplication at an early phase of infection.

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Chapter IV

Role of the Capsule in Pathogenicity of *Pasteurella multocida* in Turkeys.

Summary

The pathogenic role of the capsule was studied with three forms of organism derived from a single source of Pasteurella multocida, strain P-1059. Organisms with thick capsules were produced on dextrose starch agar. Decapsulated organisms were prepared by the treatment with hyaluronidase. A non-encapsulated mutant (T-325) was spontaneously developed from P-1059 by serial passages in tryptose broth. The encapsulated organisms were highly virulent in turkeys, whereas the non-encapsulated mutant showed significantly lower virulence. Enzymatic decapsulation, however, failed to decrease the virulence. Treatments with normal or immune turkey serum did not significantly alter the in vivo pathogenicity of either encapsulated or decapsulated form of P-1059. All three forms of organisms were able to grow in fresh normal turkey plasma in vitro. Normal turkey whole blood was bacteriostatic against T-325, but not against both encapsulated and decapsulated forms of P-1059. Fresh immune plasma was bactericidal against all three forms. The encapsulated P-1059 was inactivated by the whole blood obtained from immune turkeys to a greater extent than the decapsulated P-1059 or the mutant T-325. The results did not support the hypothesis that the virulence of P. multocida is associated with the anti-phagocytic activity of capsular hyaluronic acid.

Introduction

Fowl cholera, an acute lethal septicemic disease in avian species, is caused by Pasteurella multocida.[1,2] The organisms are usually encapsulated when isolated from the hosts. Based on the antigenicity of capsular materials, five distinct capsular types--A, B, D, E and recently F--have been recognized.[2,3] Most avian isolates associated with acute fowl cholera belong to type A.[3,4] Strains of type A P. multocida have been shown to produce a large amount of capsular hyaluronic acid[5,6] in addition to yet unidentified substance(s) specifying their serotype.

A close relation between virulence and encapsulation of P. multocida has been noticed by many researchers.[7-12] Highly virulent P. multocida strains are usually encapsulated, while non-encapsulated strains tend to be less virulent. Heddleston et al., using non-encapsulated mutants spontaneously developed from an encapsulated strain of avian P. multocida, demonstrated that the loss of ability to produce capsule resulted in a marked loss of virulence.[12] However, the presence of morphological capsules does not necessarily determine the degree of virulence.[13] Conversely, non-encapsulated P. multocida showing high virulence to mice has been noted by Carter.[14] Hence, the exact role of capsule in the pathogenesis of fowl cholera is not clear.

The capsule of type A P. multocida may act as an anti-phagocytic factor. Several studies showed that encapsulated strains of P. multocida were resistant to phagocytosis by bovine or rabbit polymorphonuclear neutrophils.[15-17] High-molecular-weight hyaluronic acid has been shown to inhibit the phagocytosis of latex particles by peritoneal macrophages.[18] Ryu et al. found an anti-phagocytic activity

in a soluble fraction extracted from type A strains of bovine P. multocida.[19] The activity was attributed to a high-molecular-weight capsular substance which was resistant to heating as well as to hyaluronidase-treatment. Truscott and Hirsh have recently isolated an outer-membrane protein from an avian strain of P. multocida, which interfered with not only phagocytosis but also the intracellular killing process of mononuclear phagocytes.[20]

The capsular substances may also act as a physicochemical barrier against humoral anti-microbial factors. Encapsulated P. multocida has been found to be more resistant than the non-encapsulated ones to the bactericidal activity by alternative complement pathway.[21] Capsular hyaluronic acid, by forming a highly negatively-charged slime layer, may possibly exhibit anti-complement activity.[22] Such activity may abortively activate complement, without forming a membrane-attack complex on the bacterial surface. Furthermore, the capsule may shield the bacterial surface antigens from the antibodies, because capsular hyaluronic acid has been shown to inhibit the agglutination of P. multocida by the antibody.[23]

The present study was conducted to examine whether the presence of capsule influenced mortality in turkeys upon intravenous inoculations of P. multocida. A single strain of P. multocida, P-1059, was used in three forms: a thickly encapsulated form, an enzymatically decapsulated form and a mutant form lacking capsule-productivity. The three forms of the organisms were also tested in vitro for their susceptibility to the humoral and cellular anti-microbial mechanisms.

Materials and Methods

Bacterial strain--Pasteurella multocida strain P-1059, a type A:3 strain of turkey origin, was kindly supplied by K. R. Rhoades, National Animal Disease Center, Ames, Iowa. In our laboratory, the strain was passaged 5 times in turkeys to maintain the virulence. For the present study, the strain was passaged again in a normal turkey. Organisms were re-isolated in a pure culture from the liver of the dead bird using dextrose starch agar[a] (DSA). A single iridescent colony was picked and propagated on another DSA plate at 37°C for 7 hours. The organisms were suspended in brain heart infusion broth[a] containing 1% sucrose, 1% sodium glutamate and 20% heat-inactivated normal turkey serum. Aliquots were sealed in glass ampules and stored at -70°C. This single lot of stock culture was used throughout the experiments. Non-encapsulated mutants were obtained according to the procedure described by Heddleston et al.[12] Briefly, the strain P-1059 was cultured in tryptose broth[a] at 37°C, and a loopful of the culture was transferred to new medium daily. The cultures were occasionally streaked on DSA plates to observe the appearance of non-iridescent colonies. A non-encapsulated mutant, designated T-325, was randomly chosen among the non-iridescent colonies on the 25th passage. The organisms were purified by another single colony-formation and stored as a single lot of frozen culture.

Culture method--Each bacterial stock was streaked on a DSA plate, and cultured overnight at 37°C in a humid incubator. Three to five colonies were picked and spread out on another DSA plate. The plate was incubated at 37°C for 3 to 4 hours to obtain the organisms during log-phase. Encapsulated P-1059 and non-encapsulated T-325 were harvested from the DSA plates with 10 ml of Dulbecco's phosphate

buffered saline containing Ca^{2+} and Mg^{2+} at pH 7.4 (PBS), and used without washing. To obtain decapsulated organisms, the encapsulated P-1059 was treated by hyaluronidase as described below. All three bacterial preparations were spectrophotometrically adjusted so that 1:10 dilutions gave the optical density at 600 nm of 0.1 (OD_{600} = 0.1 x 10; corresponding to approximately 2 x 10^9 CFU/ml), and subsequently diluted with PBS to obtain appropriate inoculum doses. The inocula were kept on ice and used within 2 hours. Actual doses were determined by plate colony-counting using heart infusion agar.[a]

Decapsulation--Bovine testicular hyaluronidase[b] was used to remove the capsule. The enzyme was dissolved at 1000 units/ml in 0.02 M phosphate buffer with 0.13 M NaCl at pH 6.3 (PB), and stored in small aliquots at -70°C. The P-1059 culture on DSA plate was briefly immersed in 0.2 ml of the hyaluronidase solution, and then harvested with 10 ml of PB. The organisms were centrifuged 10,000 x g for 10 minutes at 4°C, and then resuspended in chilled PB. The turbidity was adjusted into OD_{600} = 0.2 x 10. The bacterial suspension was mixed with an equal volume of hyaluronidase (200 units/ml in PB) and incubated at 37°C for 20 minutes. Decapsulation was checked by capsular staining (see below) or by agglutination test, in which one drop of bacterial suspension was mixed on a glass slide with one drop of turkey anti-P-1059 serum.

Treatment of inocula--In some experiments, the encapsulated and decapsulated forms of P-1059 were treated with pooled sera from normal or immune turkeys. The serum samples were obtained from turkeys used in the experiment described below. Specific antibody titers against a P. multocida antigen were determined in an ELISA using a protective

antigen (2.5S) purified from the strain P-1059 as described in our previous report.[24] The normal serum was antibody negative (<1:30), while the immune serum had a titer of 1:12,000. Each bacterial suspension was diluted into approximately 10^7 CFU/ml, and 0.2 ml was mixed with 1.8 ml of either normal or immune serum. The mixture was incubated on ice for 60 minutes, and subsequently diluted into appropriate inoculum doses. These treatments did not significantly change the bacterial numbers.

Mortality test in turkeys--Nicholas Large White turkeys at 4 to 6 weeks of age were intravenously (i.v.) injected with each inoculum. Mortality was recorded daily, and survivors at 14 days after the infection were euthanatized and examined at necropsy. Isolation of P. multocida was attempted from the livers of dead and surviving birds. Since some birds injected with T-325 developed chronic joint infection, P. multocida isolation was also attempted from the affected joints.

Blood and plasma samples--The samples were taken from normal or immune turkeys at 10 to 14 weeks of age. The immune turkeys had been vaccinated twice with the protective antigen of strain P-1059 (2.5S antigen) as described previously.[24,25] They were subsequently challenge-exposed with 1.2 x 10^3 CFU of P. multocida strain P-1059, and survivors were used for the experiments between 2 and 4 weeks after the challenge infection. Approximately 20 ml of blood was drawn from a wing vein using a syringe containing 50 µl of 1000 units/ml heparin solution.[c] Plasma samples were obtained from the blood by centrifuging 500 x g at 4°C for 15 minutes. Both blood and plasma samples were kept on ice and used within 2 hours. Heat-inactivation of plasma was carried out at

56°C for 30 minutes followed by a brief centrifugation to remove any insoluble material.

Bacterial growth in blood and plasma--When encapsulated P-1059 or non-encapsulated T-325 strain was tested, 50 µl of each bacterial suspension (about 3 x 10^5 CFU/ml) was added to 3 ml of plasma or whole blood samples. For testing the decapsulated form of P-1059, 0.3 ml of hyaluronidase (1000 units/ml) was also added to the mixture. A half-milliliter aliquots were dispensed in several polypropylene test tubes (17 x 100 mm) on ice. After being loosely capped, the tubes were transferred to a shaking water bath[d] at 37°C (140 oscillation/min). The tubes were taken out after various incubation periods and placed on ice. One hundred microliter of diluted or undiluted samples, in duplicate, were plated on heart infusion agar to enumerate the number of viable bacteria.

Capsular staining--Capsules were observed by Maneval's negative staining method.[26,27] The bacterial suspension was mixed with 1% Congo red[e] and a thin smear film was made on a slide glass. After air drying, it was stained for 2 minutes in Maneval's solution consisting of acid fuchsin.[e]

[a]Difco Laboratories, Detroit, Mich.

[b]Type IV-S, 980 NF units/mg, Sigma Chemical Co., St. Louis, Mo.

[c]Grand Island Biological Co., Grand Island, NY.

[d]Model 25, Precision Scientific Inc., Chicago, Ill.

[e]J.T. Baker Chemical Co., Phillipsburg, NJ.

Results

Bacterial morphology--Figure 1 shows the light microscopic views of the three bacterial forms derived from the strain P-1059. Strain P-1059 formed a thick capsule when it was grown on DSA plates (Fig IV.1A). Sizes of the capsule were influenced by such factors as humidity, aeration and carbohydrate content of media. Smaller capsules were produced, for instance, when the organism was grown in broth media without shaking or on an agar medium containing a poor carbohydrate source. To remove the capsule, the encapsulated organism was treated with various amounts of hyaluronidase (Table IV.1). Decapsulation was achieved at the concentrations above 20 units/ml. The treatment did not significantly change the bacterial viability. Removal of the capsule was verified by capsular staining (Fig IV.1B) or by a slide agglutination test with an anti-P-1059 serum (Table IV.1). The mutant T-325 was apparently non-encapsulated (Fig IV.1C) and readily agglutinable with the anti-P-1059 serum.

Virulence in turkeys--Virulence of the encapsulated or decapsulated form of P-1059 as well as the mutant T-325 was tested in 5-week-old turkeys by i.v. inoculation (Table IV.2). The encapsulated organisms caused 100% mortality at a dose of 1.8×10^2 CFU. Using a comparable inoculum dose, the hyaluronidase-treated organisms remained fully virulent despite the loss of capsule. In contrast, the non-encapsulated mutant caused a significantly lower mortality at a dose of 3.2×10^6 CFU, but was lethal at challenge dose of 10^9 CFU. Colonies observed on the primary isolation cultures obtained from the birds infected with the mutant T-325 were predominantly of non-iridescent type (blue type), but a few iridescent colonies were also obtained from some

birds. Subsequent capsular staining demonstrated that organisms from the iridescent colonies were encapsulated, indicating that the mutant phenotype was reversible in vivo.

Treatments of inocula with turkey sera--To investigate whether the presence of capsule affects the sensitization of bacteria to specific antibody, the encapsulated or decapsulated P-1059 was incubated with normal or immune turkey serum. The treatments were carried out at a low bacterial concentration (about 10^6 CFU/ml) on ice to insure that the decapsulated organisms did not agglutinate with antibody or reform the capsule. The inocula were subsequently diluted more than 1000-fold to minimize the carry-over of specific antibodies, and injected i.v. into 4- or 6-week-old turkeys. Their mortality rates tested in two trials were shown in Table IV.3. In both trials, there were no statistically significant differences between the normal and the immune serum treatments ($P>0.05$) either with the encapsulated or with the decapsulated organisms at any dose. At very small inoculum doses (about 10^2 CFU), however, the decapsulated organisms caused considerably lower mortality than encapsulated ones did.

Bacterial growth in blood and plasma--The three forms of organisms were tested in vitro for their susceptibility to various antimicrobial factors existing in turkey blood. Heat-inactivated samples of both normal and immune turkey plasma did not show any inhibitory effect on the growth of all three forms of organisms (Fig IV.2). Both encapsulated and decapsulated forms of P-1059 freely multiplied in the fresh plasma, as well as in the whole blood obtained from normal turkeys (Fig IV.3). The mutant T-325 could grow in fresh normal turkey plasma to a certain extent, but the growth was inhibited in the whole blood

from normal birds. When fresh immune turkey plasma was used for the tests, the numbers of all three forms of organisms similarly decreased during the first 60 minutes of incubation (Fig IV.4). On the other hand, the whole blood from immune birds showed a greater level of bactericidal activity against the encapsulated P-1059 than against the decapsulated P-1059 or the non-encapsulated T-325.

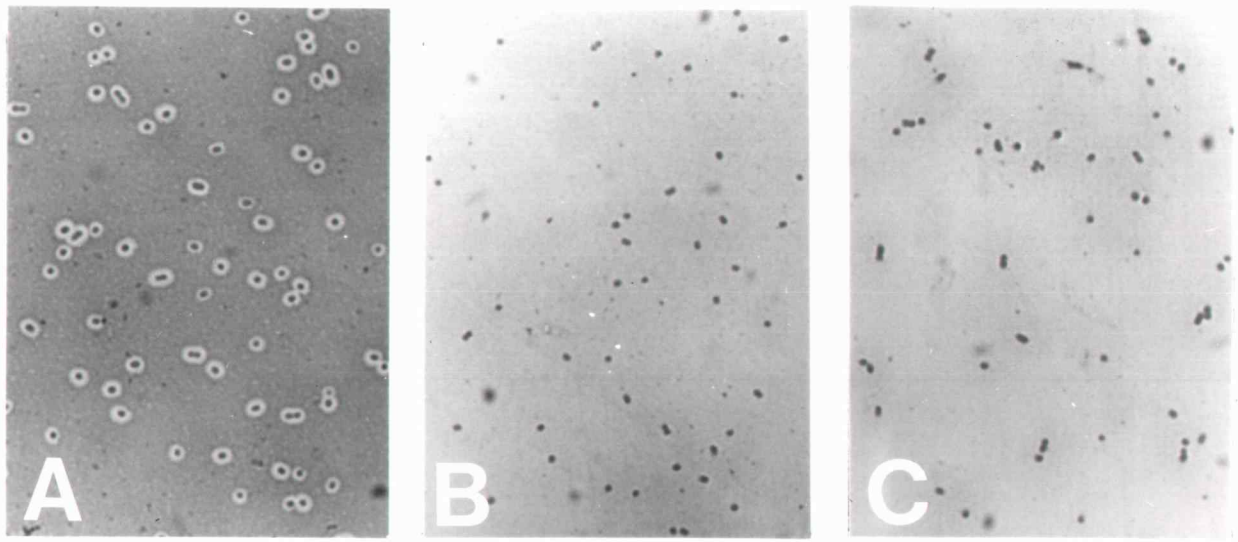

Fig IV.1--Three forms of P. multocida observed by light microscopy after capsular staining: encapsulated P-1059 strain (A), hyaluronidase-treated P-1059 (B) and non-encapsulated mutant strain T-325 (C).

TABLE IV.1--Effects of hyaluronidase-treatment on the capsular morphology of P. multocida strain P-1059, and on the agglutination by anti-P-1059 serum

Amount of hyaluronidase* (units/ml)	Number$^{\Phi}$ of bacteria (CFU/ml)	Capsule‡	Agglutination§ by Anti-P-1059
0	2.3×10^9	+	Negative
4	2.0×10^9	$\pm$	+
20	1.8×10^9	-	++
100	1.6×10^9	-	+++
500	2.0×10^9	-	+++
Untreated$^{\Pi}$	2.2×10^9	+	Negative

* Encapsulated P-1059 was treated with various amounts of hyaluronidase at 37°C for 60 minutes. $^{\Phi}$ Viable bacterial number was determined after the treatment. ‡ Observed by negative staining: large capsules on most bacterial cells (+), large capsules on some cells ($\pm$), none of the cells had large capsule (-). § Slide agglutination test with turkey anti-P-1059 serum: coarse granules with 2 minutes (+++), apparent agglutination with 5 minutes (++), fine granules after 5 minutes (+). $^{\Pi}$ The bacteria before the hyaluronidase treatment.

TABLE IV.2--Mortality rates in 5-week-old turkeys after i.v. inoculation with the encapsulated or decapsulated form of strain P-1059, as well as with the non-encapsulated mutant T-325

Inoculum	Dose (CFU)	Mortality (dead/total)	MDT* (days)	Isolation of *P. multocida*[ϕ]
Encapsulated P-1059	1.8×10^2	5/5	1.4	5/5
	1.8×10^3	5/5	1.2	5/5
Decapsulated P-1059[ǂ]	3.4×10^2	5/5	1.8	5/5
	3.4×10^5	5/5	1.0	5/5
Non-encapsulated T-325	3.2×10^3	0/5	-	1/5[§Π]
	3.2×10^6	1/5	3.0	2/5[§Π]
	3.2×10^9	5/5	1.0	5/5[§]
Non-infected[¶]	0	0/5	-	0/5

* Mean death time. [ϕ] Number of *P. multocida*-positive/number of total. [ǂ] The organisms were treated with hyaluronidase, and the inoculum dose was determined after the treatment. [§] A few iridescent *P. multocida* colonies were observed on one of the primary isolation plates. [Π] One bird in each group developed chronic disease, and *P. multocida* was isolated from the hock joint but not from the liver. [¶] Non-infected birds housed in the same experimental unit.

TABLE IV. 3--Influence of the treatment of inoculum with normal or immune turkey serum on the mortality in turkeys caused by the encapsulated or decapsulated P. multocida strain P-1059

Inoculum	Treated* with	Dose[φ] (CFU)	Mortality (dead/total)	MDT[‡] (days)	Isolation of P. multocida[§]
Trial 1. (with 6-week-old turkeys)					
Encapsulated P-1059	NS	9.6×10^1	4/4	1.0	4/4
		2.9×10^3	5/5	1.0	5/5
	IS	8.9×10^1	6/9	1.3	6/9
		2.7×10^3	5/5	1.0	5/5
Decapsulated[Π] P-1059	NS	7.3×10^1	2/4	1.5	2/4
		2.2×10^3	5/5	1.0	5/5
	IS	7.2×10^1	4/10	1.3	4/10
		2.2×10^3	5/5	1.2	5/5
Non-infected	—	0	0/4	—	0/4
Trial 2. (with 4-week-old turkeys)					
Encapsulated P-1059	NS	1.8×10^2	9/10	1.2	9/10
	IS	1.7×10^2	7/10	1.7	7/10
Decapsulated[Π] P-1059	NS	1.2×10^2	4/10	2.0	4/10
	IS	1.3×10^2	3/10	5.0[†]	3/10

* The organisms were treated in normal turkey serum (NS) or in immune turkey serum (IS) at the concentration about 3×10^6 CFU/ml. [φ] Inoculum doses were determined after the serum treatments. [‡] Mean death time. [§] Number of P. multocida-positive/number of total. [Π] The strain P-1059 was decapsulated by hyaluronidase before the serum treatment. [†] One bird died 11 days after the injection.

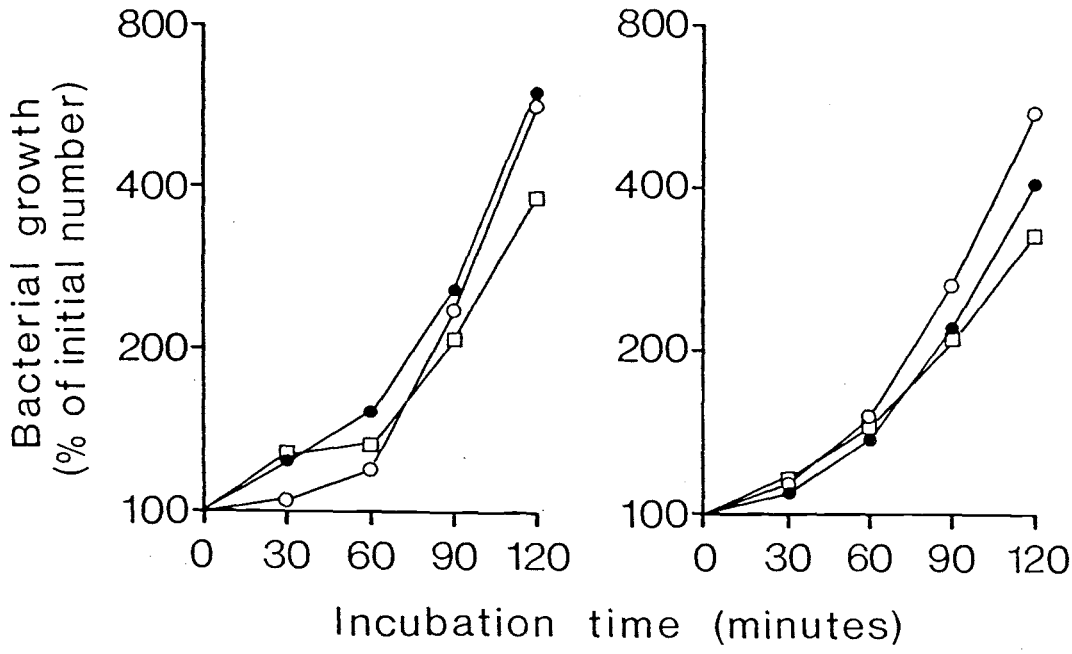

Fig IV.2--Bacterial growth at 37°C in heat-inactivated normal turkey plasma (left), or in heat-inactivated immune turkey plasma (right), tested in a single experiment with the three forms of P. multocida organisms: encapsulated P-1059 (●), decapsulated P-1059 (O) and non-encapsulated mutant T-325 (□).

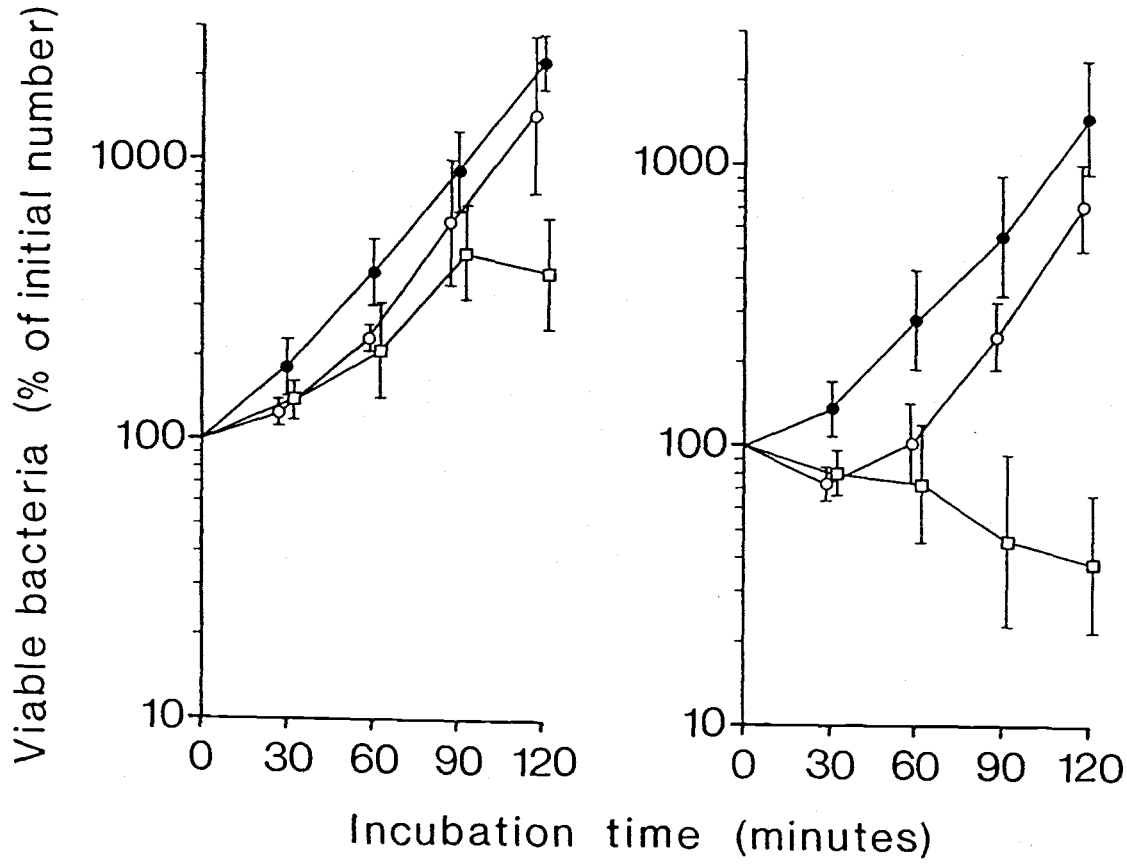

Fig IV.3--Changes of viable bacterial number at 37°C in fresh normal turkey plasma (left), or in normal turkey whole blood (right). In both experiments, encapsulated P. multocida strain P-1059 (●), decapsulated P-1059 (O) and non-encapsulated mutant T-325 (□) were tested with three samples obtained from different turkeys. The changes were expressed by percentages to the initial number, and the mean ± standard deviation was indicated.

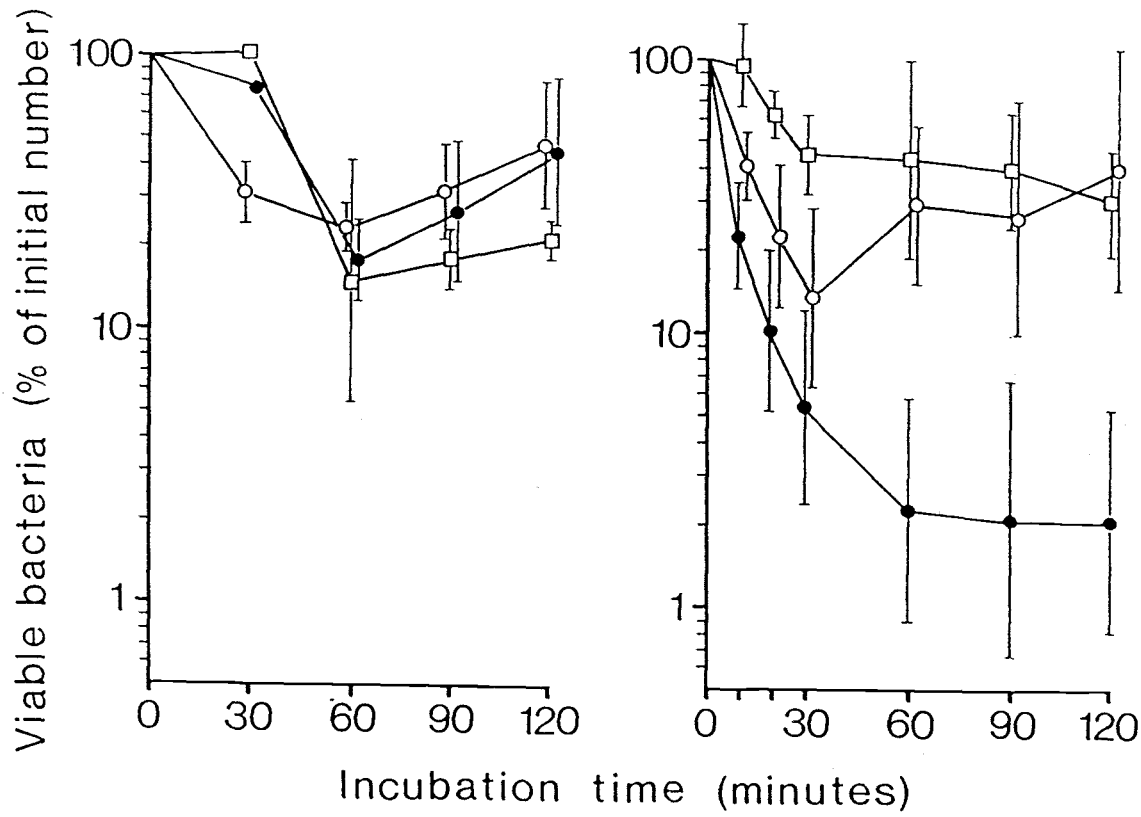

Fig IV.4--Inactivation of encapsulated P. multocida strain P-1059 (●), decapsulated P-1059 (O) and non-encapsulated mutant T-325 (□) in fresh immune turkey plasma (left), or in immune turkey whole blood (right). The results were obtained from three independent experiments with different turkey samples. Decrease in viable bacterial number was expressed by percentage to the initial number, and the mean ± standard deviation was indicated.

Discussion

Relationship of virulence, encapsulation and colony morphology of P. multocida has been studied by many workers since the early years of this century.[7-14] The results, however, are somewhat confusing, because individual workers used different terminology to describe the colonial morphology, and because P. multocida strains of various capsular types were examined for their virulence in various animal species including those other than original host species. Nonetheless, some observations are common; highly virulent strains of P. multocida are encapsulated, but not all encapsulated strains are highly virulent. In contrast, non-encapsulated strains of P. multocida are generally of low virulence. On an appropriate agar medium, encapsulated, avian strains of P. multocida develop large iridescent (fluorescent) colonies, whereas non-encapsulated strains form smaller non-iridescent (blue type) colonies.[1,8,12]

Using the Maneval's negative staining method, the capsules of strain P-1059 were observed as large unstained regions around the organisms (Fig IV.1). Neither hyaluronidase-treated P-1059 strain nor the mutant T-325 had large capsules, but many organisms were surrounded by very small, microcapsule-like haloes. However, whether the haloes are due to the presence of some capsular materials other than hyaluronic acid or simply due to an artifact caused by bacterial shrinkage is not clear, because Maneval's method does not stain the capsule itself.

The ability to produce capsule appeared to be closely related to the virulence of P. multocida (Table IV.1). Removal of the capsule by hyaluronidase-treatment, however, did not result in the loss of virulence. This is not surprising because the decapsulated organisms

may have reformed the capsules after being injected IV into turkeys. How soon the bacteria could regenerate the capsule was not examined. Despite our precautions, a few organisms may have been injected outside the blood vessel, giving them a better chance to regain the capsule. Moreover, the inoculum may have contained a small number of encapsulated organisms despite the hyaluronidase-treatment, although this is unlikely at such a low inoculum dose as 10^2 CFU doses. A more likely possibility is that the organism may have some unknown virulence factors, besides hyaluronic acid, that are missing in the mutant T-325. Possible candidates for such factors are the capsular substance responsible for the serotype-specificity and some outer-membrane proteins.

The non-encapsulated mutant T-325 was spontaneously developed from the strain P-1059 during repeated passages in the broth culture. A few non-iridescent and some intermediate-type colonies were seen as early as the 12th passage. The mutant T-325 did not change the colonial morphology as long as it was subcultured on DSA plates. However, the present study demonstrated that the mutant could transform *in vivo* into the encapsulated form, even by a single passage in a turkey. Similar observations have been reported by Heddleston *et al.*[12] The reversion frequency *in vivo* was not high, because most primary isolation cultures developed only non-iridescent type colonies. A few primary cultures contained a very small number of iridescent colonies (including sectored and intermediate types). Those revertants probably arose due to the selective pressure of host defense mechanisms, although whether or not those revertants fully regained the original level of virulence was not examined. We designated the strain T-325 as "mutant," but this term may not be appropriate without examining the genetic background.

Considering the facts described above, this "mutation" may be not due to simple defect in a certain structure gene, but perhaps due to alteration in some regulatory genes.

The treatment of encapsulated P-1059 with immune turkey serum did not significantly reduce the virulence, even with very small doses of inoculum (Table IV.3). Several explanations are possible. For example, the capsule may inhibit the binding of the antibodies to the bacterial surface antigens. If so, the antibody can neither opsonize the bacteria nor cause the bacteriolysis in association with complement. This may also explain the reason why the encapsulated organisms do not agglutinate in the antiserum unless they are pre-treated with hyaluronidase. However, the possibility is quite unlikely because the in vitro results indicated that the encapsulated organisms were significantly inactivated in both fresh plasma and whole blood from immune turkeys (Fig IV.4). Alternatively, the antibodies may freely permeate the capsule-layer and bind to the bacterial cells, but they may not act as an efficient opsonin because of the presence of serologically inactive capsular substances, such as hyaluronic acid. To test this possibility, decapsulated form of P-1059 was opsonized with immune serum and then injected into turkeys. At very small inoculum doses, the decapsulated organisms caused apparently lower mortality than the encapsulated ones. The results, however, were quite equivocal because the decapsulated organisms treated with normal serum also caused lower mortality.

The encapsulated P-1059 was highly resistant to both cellular and humoral defense mechanisms existing in normal turkey blood (Fig IV.3).

The resistance against phagocytic cells has been presumed to be due to the anti-phagocytic activity of capsular hyaluronic acid.[15,17] Our results, however, were not consistent with the hypothesis, because not only encapsulated but also decapsulated organisms freely multiplied in normal turkey whole blood. Since the decapsulated P-1059 was tested in the presence of hyaluronidase, reproduction of capsular hyaluronic acid is probably not the case. However, the addition of the enzyme may have caused adverse effects on the phagocytes. Furthermore, the heparin, which was contained in the blood sample as anticoagulant (approximately 2 units/ml), may have inhibited the phagocytosis activity.[28] This possibility, however, may be excluded because the same heparinized whole blood significantly inhibited the growth of non-encapsulated mutant T-325.

Snipes and Hirsh[21] have recently reported that a non-encapsulated mutant derived from strain P-1059 was inactivated by fresh normal turkey plasma, indicating that the capsule of P. multocida was important for the resistance against the bactericidal activity mediated by alternative complement pathway. Our results, however, differed somewhat from theirs; the mutant T-325 was able to grow in fresh normal plasma into a certain extent. Whether the difference is due to the distinct characteristic between the two mutants or because of some technical reasons is not known.

The presence of capsule appeared not to affect the bactericidal activity of fresh immune turkey plasma, because all three forms of organisms were equally susceptible (Fig IV.4). Since the immune plasma lost the activity upon heating, complement is probably essential for the inactivation of P. multocida. However, the inactivation process may

also involve several other serum proteins, such as lysozyme and especially iron-sequestering proteins (mainly transferrin).[29,30] Bullen et al.[31] showed that the bacteriostatic effect of rabbit immune serum against P. multocida was totally abolished by addition of iron compounds, indicating the participation of iron-binding proteins. They have also demonstrated that passively immunized mice lost their protection against P. multocida by the injection of various iron compounds.[32] Hence, the host's mechanism, by which iron compounds are sequestered from bacterial pathogens, seems to be an important defense system against P. multocida.

The encapsulated P-1059 strain was inactivated in the whole blood from immune turkeys at a greater level than the decapsulated form of P-1059 or the non-encapsulated mutant was. In the presence of suitable opsonins, the phagocytic cells may preferentially ingest the encapsulated organisms by recognizing certain capsular antigens which are removable by hyaluronidase-treatment or missing in the mutant T-325. Alternatively, the opsonization of capsule may somehow render the bacteria more susceptible to the intracellular bactericidal mechanisms. Quantitative phagocytosis/killing assay with fractionated phagocytes will be needed to differentiate these possibilities.

The primary aim of this study was to test the hypothesis that the capsular hyaluronic acid acts as a major virulence factor of P. multocida. However, the results presented above do not support this hypothesis. Even though the virulence of P. multocida is associated with the ability to produce some capsular substances, hyaluronic acid is probably not the substance.

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Chapter V

Pathogenesis of Fowl Cholera: Influence of Encapsulation on the Fate of *Pasteurella multocida* after Intravenous Inoculation into Turkeys

Summary

The role of the capsule in the pathogenesis of fowl cholera was studied in turkeys. An avian strain of Pasteurella multocida, P-1059, was used in three forms: an encapsulated form, an enzymatically decapsulated form, and a mutant form lacking capsule-productivity (strain T-325). They were intravenously inoculated into normal or immune turkeys, and the numbers of viable bacteria in the blood, liver and spleen were enumerated during a period of 120 minutes post-inoculation (PI). In both normal and immune birds, the three forms of organisms were rapidly removed from the bloodstream at similar rates, and trapped in the liver and spleen. In the liver of normal birds, the non-encapsulated mutant T-325 was readily inactivated, but the encapsulated P-1059 strain was not. When the decapsulated form of P-1059 was used, the bacterial counts in the liver temporarily decreased at 60 minutes PI. In immune birds, all three forms of organisms were equally inactivated in the liver. In the spleen, however, the numbers of bacteria did not change throughout 120 minutes PI with all three forms of organisms in both normal and immune turkeys. The results indicated that the blood-borne P. multocida were readily trapped by reticulo-endothelial phagocytes. The trapping process was not affected by encapsulation of the organism or by the immune status of turkey. Both factors, however, appeared to greatly influence the subsequent killing of P. multocida by hepatic, but not splenic, phagocytes.

Introduction

Capsular type A strains of Pasteurella multocida, characterized by the production of hyaluronic acid as a major component of the capsule[1-3] have been recognized as the primary cause of fowl cholera.[1,4] Many strains of P. multocida isolated from acute cases of fowl cholera are highly virulent in susceptible avian species. Even when a very small number (30 to 100 organisms) of such strains are inoculated into turkeys, nearly 100% mortality results within 48 hours. Histopathological examination of the infected birds reveals typical lesions of acute septicemia, characterized by generalized passive hyperemia and massive dissemination of bacteria in the liver and spleen as well as the blood vascular system throughout the body.[5] The cause of death is presumed to be due to endotoxic shock.[5-7]

Relatively little is known about the virulence factors of P. multocida. As the histopathological findings indicate, the virulence of P. multocida is ascribed primarily to its ability to invade and grow in the host tissues. Since most avian strains of P. multocida appear not to produce any potent exotoxin in the culture medium,[6,8] this ability probably resides in some specialized bacterial surface components, such as capsular polysaccharides, outer-membrane proteins and lipopolysaccharides. In natural infections, the portal of entry for P. multocida is probably the pharynx and/or upper respiratory tract.[9,10] Virulent strains of P. multocida, however, appear to invade the bloodstream soon after the colonization by some unknown mechanisms. Maheswaran et al.[11] demonstrated that when a small number of virulent P. multocida were endotracheally inoculated into turkeys, the bacteria

emerged in the blood and spleen as early as 6 hours after the inoculation.

The virulence of P. multocida is closely associated with encapsulation of the organism.[1,4,6,12] Highly virulent strains of P. multocida are usually encapsulated, whereas non-encapsulated strains tend to be less virulent. More convincing evidence is that the loss of ability to produce the capsule generally accompanies the loss of virulence.[12] The capsule seems to play an important role after the invasion of the organisms into the bloodstream. Snipes et al.[13] demonstrated that an encapsulated strain of P. multocida inoculated intravenously into turkeys was able to multiply in the blood vascular systems to a greater extent than a non-encapsulated strain. Since the capsular hyaluronic acid has been shown to act as an anti-phagocytic factor,[14,15] once the encapsulated virulent organisms enter the host tissues, they may freely multiply in an extracellular environment,[6] resulting in fatal septicemia. Pabs-Garnon and Soltys,[16] on the other hand, showed that P. multocida inoculated intravenously into turkeys were initially localized in the liver and spleen. The bacteria continued to multiply in these organs, and were abruptly released into the bloodstream at the terminal phase of infection, implicating possible multiplication of P. multocida in the cells of reticulo-endothelial system in liver and spleen.

The objective of this study was to investigate the role of the capsule in the pathogenesis of P. multocida infection in turkeys. Three forms of P. multocida--an encapsulated form, an enzymatically decapsulated form, and a non-encapsulated form of mutant derivative--were intravenously inoculated into turkeys, and the numbers of viable

bacteria in the blood, liver and spleen were enumerated during 120 minutes PI. The experiments were carried out in both normal and immune birds to investigate the influence of specific immunity on the fate of bacteria.

Materials and Methods

Organisms--Pasteurella multocida strain P-1059 (serotype 3:A), an encapsulated, highly virulent strain of turkey origin, was kindly supplied by K. R. Rhoades, National Animal Disease Center, Ames, Iowa. To enhance the capsule-productivity, the strain was passed 6 times in vivo in turkeys. Organisms were re-isolated from the liver and stored at -70°C as previously described.[17] A non-encapsulated, avirulent mutant strain, designated T-325, was developed in our earlier study,[17] in which the mutant spontaneously arose from the strain P-1059 during the repeated subculture in a broth medium. For each experiment, either strain P-1059 or T-325 was propagated on dextrose starch agar[a] at 37°C for 3 to 4 hours. Decapsulation of strain P-1059 was carried out with 100 units/ml of hyaluronidase[b] at 37°C for 20 minutes as described previously.[17]

Turkeys--Wrolstad Medium White turkeys of mixed sex were used. The experiments were carried out with both normal and immune turkeys at the age between 11 and 15 weeks old. The immune turkeys were prepared at two different periods in an identical manner. A total of 60 turkeys were subcutaneously immunized twice, at 6 and 8 weeks of age, with an oil-emulsified vaccine consisting of Freund's incomplete adjuvant[a] and a protective antigen (designated 2.5S antigen) purified from the strain P-1059 as described previously.[18,19] The birds were intramuscularly challenge-exposed at 10 weeks old with approximately 10^3 colony-forming units (CFU) of the strain P-1059. During 14 days after the challenge, about 40% of birds died. The survivors were used for the experiments as the immune turkeys between 2 and 5 weeks from the challenge infection. Both normal and immune birds were tested prior to use for

their serum antibody titers against the 2.5S antigen by an enzyme-linked immunosorbent assay as described in our earlier report.[19] None of the normal turkeys had significant antibody titers (<1:30), whereas the immune birds had a mean antibody titer of 1:12,000 (ranging from 1:3,000 to 1:50,000).

Inoculum and blood sampling--Each experiment was carried out in a group consisting of four or five turkeys using one of three forms of P. multocida. Organisms were suspended in Dulbecco's phosphate buffered saline at pH 7.4 (PBS), and the turbidity was adjusted into an optical density value of 0.30 at 600 nm, which corresponded to approximately 6×10^8 CFU/ml. When dose effect was studied, concentrations 10 times higher and 10 times lower were also prepared. The organisms were kept on ice and used within 2 hours. Actual inoculum doses were determined by plate colony-counting with heart infusion agar.[a] One milliliter of bacterial suspension was intravenously inoculated into each bird. Approximately 0.8 ml of blood samples were periodically drawn from the wing veins at various times post-inoculation. A half milliliter of each sample was immediately added to a chilled test tube containing 1.0 ml of heparin solution (10 units/ml in PBS) and kept in an ice box.

Necropsy procedure--Immediately after the last bleeding, each bird was sacrificed by electrocution and the body weight was measured. The bird was quickly dipped in a disinfectant solution and subjected to necropsy. A portion of the liver (about 4 grams) and whole spleen were quickly removed from each bird, and individually inserted into a sterile plastic bag.[c] Each specimen was weighed and immediately placed on ice. The weight of plastic bag was subtracted from the weight of each specimen. All procedures were accomplished within 2 minutes following

death. The rest of the liver was subsequently taken out to obtain the whole organ weight.

Enumeration of bacteria--Samples of liver and spleen were briefly mashed by squeezing the plastic bag, and 10 ml of chilled PBS was added to each bag. The tissues were further homogenized for 1 minute by Stomacher[d] and placed on ice. Serial 10-fold dilutions of each sample were made with cold PBS, and 100 µl of appropriate dilutions, in duplicate, were spread on heart infusion agar plates. The plates were briefly dried in a laminar flow cabinet for about 10 minutes, and then placed in an incubator at 37°C. The whole procedure was completed within 6 hours after the sampling. Bacterial viability did not significantly decrease during this period. Colony-counting was carried out on the next day and again on the day after. Since the total blood volume of an adult turkey has been estimated at approximately 8% of the body weight,[20,21] the bacterial concentration in the blood (CFU/ml) was converted to the number/total blood volume, and, in turn, to the recovery rate relative to the inoculum dose using the following formula:

$$\text{Recovery (\%)} = \frac{\text{CFU/ml x body weight x 0.08}}{\text{CFU of the inoculum}} \times 100$$

The recovery rates from the liver and spleen were calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{CFU/g x organ weight}}{\text{CFU of the inoculum}} \times 100$$

Statistical analysis--The recovery rates were transformed into $\log_{10}$ values and the mean and standard deviation were calculated in each group. Differences among the means were examined in one-way tables of analysis of variance using Fisher's protected LSD test.

[a] Difco Laboratories, Detroit, Mich.

[b] Sigma Chemical Co., St. Louis, Mo.

[c] Stomacher '80' Bag, Tekmar Company, Cincinnati, Ohio.

[d] Model STO-80, Tekmar Company.

Results

Clearance of P. multocida from bloodstream--The encapsulated or decapsulated form of P-1059, as well as the non-encapsulated mutant T-325 was intravenously inoculated into normal or immune turkeys. Within the first 10 minutes PI, more than 99% of the inoculum was removed from the bloodstream (Fig V.1). The clearance rates were similar with all three forms of organisms and in both normal and immune birds. The clearance between 10 and 120 minutes PI, was examined in separate experiments (Fig V.2). In normal birds, the clearance rates became slower after 10 minutes PI, and reached the lowest levels at 30 minutes PI, when the bacterial numbers in the blood were about 1/3000 of the inoculum doses with all three forms. From 30 to 120 minutes PI, low levels of bacteremia ($10^{2.5}$ to $10^{3.5}$ CFU/ml) were observed with all three forms of organisms. In immune turkeys, the encapsulated P-1059 strain was cleared to a greater extent than in normal birds. The patterns of clearance with decapsulated P-1059 strain and non-encapsulated mutant T-325 were similar in both normal and immune birds.

Distribution and fate of P. multocida in normal birds--The recovery rates (% of inocula) of viable bacteria from the blood, liver and spleen of normal turkeys were determined at 10, 60 or 120 minutes PI (Fig V.3). At 10 minutes PI, the distribution patterns were similar with all three forms of organisms: roughly 25%, 2% and 0.2% of the inocula were recovered from the liver, spleen and blood, respectively. When comparisons were made on the concentration basis (CFU/ml or g), the spleen contained slightly higher number of bacteria than the liver, while the concentration in the blood was at least 500 times lower than that in the liver or in the spleen. Between 10 and 120 minutes PI,

the bacterial recovery from the liver decreased approximately 800-fold with the non-encapsulated strain T-325. In contrast, the recovery of the encapsulated P-1059 from the liver did not significantly change throughout 120 minutes PI ($P>0.10$). When the decapsulated form of P-1059 was used, the bacterial recovery from the liver temporarily decreased at 60 minutes PI. The bacterial recovery in the spleen, on the other hand, did not significantly change during 120 minutes PI with all three forms ($P>0.05$).

Distribution and fate in immune birds--Similar experiments were carried out in immune turkeys (Fig V.4). At 10 minutes PI, no significant difference was observed among the three forms of organisms in their recovery rates from the blood, liver and spleen ($P>0.05$). The patterns of bacterial distribution at this sampling time were similar in both immune and normal birds. Between 10 and 120 minutes PI, the bacterial numbers in the liver decreased about 300-fold with all three forms. The substantial decrease was also observed in the blood, but it was not as great as in the liver. In the spleen, by contrast, all three forms showed no significant differences in their recovery rates between 10 and 120 minutes PI ($P>0.90$). At 120 minutes PI, the bacterial concentration (CFU/g) in the spleen was more than 1,000 times higher than in the liver and 10,000 times greater than in the blood with all three forms of organisms.

Dose effect--The clearance experiment was carried out with the encapsulated form of strain P-1059 at three different inoculum doses (Fig V.5). At the initial phase, the bacteria appeared to be removed from the blood at faster rates with increasing dosage, but the patterns of clearance were similar at any dose. The inoculum dose did not

significantly change the rates of bacterial recovery from the liver and spleen at 120 minutes PI ($P>0.40$).

Growth of strain P-1059 in normal turkey--The encapsulated form of strain P-1059 (approximately 6 x 10^8 CFU) was intravenously inoculated into normal turkeys, and the bacterial numbers in the blood, liver and spleen were examined up to 7 hours PI (Fig V.6). In the liver and spleen, the bacteria grew logarithmically after 2 hours PI. The bacterial concentrations in these two tissues were always approximately equal and significantly higher than in the blood. The number of bacteria in the blood, however, increased at a faster rate than in the liver or in the spleen: approximately 60,000-fold increase was observed in the blood between 2.5 and 7 hours PI, which corresponded to a doubling-time of every 17 minutes. The growth rates were similar in both liver and spleen (doubling time of about 27 minutes). The birds showed general signs of fowl cholera as early as 4 hours PI and were just about to die at 7 hours PI. A large number of encapsulated organisms were observed in the blood (Fig V.7), liver and spleen samples taken at the terminal stage.

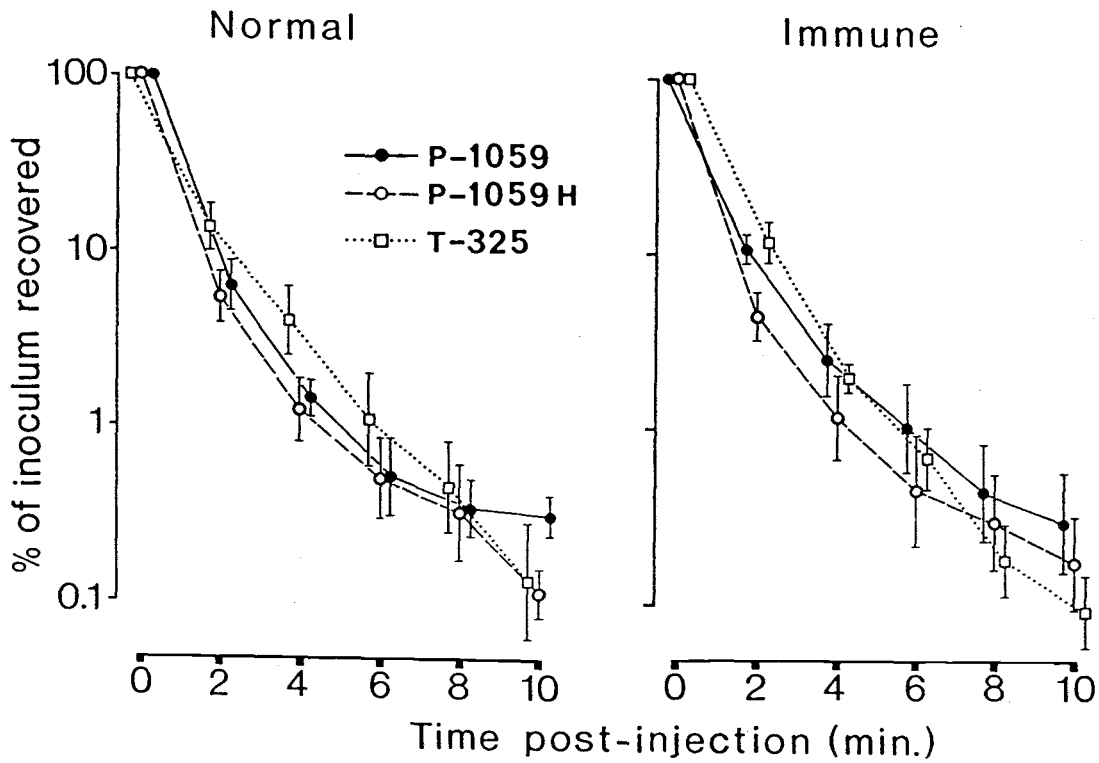

Fig V.1--Clearance of P. multocida from the bloodstream during 10 minutes PI. Encapsulated P-1059 strain (P-1059), decapsulated P-1059 strain prepared by hyaluronidase-treatment (P-1059H), and non-encapsulated mutant T-325 were intravenously inoculated into normal or immune turkeys, and the numbers of bacteria in the blood were determined. Four birds were used in each group. The data are expressed as the mean ± standard deviation.

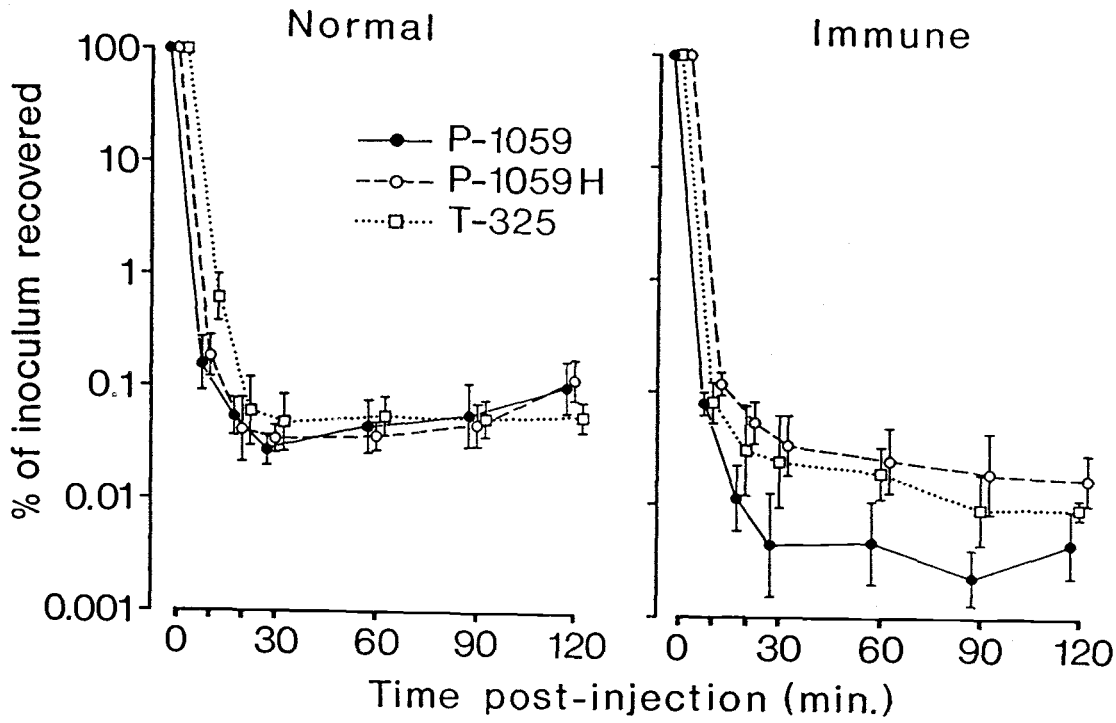

Fig V.2--Bacterial recovery from the blood during 120 minutes PI. Encapsulated P-1059 strain (P-1059), decapsulated P-1059 strain prepared by hyaluronidase-treatment (P-1059H), and non-encapsulated mutant T-325 were intravenously inoculated into normal or immune turkeys. Five birds were used in each group. The data are expressed as the mean $\pm$ standard deviation.

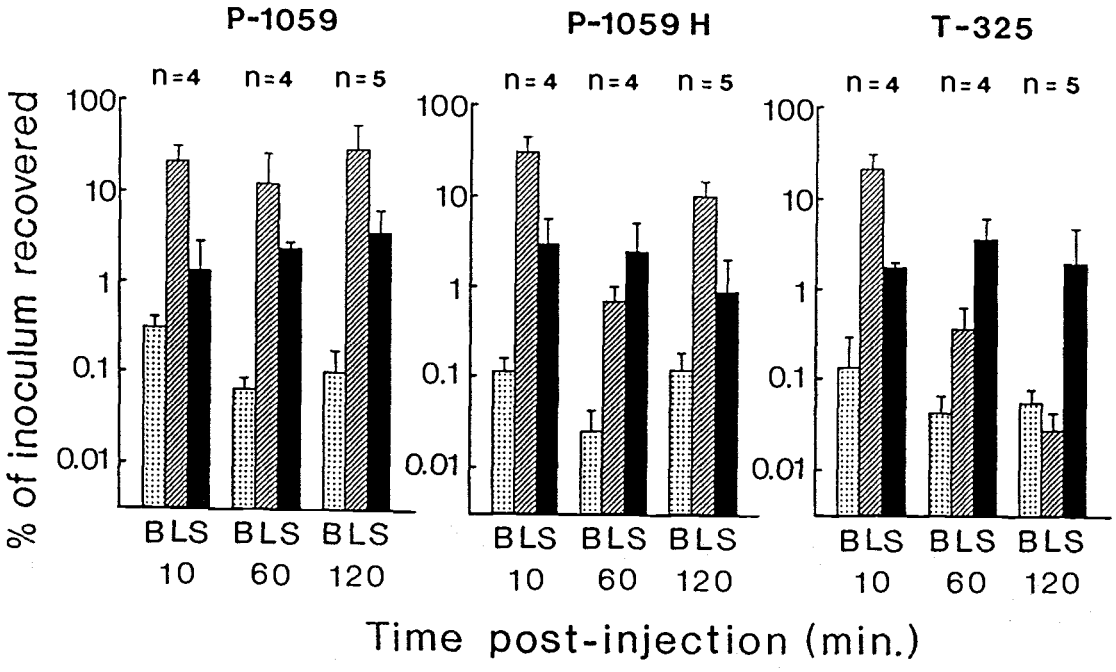

Fig V.3--Bacterial recovery from the blood (B), liver (L) and spleen (S) of normal turkeys at 10, 60 or 120 minutes after the intravenous inoculation of encapsulated P-1059 strain (P-1059), decapsulated P-1059 strain prepared by hyaluronidase-treatment (P-1059H), or non-encapsulated mutant T-325. The number of birds (n) used at each sampling period is indicated. The data are expressed as the mean and its standard deviation.

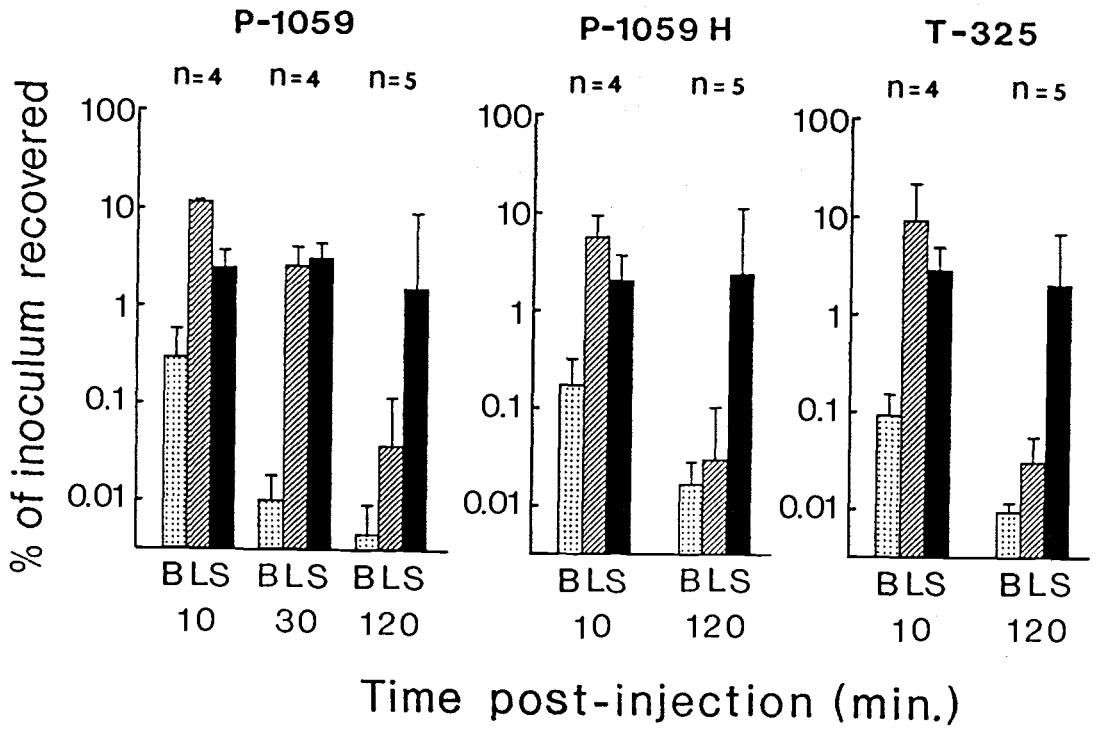

Fig V.4--Bacterial recovery from the blood (B), liver (L), and spleen (S) of immune turkeys at 10, 30, or 120 minutes after the intravenous inoculation of encapsulated P-1059 strain (P-1059), decapsulated P-1059 strain prepared by hyaluronidase-treatment (P-1059H), or non-encapsulated mutant T-325. The number of birds (n) used at each sampling period is indicated. The data are expressed as the mean and its standard deviation.

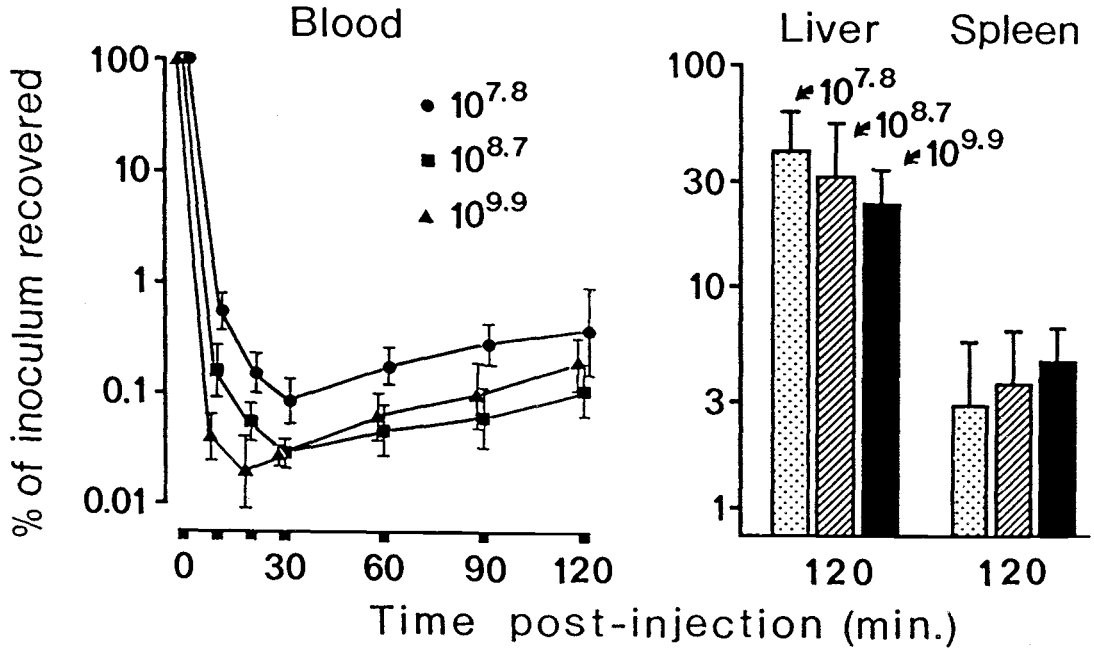

Fig V.5--Effect of inoculum dose on the clearance of P. multocida from the bloodstream (left), and on the recovery rates from the liver and spleen at 120 minutes PI (right). Normal turkeys were intravenously inoculated with $10^{7.8}$, $10^{8.7}$ or $10^{9.9}$ CFU of encapsulated P-1059 strain. Four birds were used for each inoculum. The data are expressed as the mean $\pm$ standard deviation.

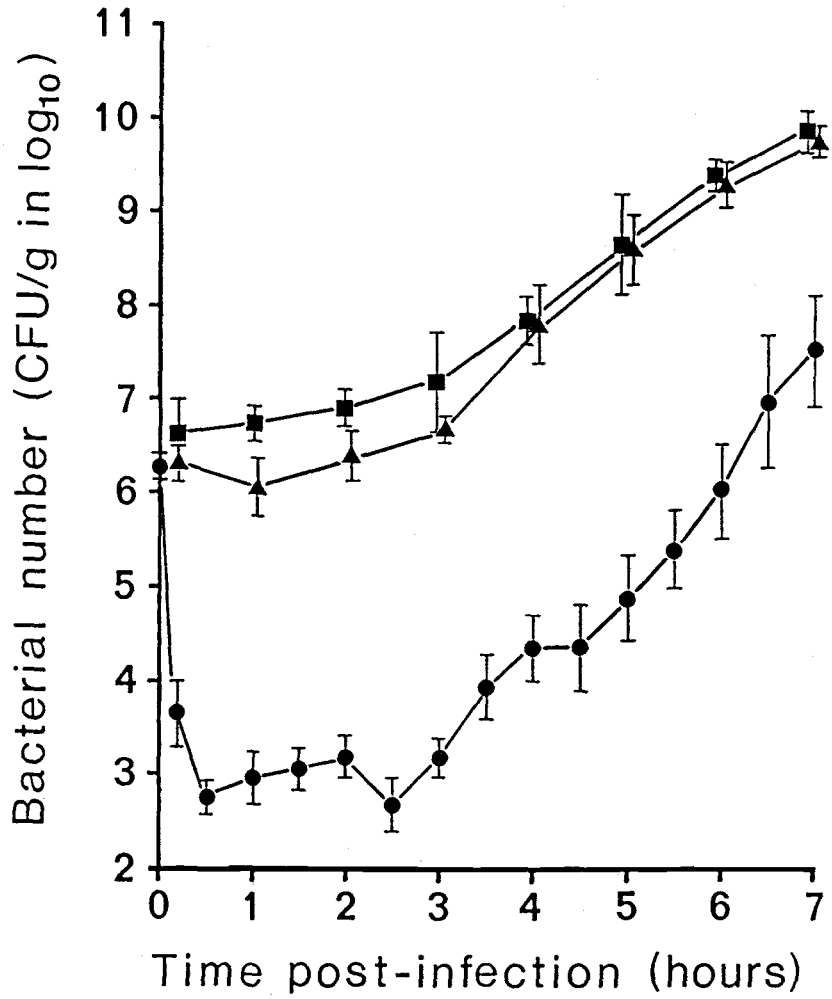

Fig V.6--Bacterial numbers in the blood (●), liver (▲) and spleen (■) of normal turkeys after the intravenous inoculation of approximately $10^{8.8}$ CFU of encapsulated P-1059 strain. Mean ± standard deviation was determined with the data from four or five samples taken at each period. The initial concentration of bacteria in the blood was estimated from the inoculum dose divided by the total blood volume of each bird (8% of the body weight).

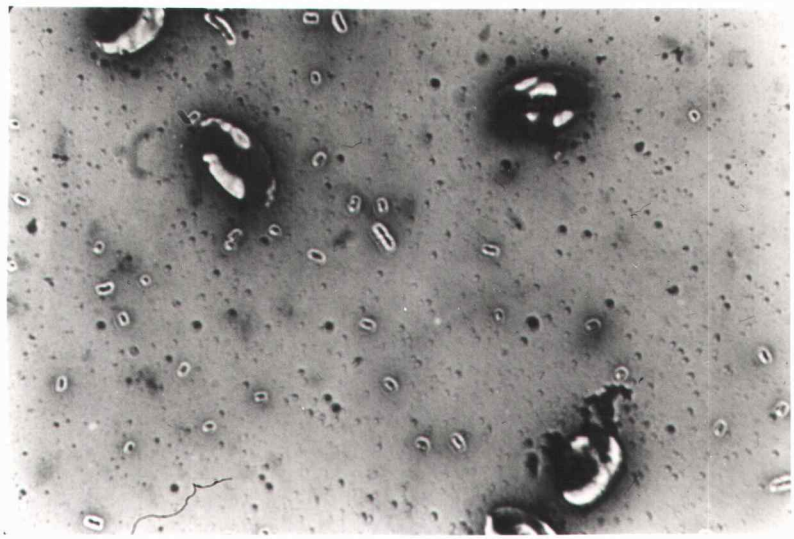

Fig V.7--Encapsulated P. multocida observed in a blood smear. The sample was obtained at 7 hours PI from one of the birds used in the experiment shown in Fig V.6. A thin smear was made on a slide glass with one drop of the blood and two drops of 1% Congo red, and stained with Maneval's solution consisting of acid fuchsin.[22]

Discussion

Pasteurella multocida must survive and overwhelm the host's intravascular defense systems to cause fatal septicemia. The capsule seems to play an important role in this process. The previous study[17] showed that the intravenous inoculation of non-encapsulated mutant T-325 caused only a 20% mortality rate in turkeys at a dose of $10^{6.5}$ CFU, whereas the encapsulated form of strain P-1059 caused a 100% mortality rate at a dose of approximately 10^2 CFU. However, the role of capsular hyaluronic acid was still unclear, because the decapsulation of strain P-1059 by hyaluronidase did not cause a decrease in mortality at a inoculum dose of about 10^2 CFU.

The present study clearly demonstrated that the blood-borne P. multocida was rapidly removed from the bloodstream, regardless of the presence or absence of the capsule (Fig V.1). Thus, the capsular substances appear not to act as an anti-phagocytic factor against the phagocytes in the blood vascular system. Since the bacterial concentrations in the liver and spleen at 10 minutes PI were more than 500 times higher than in the blood, the organisms were presumed to be trapped by the reticulo-endothelial (RE) phagocytes in these two organs. No effort was made to obtain histological evidence for the interaction between P. multocida and the RE phagocytes, because the inoculum dose used in the experiments may not have been high enough to microscopically detect the bacteria in the tissues at an early clearance phase. Whether heterophils, besides RE phagocytes, are also involved in the intravascular clearance of P. multocida is not known. However, it has been shown with other bacterial species that intravenous injection of a large number of bacteria induces adherence of circulating leukocytes on

the capillary endothelium (especially in lung), creating secondary sites for trapping the blood-borne bacteria.[23] Providing an immovable tissue surface for polymorphonuclear neutrophils has been recognized as an important factor for the phagocytes to be capable of ingesting virulent, encapsulated gram-positive cocci.[24]

The initial, rapid clearance of P. multocida halted at 30 minutes PI, and a low level of bacteremia continued up to 120 minutes PI (Fig V.1). Although similar phenomena have been observed with many other bacterial species, the reason for this persistent bacteremia is not clearly understood.[23] Since a high inoculum dose (approximately 10^9 CFU) was used for the tests, it may have exceeded the host's blood clearance capacity. However, this possibility is quite unlikely because the trapping capacities of liver and spleen appeared not to be saturated even with a inoculum dose of 10^{10} CFU (Fig V.5). A small number of the bacteria captured by the RE phagocytes may have escaped before being completely internalized. Furthermore, the overwhelming bacteria in the phagocytes may have impaired their phagocytic function. Another possibility is that a small population of the bacteria may have been associated with free phagocytes in the circulation.

At 10 minutes PI, the total number of bacteria recovered from the liver, spleen and blood was only about 30% of the inoculum dose (Fig V.3). Whether the other 70% was already inactivated or trapped in some other tissues, such as lung and bone marrow, is not known. The recovery rates from the liver and spleen are probably an underestimate, because our method for bacterial enumeration does not accurately reflect the number of intracellular bacteria. The homogenized tissues were cultured without lysis, because the viability of bacteria decreased when the

samples were lysed with water. The liver and spleen, at 10 minutes PI, contained roughly equal numbers of bacteria per gram of tissue. Thus, both hepatic and splenic macrophages appeared to be equally avid in sequestering the blood-borne P. multocida. Quantitatively, however, the liver seems to be the primary organ for the clearance because of its relatively larger size. In fact, the liver collected about 10 times more bacteria than the spleen within the first 10 minutes PI.

Encapsulation seemed to be essential for the survival of P. multocida after being entrapped in the liver (Fig V.3). The capsular substances may function as a resistance factor against the intracellular bactericidal mechanisms of the hepatic phagocytes. Decapsulation of strain P-1059 caused a significant decrease in the rate of bacterial recovery from the liver at 60 minutes PI ($P<0.001$), but the organisms seemed to be rejuvenated at 120 minutes PI probably due to reformation of the capsules.

At later stages of the infection, the encapsulated form of P. multocida re-emerged in the blood circulation at high numbers (Fig V.6 and 7). It is possible that the bacteria grew in the bloodstream in situ, because the previous study[17] showed that the strain P-1059 was able to freely multiply in fresh turkey blood in vitro. However, since the bacterial numbers (CFU/g) in the liver and spleen were consistently at least 1000 times higher than in the blood, it seems more likely that the intracellular multiplication of bacteria disrupts the RE phagocytes, releasing them back into the blood circulation.[16] This may also explain the reason for the faster rate of bacterial increase in the blood than in the liver or in the spleen. Some histopathological evidence for the

intracellular multiplication of P. multocida in RE phagocytes has recently been reported.[25]

Specific immunity did not change the rate of bacterial uptake in the liver and spleen, but did influence the subsequent fate of bacteria in the liver (Fig V.2 and 4). Immunity appeared to be essential for the hepatic phagocytes to be capable of inactivating the encapsulated form of organisms. Although the precise mechanism is not known, both specific antibody[26-28] and activated macrophages[29,30] may have been involved in this immunity. Immunity appeared to enhance the clearance of P. multocida from the blood only when the organisms were encapsulated. In the presence of specific opsonins, the capsular antigens may serve as a better target than the somatic antigens for recognition by the RE phagocytes.

A striking difference was observed in the bactericidal activity between the liver and the spleen (Fig V.3 and 4). No sign of bacterial inactivation was observed in the spleen even when the non-encapsulated, avirulent strain T-325 was tested in the immune birds. Phagocytes in the spleen appear to be less capable of killing P. multocida than those in the liver. The function of RE phagocytes in liver (Kupffer cell) and spleen (reticular sheath macrophage) has long been recognized as a "filter" for pathogenic microorganisms and other foreign particles invading the blood.[23] However, little information is available as to whether there is a fundamental difference in bactericidal capability between these two populations of macrophages. Poor bactericidal activity may possibly be related to the function of spleen as a lymphoid organ; the splenic macrophages not only act as a phagocytic filter but also serve as antigen-presenting cells for lymphocytes.

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Chapter VI

Immune Defense Mechanism against Blood-borne *Pasteurella multocida* in Turkeys

Summary

Humoral and cellular immune defense factors involved in controlling the blood-borne Pasteurella multocida were investigated in turkeys by the passive transfer of immune serum or by the treatment with macrophage-activating agents. The treated or untreated birds were intravenously inoculated with a virulent strain of P. multocida, and the numbers of viable bacteria in the blood, liver and spleen were enumerated. In untreated birds, the bacteria were rapidly removed from the blood, and the majority were recovered from the liver and spleen at 120 minutes post-inoculation (PI). Neither the transfer of immune serum nor the treatment with macrophage-activating agents enhanced the clearance of bacteria from the blood. The number of bacteria recovered from the liver at 120 minutes PI was slightly lower in the birds treated with macrophage-activating agents, and significantly lower in those given immune serum than in the untreated birds. None of the treatments, however, significantly changed the number of bacteria recovered from the spleen at 120 minutes PI. The results suggest that the phagocytes in the liver, but not in the spleen, play a crucial role in the intravascular defense against P. multocida under the presence of specific antibodies.

Introduction

Fowl cholera, a severe septicemic disease of avian species, is caused by Pasteurella multocida. In natural infections, the organism probably enters the host from the pharynx and/or upper respiratory tract.[1,2] Following the invasion, the bacteria seem to spread via bloodstream, and initially localize in the liver and spleen.[3,4] The bacteria grow primarily in these two organs, and are abruptly released into the bloodstream at the terminal stage, resulting in fatal septicemia.[4] At the time of death, the numbers of bacteria in the liver and spleen usually exceed 10^9 per gram of tissue.

Immunity against fowl cholera can be induced by either inactivated or live vaccines. Although the nature of immunity is not clearly understood, both humoral and cellular factors may play roles. Involvement of humoral factors has been demonstrated by passive immunization experiments with immune serum[5,6] or the IgG antibody.[7] On the other hand, Baba[8] reported that immunity was adoptively transferable into susceptible birds by administration of the spleen cells from immune birds. He further demonstrated that the peritoneal macrophages obtained from immunized, but not unimmunized, birds were capable of inhibiting the intracellular multiplication of P. multocida.

Whether hosts permit or prevent the development of fatal septicemia may depend on the bactericidal capability of intravascular defense systems. Our previous study showed that the bacteria, when inoculated intravenously, were rapidly removed from the bloodstream in both immune and susceptible birds.[3] Since the majority of bacteria were recovered from the liver and spleen shortly after the inoculation, the bacteria were presumed to be captured by reticulo-endothelial phagocytes

in these organs. Removal of bacteria from the blood, however, does not assure the elimination of bacteria. Specific immunity is prerequisite for the subsequent inactivation of bacteria.

The objective of this study was to investigate the possible factors involved in the intravascular defense against P. multocida at an early stage of intravenous infection in turkeys. Involvement of humoral or cellular defense factor was tested by the passive transfer of immune serum or by the treatment with macrophage-activating agents. The effects of these treatments were examined on the clearance of bacteria from the bloodstream and on the recovery rates of viable bacteria from the liver and spleen at 120 minutes PI.

Materials and Methods

Bacterial strain--Pasteurella multocida strain P-1059 (serotype 3:A), a highly virulent, encapsulated strain of turkey origin, was used. The strain was kindly supplied by K. R. Rhoades, National Animal Disease Center, Ames, Iowa. To maintain the virulence, the strain was passed several times in turkeys. Organisms were re-isolated from the liver and stored at -70°C as described previously.[9]

Immune serum--Thirty Nicholas Large White turkeys were subcutaneously immunized twice, at 7 and 9 weeks of age, with an oil-emulsified vaccine consisting of Freund incomplete adjuvant[a] and a protective antigen (designated 2.5S antigen; 250 μg/dose) purified from the strain P-1059 as described previously.[10,11] The birds were intramuscularly challenge-exposed at 11 weeks of age with 1.1×10^3 colony forming units (CFU) of P. multocida strain P-1059. During 14 days after exposure, seven birds died of fowl cholera. Five surviving birds were used for a clearance experiment on day 16 after exposure (group 9). Serum samples were collected from all other surviving birds on day 14 and again on day 21 after exposure. All sera were pooled, sterilized by 0.45 μm pore-size filters, and stored at -20°C. The pooled serum had an anti-2.5S antigen titer of 1:12,500 when it was tested in an enzyme-linked immunosorbent assay (ELISA) described in our previous report.[10]

Treatments of turkeys--Clearance experiments were carried out in nine groups, each consisting of four or five Wrolstad Medium White turkeys. Only in group 9, Nicholas Large White turkeys were used. Untreated birds were used in groups 1 and 4. The birds belonging to other groups were treated in various manners as summarized in

Table VI.1. Two macrophage-activating agents were used; the birds in group 2 were treated with Mycobacterium butyricum[a] (killed dry cells), and those in groups 3 and 8 were treated with Propionibacterium acnes[b] (previously termed Corynebacterium parvum; heat killed cells in lyophilized form). Both M. butyricum and P. acnes were suspended at 10 mg/ml in 0.85% NaCl solution containing 1% gelatin and 0.1% Tween 80, and a dose of 2.5 ml was inoculated intravenously at 5 days before the clearance experiments. Immune serum was intravenously administered at 3 ml/kg of body weight either 30 minutes before (in groups 5, 7 and 8) or 30 minutes after (in group 6) inoculation of P. multocida. The immune birds used in group 9 has been described earlier. Serum samples were obtained from every bird prior to each clearance experiment, and the antibody titers against the 2.5S antigen were determined by the ELISA.

Inoculum preparations--For each clearance experiment, the strain P-1059 was streaked on a dextrose starch agar[a] plate, and incubated overnight at 37°C. Three to five colonies were picked and spread out on another plate. The plate was incubated at 37°C for 3 to 4 hours, and harvested in Dulbecco's phosphate buffered saline at pH 7.4 (PBS). For groups 1, 2, 3, 5, 6 and 9, bacterial suspensions were diluted with PBS to give an optical density value of 0.30 at 600 nm, which corresponded to approximately 6×10^8 CFU/ml. For groups 4, 7 and 8, bacterial concentrations were adjusted at the optical density value of 0.60 and the suspensions were mixed with equal volume of immune serum (diluted 1:3 with PBS). The mixtures were incubated at 37°C for 20 minutes. This treatment did not cause any agglutination of bacteria. Each

inoculum was kept on ice and used within 2 hours. Actual inoculum doses determined by plate colony-counting with heart infusion agar[a] are listed in Table VI.1.

Clearance experiments--Nine experiments were carried out separately, yet in an identical manner. The treated or untreated birds were intravenously inoculated with immune serum-sensitized or unsensitized bacteria. Blood samples were collected from each bird at 10, 20, 30, 60, 90 and 120 minutes PI. Immediately after the last bleeding, the birds were euthanatized and a portion of the livers (about 4 grams) and the whole spleens were quickly removed. The number of viable bacteria in each specimen was determined by plate colony-counting with heart infusion agar plates. Details of specimen sampling, tissue homogenization and bacterial enumeration method have been described.[3] Bacterial concentration of each blood sample (CFU/ml) was converted to the number/total blood volume, and, in turn, to the recovery rate relative to the inoculum dose using the following formula:

$$\text{Recovery (\%)} = \frac{\text{CFU/ml x body weight x 0.08}}{\text{CFU of the inoculum}} \times 100$$

The total blood volume was estimated at 8% of the body weight.[12] Recovery rates from the liver and spleen were calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{CFU/g x organ weight}}{\text{CFU of the inoculum}} \times 100$$

Passive protection test--Protective potency of the immune serum was tested in 31-day-old Nicholas Large White turkeys. The birds were intramuscularly inoculated with various doses of the immune serum. After 24 hours, the birds were intravenously challenge-exposed with 1.2×10^3 CFU of P. multocida P-1059. Mortality was recorded daily,

and survivors at day 14 after exposure were euthanatized. All birds were examined at necropsy, and isolation of P. multocida was attempted from the livers using dextrose starch agar plates.

Statistical analysis--The recovery rates were transformed into $\log_{10}$ values and the mean and its standard deviation were calculated in each group. Differences among the mean recovery rates were examined in one-way tables of analysis of variance using Fisher's protected LSD test. Differences in mortality rates were tested by Fisher's exact test.

[a]Difco Laboratories, Detroit, Mich.

[b]Ribi ImmunoChem Research, Inc., Hamilton, Mont.

Results

Clearance of P. multocida from the bloodstream--Nine experiments were conducted with untreated or treated turkeys (Table VI.1). The results are shown in Fig VI.1. In all the treated and untreated birds, more than 99.9% of inoculum was disappeared from the bloodstream within 30 minutes PI. Clearance of bacteria, however, was slower in the M. butyricum-treated or in the P. acnes-treated birds (groups 2 and 3, respectively) than in the untreated ones (group 1). Clearance rate was slightly enhanced when the bacteria had been sensitized with immune serum (group 1 versus 4). The passive transfer of immune serum did not enhance the rate of clearance within 30 minutes PI (group 1 versus 5), although the numbers of bacteria in the blood at 90 and 120 minutes PI were significantly lower in group 5 than in group 1 ($P<0.01$). The clearance pattern did not change whether the immune serum was given to the birds at 30 minutes before or 30 minutes after inoculation of P. multocida (groups 5 and 6). The level of bacteremia between 30 and 120 minutes PI was significantly lower in the immune than in the untreated group (group 1 versus 9, $P<0.01$). The clearance pattern in the immune group was similar to that in group 8, in which the birds were treated with both P. acnes and immune serum, and then inoculated with immune serum-sensitized bacteria.

Bacterial recovery at 120 minutes PI--Figure VI.2 shows the recovery rates (% of inoculum) of viable bacteria from the blood, liver and spleen at 120 minutes PI. Major differences among the nine groups were observed in the recovery rate from the liver. The mean recovery rate from the livers of immune birds was 800-fold less than that of

untreated birds (group 1 versus 9). On the concentration basis (CFU/g), the numbers of bacteria in the liver and in the spleen of the untreated birds were about 1,000 and 3,000 times higher, respectively, than that in the blood. In the immune birds, by contrast, the concentrations in the liver and in the blood were 1,000 and 30,000 times less, respectively, than that in the spleen. The recovery rate from the liver of M. butyricum- or P. acnes-treated birds was slightly lower than that of untreated birds (group 1 versus 2 or 3). The difference between groups 1 and 3 was significant ($P<0.01$), but not between groups 1 and 2 ($P>0.05$). Sensitization of P. multocida with immune serum significantly reduced the bacterial recovery from the liver (group 1 versus 4, $P<0.001$). Recovery rate from the liver was also reduced when immune serum was administered into turkeys at 30 minutes before or even at 30 minutes after inoculation of P. multocida (groups 5 and 6). Recovery from the liver was further reduced when the sensitized bacteria were inoculated into the birds which had received immune serum (group 7). The lowest recovery, next to the immune group, was obtained when the birds which had been treated with both P. acnes and immune serum were inoculated with the sensitized bacteria (group 8). Significant differences were also observed among the recovery rates from the blood, but the differences were not as great as those among the livers. None of the treatments significantly changed the rate of bacterial recovery from the spleen ($P>0.90$). The birds in groups 2, 3 and 8 had enlarged spleens, and their antibody titers in ELISA were slightly higher than those of untreated birds (Table VI.1). The immune birds had high titers of antibody (Table VI.1).

Passive protection--The birds which had received the immune serum were protected against the intravenous challenge infection with P. multocida strain P-1059 (Table VI.2). Protection rates were roughly proportional to the dose of immune serum. A fifty percent protective dose of the immune serum was estimated at approximately 1 ml/kg of body weight.

TABLE VI.1--A design for clearance experiments with P. multocida strain P-1059 in turkeys

Group number	Treatment of turkeys	Inoculum		Turkeys			
		Sensitized with	Dose (log_{10})	Number used	Age (weeks)	S/B ratio*	Antibody titer$^{\Phi}$
1	None	None	8.7	5	11	0.9	1.01 ± 0.16
2	M. butyricum‡	None	8.8	5	14	1.3	1.67 ± 0.11
3	P. acnes‡	None	8.4	4	15	1.8	1.86 ± 0.21
4	None	IS$^{\sqcap}$	9.1	5	12	1.0	1.14 ± 0.17
5	IS (-30 minutes)§	None	8.5	4	10	1.0	0.88 ± 0.16
6	IS (+30 minutes)†	None	8.7	4	10	0.9	1.19 ± 0.08
7	IS (-30 minutes)§	IS$^{\sqcap}$	9.1	4	15	0.9	0.98 ± 0.40
8	P. acnes‡ and IS (-30 minutes)§	IS$^{\sqcap}$	8.8	4	9	1.5	1.76 ± 0.25
9	Immune**	None	9.0	5	13	0.9	4.31 ± 0.30

* Ratio of the mean spleen weight relative to the mean body weight (g/kg).

$^{\Phi}$ Serum samples were obtained about 1 hour prior to the clearance tests, and the antibody titers (mean ± standard deviation in log_{10}) against the 2.5S antigen of P. multocida P-1059 were determined by ELISA. Titers below 1.50 were considered to be negative.

‡ Inoculated intravenously, at 25 mg/dose, at 5 days before the clearance tests.

$^{\sqcap}$ Organisms were incubated with immune serum (IS) prior to inoculation.

§ Immune serum was administered intravenously, at 3 ml/kg of body weight, 30 minutes before inoculation of P. multocida.

† Immune serum was administered 30 minutes after inoculation of P. multocida in the identical manner.

** Survivors on day 16 after challenge infection with P. multocida P-1059.

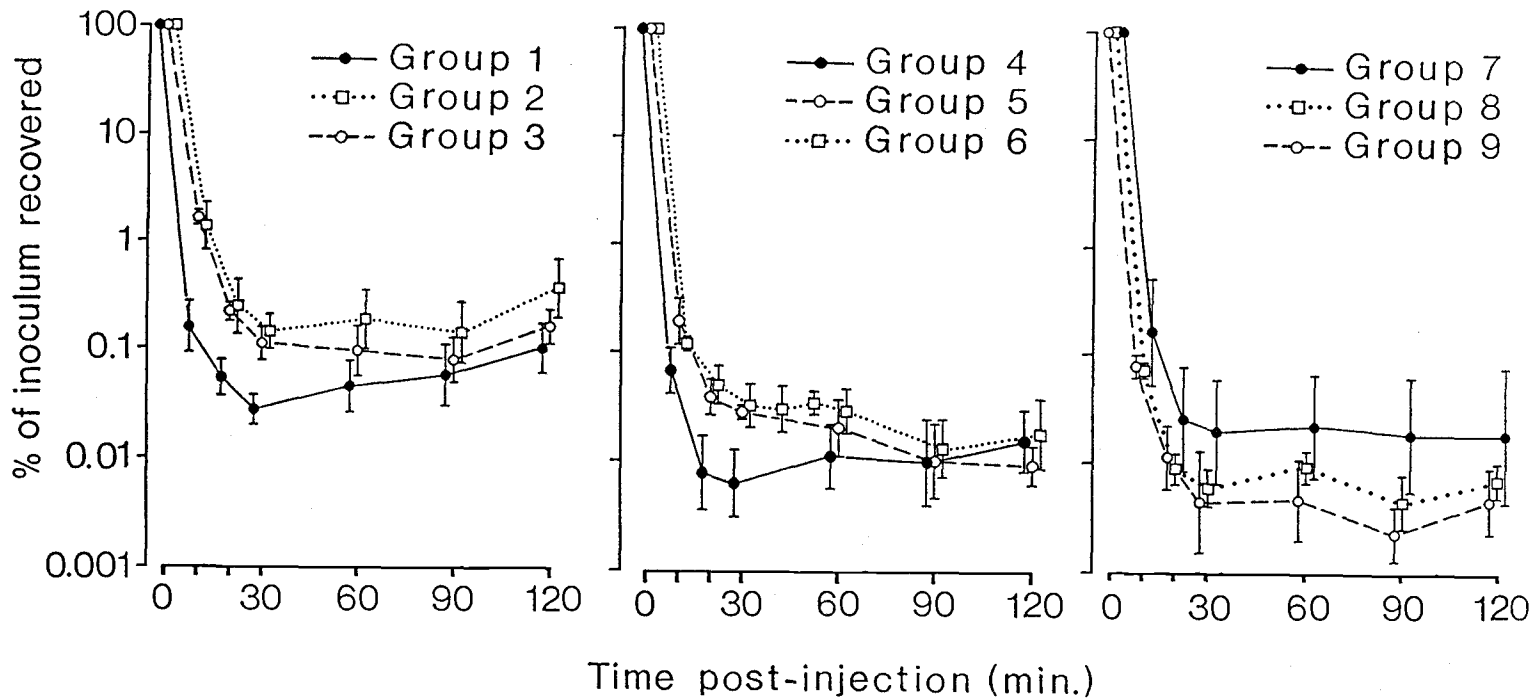

Fig VI.1--Clearance of P. multocida from the bloodstream in the nine groups of turkeys. Vertical bars on each mean indicate standard deviation. The group numbers correspond to those shown in Table VI.1.

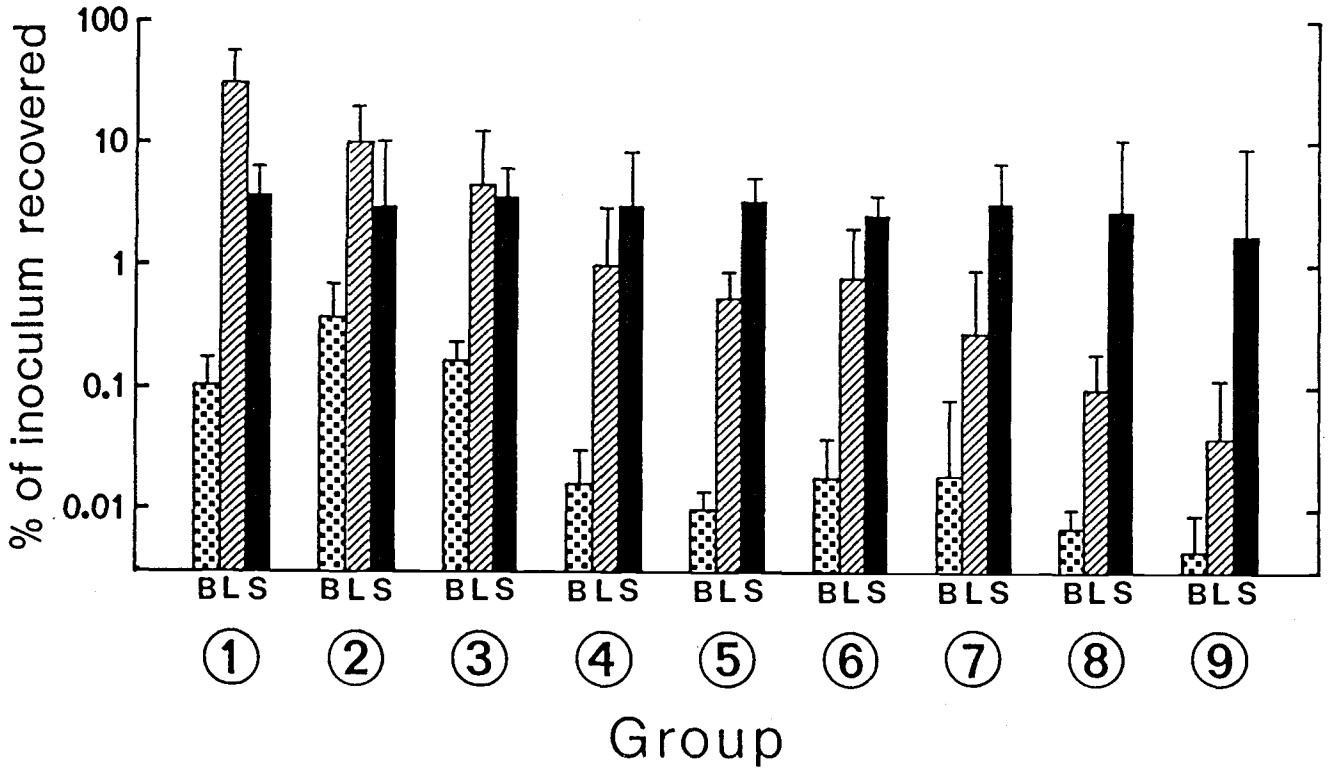

Fig VI.2--The rates of bacterial recovery from the blood (B), liver (L) and spleen (S) at 120 minutes after the intravenous inoculation of P. multocida. Vertical bars on each mean indicate standard deviation. The group numbers correspond to those shown in Table VI.1.

TABLE VI.2--Protective potency of the immune serum tested by the administration into 31-day-old turkeys followed by intravenous challenge infection with P. multocida strain P-1059

Group	Serum sample*	Dose (ml)	Mortality$^{\Phi}$	MDT‡	Isolation of P. multocida$^{\Pi}$
1	Immune serum	3.0	1/10§	9.0	1/10
2	Immune serum	1.0	3/10§	9.3	4/10
3	Immune serum	0.3	7/10	5.6	7/10
4	Immune serum	0.1	8/10	5.0	8/10
5	Normal serum	3.0	10/10	1.6	10/10

* Intramuscularly administered into the birds, which weighed 691 ± 80 g (mean ± standard deviation), at 24 hours before the intravenous challenge with 1.2×10^3 CFU of P. multocida strain P-1059.

$^{\Phi}$ Number of birds dead/total infected.

‡ Mean death time (days).

$^{\Pi}$ Number of P. multocida-positive/total examined.

§ Significantly different from group 5 ($P<0.01$).

Discussion

Rapid removal of P. multocida from the bloodstream was observed not only in the immune but also in the untreated birds. The clearance process, therefore, appears to be essentially independent of specific immunity. The clearance rate in the birds which had been treated with macrophage-activating agents was slower than that in the untreated birds. In the treated birds, some bacteria may have been associated with the monocytes in the bloodstream, because intravenous inoculation of P. acnes has been shown to induce activation of reticulo-endothelial phagocytes as well as monocytes in the peripheral blood.[13] Whether specific antibody serves as an effective opsonin for the phagocytes in blood vascular system is not clear. The passive transfer of immune serum did not enhance the clearance rate within 30 minutes PI. The organisms may have been removed too rapidly to be sufficiently opsonized by the antibody. In fact, clearance rate was slightly accelerated when the bacteria had been incubated with the immune serum prior to the inoculation (in group 4, but not in group 7). However, even if specific antibody acts as an effective opsonin, such a role has only a secondary importance, because the clearance capability in the absence of specific antibody was effective enough to remove more than 99.9% of the inoculum from the bloodstream within 30 minutes.

Effects of various treatments were most evident on the recovery rate of bacteria from the liver at 120 minutes PI. This sampling period was determined based on the results of our previous study,[3] which demonstrated that the number of bacteria in the liver of untreated birds were relatively constant between 10 and 120 minutes PI. In immune

birds, by contrast, the bacterial number in the liver decreased about 300-fold during this period. It has also been shown that the presence or absence of immunity did not significantly change the distribution of bacteria among the liver, spleen and blood at 10 minutes PI. Therefore, the difference between the untreated and each treated group primarily reflects the extent of bactericidal activity in the liver in each group.

The bacterial recovery from the liver was reduced into a greater extent by the transfer of immune serum than by the treatment with macrophage-activating agents. Thus, specific antibodies may be more important than activation of macrophages for the bactericidal activity in the liver. In the immune birds, however, both factors appeared to work synergistically. The birds that had been treated with both immune serum and P. acnes (group 8) had low numbers of bacteria in the livers, which were comparable with those of the immune birds.

The mode of action by which the specific antibody facilitates the bactericidal activity in the liver is not clear. Our working hypothesis was that the hepatic phagocytes are capable of killing P. multocida only when the bacteria have been sensitized (or opsonized) with specific antibody before or during the phagocytosis. The results obtained in groups 4, 5 and 7 support this hypothesis, but the result in group 6 does not. The birds in group 6 received the immune serum at 30 minutes after the inoculation of P. multocida. Judging from the clearance profile, there seemed to be enough time for the hepatic phagocytes to engulf the bacteria by the time of immune serum administration. If so, the antibodies should not have access to the intracellular bacteria. Nonetheless, the bacterial recovery from the liver in this group was

significantly lower than that in the untreated group. The immune serum may have possibly contained some unknown factors, besides the specific antibodies, which externally enhance the bactericidal activity of hepatic phagocytes. Further trials should be done with the purified immunoglobulin fraction at various post-inoculation periods.

None of the treatments facilitated the inactivation of bacteria in the spleen within 120 minutes PI. In contrast to the hepatic phagocytes, the splenic macrophages appear to be poorly capable of inactivating P. multocida at an early period of infection. Similar findings have previously been reported.[3] However, since the passively immunized birds were protected against intravenous challenge infection, the inactivation of bacteria in the spleen may have been observed at later periods.

The results of passive protection test clearly indicate the importance of humoral factors for the protection against the blood-borne P. multocida, but it does not necessarily exclude the importance of cellular factors. It is possible that the specific antibody, in association with complement, directly inactivated the bacteria without the aid of phagocytes.[9] However, if solely the humoral factors were responsible for the killing of bacteria, the decrease in bacterial recovery should have been observed not only in the liver but also in the spleen.

The evidence presented here suggests that the phagocytes in the liver, but not in the spleen, play a crucial role in controlling the blood-borne P. multocida at an early phase of infection. Neither specific antibody nor macrophage activation is required for the initial, rapid removal of the bacteria from the blood stream. The presence of

specific antibody, however, appears to be essential for the hepatic phagocytes to be capable of inactivating the bacteria. Activation of macrophages may also contribute to enhancing the bactericidal activity in the liver.

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