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Biological activities of fractions of type A *Pasteurella multocida*

by

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TABLE OF CONTENTS

GENERAL INTRODUCTION 1

LITERATURE REVIEW 3

Historical Background 3
Characteristics of Pasteurella multocida 5
Typing Systems for Pasteurella multocida 6
Antigens of Pasteurella multocida 10
Antigenic Analysis of Pasteurella multocida 22
Immunization of Cattle against Pasteurella Infection 24
Cellular Immunity against Pasteurella multocida 28

SECTION I. EFFECT OF TYPE A PASTEURELLA MULTOCIDA FRACTIONS ON BOVINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS 31

Summary 31
Introduction 32
Materials and Methods 33
Results 41
Discussion 53
Literature Cited 58

SECTION II. IMMUNOGENICITY OF POTASSIUM THIOCYANATE EXTRACT OF TYPE A PASTEURELLA MULTOCIDA 62

Summary 62
Introduction 63
Materials and Methods 65
Results 73
Discussion 82
Literature Cited 86

GENERAL SUMMARY 92

LITERATURE CITED 94

ACKNOWLEDGMENTS 108
GENERAL INTRODUCTION

Bovine respiratory disease is an acute infectious disease of young cattle characterized by fever, nasal discharge, coughing, and fibrinous pneumonia followed in some cases by death (2, 57, 58). The economic loss to the cattle industry in the United States from the disease was reported to be millions of dollars annually, excluding cost of treatment or preventive measures (1). Studies on etiology of the disease have indicated that the disease may be the result of a complex interaction or cumulative effect of viruses, bacteria, mycoplasma, chlamydiae and environmental factors (33, 101).

*Pasteurella multocida* (P. multocida) is a major etiological agent in bovine respiratory disease (25, 32), with capsular type A strains most frequently isolated in North America. Some encapsulated strains of *P. multocida* are known to be highly pathogenic in experimental animals and the presence of the mucoid capsule is important for virulence (25, 34). The capsule of type A strains is primarily composed of hyaluronic acid (26, 28) which serves as a framework for the attachment of polysaccharides, proteins and lipids (25).

The importance of *Pasteurellae* in bovine respiratory disease has led to the development and use of various bacterin products, either commercial or experimental, for immunization. The efficacy and safety of most of these products still remains in question (9, 42, 49, 64, 71). There is evidence that the use of bacterins which are currently available may in fact be detrimental to the health of the animal (9,
Recently, efforts have been made to find effective immunizing agents to prevent infection by \( P. \) \( \textit{multocida} \) including viable vaccinal strains such as attenuated avirulent strains (14, 67, 102), streptomycin-dependent mutants (30, 31), and a chemically altered strain (63, 100). Also, lysates of turkey-grown bacteria (18, 87, 88, 89) as well as isolated cellular fractions of the organism such as endotoxin (54, 84, 85, 86), free endotoxin (43, 53, 84), ribosomal proteins (4), saline extract (62, 98), potassium thiocyanate (KSCN) extract (44, 75, 76, 77) and culture filtrate (96, 97) have been studied. Although the immunogenicity of several of these preparations has been proved in experimental animals, identification of the protective factor(s) resident in the various preparations was not determined.

The objective of this investigation was to characterize the biological activities of fractions of type A \( P. \) \( \textit{multocida} \). Specific aims were: (1) to identify and characterize inhibitory factors for phagocytic cells present in the type A \( P. \) \( \textit{multocida} \) capsule and determine its effect on specific aspects of bovine polymorphonuclear leukocyte (PMN) function, and (2) to evaluate immunogenicity of the KSCN extracts obtained from several \( P. \) \( \textit{multocida} \) strains in mice and identify a protective component(s) in the KSCN extract on the basis of antigenic analysis.
A disease process now known as pasteurellosis was reported long before the isolation of the causative microorganisms. During the 18th century, large epornitics in poultry were reported throughout European countries and were first studied in France by Chabert in 1782 (46). Maillet in 1836 termed the disease fowl cholera in connection with severe losses of fowl (46).

Renault and Delafond, in the middle of the 19th century, presented the first experimental evidence of the transmissibility of the disease by the injection of the infected blood, secretions and tissues to poultry (50). The presence of a bipolar staining, non-motile organism in the blood of affected birds was first reported by Rivolta in 1877 and Perroncito in 1878 (50). Toussaint confirmed these observations in 1879, was the first to isolate the microorganism from the blood of an affected chicken, and cultivated it in neutralized urine (46). Pasteur in 1880 grew the organism in pure culture in chicken broth and examined the virulence and immunological properties of the organism in chickens (50). He observed that chickens which recovered from the disease or were immunized with aged live organisms, which were found to be avirulent for chickens, were protected against a lethal challenge dose of fresh-cultured, virulent
live organisms. However, the immunity could be overwhelmed by a large challenge dose of bacterin.

After isolation of the organism from birds, many investigators found that this organism was a very versatile pathogen capable of infecting cattle, buffalo, sheep, swine, rabbits, horses, cats, dogs, rats and mice (73). Moore in 1895 found the organism on the mucous membrane of the respiratory tract of apparently normal cattle, sheep, swine, dogs and cats (19). The importance of the organism in human infection was recently recognized in relation to infected animal bites (41).

The isolation of different strains by various individuals from multiple animal hosts and at different times resulted in a variety of terms being suggested to name this organism (73). Kitt in 1885 suggested the term Bacterium bipolar multocidum to name the bipolar organism on the basis of its infectivity for a variety of hosts. Trevisan in 1887 used the name "Pasteurella" in honor of Pasteur and listed 3 species; P. cholerae-gallinarum, P. davainei and P. suilla. Flugge in 1896 related the organisms to species by names such as Bacillus boviseptica, Bacillus suiseptica and so on. These classifications represent the early tendency to form a species name on the basis of the affected host. In following this procedures in detail, Lignieres listed species names, such as Pasteurella bovine for wild animal, buffalo and cattle strains, P. aviaire for avian strains, P. ovine for sheep strains, P. porcine for pig strains, etc. Since all of the strains of the organisms isolated from the different
animals were so similar in their morphology and cultural characteristics, an inclusive name was suggested again as a consequence of the trend to use one name for a variety of strains. The name Pasteurella septica suggested by Topley and Wilson in 1936 was employed widely in some European countries. The name Pasteurella multocida proposed by Rosenbusch and Merchant in 1939 (92) has acquired almost universal recognition at the present time.

Characteristics of Pasteurella multocida

According to the Bergey's manual (95), P. multocida is a Gram-negative facultative anaerobic bacterium with non-motile and bipolar-staining properties. Organisms from infected animal tissues usually appear as coccobacilli or short rods (1.4 ± 0.4 by 0.4 ± 0.1 um). Strains isolated from healthy animals are often pleomorphic with longer bacillary forms and are occasionally filamentous. With electron microscopic examination of the organism, Brogden (16) observed small spherical blebs on the surface of the cell and filamentous appendages which closely resembled the structures present on Bordetella species. P. multocida can be distinguished from other Pasteurella species (P. hemolytica, P. pneumotrophica, P. ureae) by the following characteristics: hemolysis on blood agar -, growth on MacConkey agar -, indol +, and urease - (25).

Many isolates of P. multocida possess a capsule which usually can
be detected by Jasmin's procedure, the Alcian blue method, India ink staining (25), or a negative staining method (16). The amount and chemical nature of the capsule seems to vary greatly from isolate to isolate. Carter's type A organisms possess the largest capsules which are composed, to a large extent, of hyaluronic acid (26, 28) and give the colonies a mucoid appearance (26). Some type D *P. multocida* also possess a hyaluronic acid capsule, but the amount is quantitatively much less than type A (29). Carter's type B and E organisms also possess a capsule but the chemical nature is a glycoprotein rather than hyaluronic acid, an acid-mucopolysaccharide (5, 79).

**Typing Systems for *Pasteurella multocida***

Methods for grouping and classification of *P. multocida* were extensively reviewed by Cornelius in 1929 (35), Carter in 1967 (25) and Brogden in 1977 (16). According to the review by Cornelius, early workers tried to establish a serological classification of the bacteria which corresponded to the zoological grouping. Matsuda in 1910 considered that a complement fixation test verified the zoological classification of pig, fowl, rabbit and calf strains. Miesser and Schern in 1910 found no agglutinatory relationship between ovine strains and other animal strains. Fitch and Nelso in 1923 could not demonstrate correspondence between a serological and a zoological grouping. Tanaka in 1926 used complement fixation methods and
demonstrated the heterogeneous nature of the zoological groups in this respect. Lal in 1927 found some evidence of cross-reactivity among different animal strains using a complement fixation test.

Cornelius (35) classified 17 of 26 strains into 4 groups using agglutinin-absorption tests and found no relationship between a serological group and the animal origin of the strains. In 1934, Ochi examined 72 hemorrhagic septicemia strains isolated from various species of animals and divided them into 4 groups on the basis of serological, immunological and pathological properties (16). Like Cornelius, he found that there was no relationship between his groups and the host of origin.

Rosenbusch and Merchant in 1939 (92) identified 3 serologically distinctive types among 114 non-hemolytic Pasteurella strains by agglutinin tests and ability of the organisms to ferment xylose, arabinose and dulcitol. Little and Lyon in 1944 (65) confirmed the existence of 3 serologically distinct types within 30 non-hemolytic Pasteurella strains by passive immunization of mice and agglutination tests. They found that type specificity, virulence and host origin of these strains were unrelated. Roberts in 1947 (90) divided P. multocida strains into four groups, I, II, III and IV, on the basis of serum protection tests in mice.

Carter (21) observed that P. multocida possesses a type specific capsular antigen, presumably polysaccharide in nature. He prepared the capsular antigen from several serologically distinctive strains of P. multocida by 2 methods: (1). Bacteria grown on an agar plate were
suspended in buffered saline and heated at 56 C for 1 h. After centrifugation, the bacterial pellet was discarded and the supernatant fluid was collected. (2). Bacteria grown on an agar plate were treated with 1 M sodium acetate and ethanol followed by subsequent extraction with phenol and saline. He also immunized rabbits and chickens with 0.25% formalized saline-treated bacteria to obtain the type specific antisera. By the use of the two extracted antigens and type specific antisera in precipitation tests, he determined that there appeared to be a type specific polysaccharide antigen consisting of, or associated with, the capsule. Based on his studies, he identified 3 capsular types, A, B and C among strains of *P. multocida*. An additional type D was demonstrated by Carter and Byrne (27) by the same methods.

Carter (22) applied an indirect hemagglutination test for typing the bacteria. By the use of his capsular antigen preparations, immune sera and erythrocytes of human O blood type for the test, he found that this technique yielded highly specific reactions and had advantages over other typing methods. The type C designation was subsequently dropped because of difficulties in recognition (23). An additional type E, which was isolated in central Africa and closely related to type B strains, was suggested later (24).

Heddleston et al. (51) introduced a gel-diffusion precipitation test for serotyping avian isolates of *P. multocida*. They prepared *P. multocida* bacterins from 5 serologically distinctive strains (an extention of a proposal by Little and Lion) to immunize chickens for
the preparation of type specific antisera. Typing antigen was obtained from bacteria suspended in 0.85% NaCl solution containing 0.3% formaldehyde and heated in a water bath at 100°C for 1 h. The supernatant fluid obtained by centrifugation of the suspension was designated a heat-stable serotyping antigen. They found that the heat-stable antigen of one serotype reacted only with the homologous serum. On the basis of the experiments, they reported that at least 5 different serotypes could be differentiated by this gel-diffusion test with heat-stable antigens. This serotyping system was further studied (15, 52) and is now composed of 16 serotypes (16).

Several non-serological typing methods for specific serotypes of P. multocida strains were suggested for the support of the previous typing systems as well as for a rapid procedure. Carter and Annau (26) found that the capsule of type A strains contains a large amount of hyaluronic acid which endows colonies with a mucoid appearance. The mucoid appearance of the colony was changed by treatment with staphylococcal hyaluronidase which depolymerizes the hyaluronic acid polymer of the capsule. By use of this method, type A strains could be identified non-serologically with staphylococcal hyaluronidase (28). Carter and Suboronto (29) identified type D strains non-serologically by the use of an acriflavine reaction that showed a coarse flocculation only with type D strains in the slide agglutination test.
Antigens of Pasteurella multocida

Characterization of the antigenic structure of P. multocida has proven difficult. The major differences in the antigenic structure among various strains were found to be present in the capsules and cell walls of the organism (25). Characteristics of the capsules and cell walls vary with different conditions of growth. Certain antigens of the organism have been studied for the investigation of their serological and immunological nature as well as their role in pathogenicity.

Carter and Annau (26) isolated capsular polysaccharides from the colonial variants of a type B P. multocida strain. The agar-grown bacteria were suspended in distilled water and heated at 56°C for 1 h, followed by centrifugation at 10,000 rpm. The supernatant fluid obtained by centrifugation was precipitated with 95% ethanol containing 0.25% sodium acetate. The precipitated polysaccharides from the fluorescent variant were found to induce a high level of protection in mice. Those of the mucoid variant were not protective. When the surviving mice were challenged with strains of other serological types, there was no protection.

Yaw and Kakavas (102) isolated a capsular polysaccharide by the method of Carter and Annau (26) from an encapsulated type I strain (Little and Lyon) which was virulent for mice and chickens. They immunized mice and chickens with the capsular polysaccharide, the heat-killed cells remaining after the saline extraction at 56°C for 1
h (20), and the heat-killed cells of an avirulent unencapsulated variant of the same strain. When the animals were challenged with the live virulent bacteria, the capsular polysaccharide was found to be protective in mice and chickens. However, the two heat-killed cell preparations could protect only chickens. They speculated that the protective antigen(s) of P. multocida may differ in various animal species.

Knox and Bain (61) extracted capsular polysaccharides from a P. multocida strain with 2.5% NaCl solution at 27 C for 2 h. This saline extract was found to be protective in mice. For the characterization of the extract, it was acidified with HCl to pH 3.8, which yielded a precipitate. The precipitate was removed by centrifugation, and the remaining supernatant fluid was treated with excess ethanol, which yielded another precipitate. The nitrogen (for protein), fructose (for polysaccharide) and heptose (for lipopolysaccharide) contents as well as immunogenicity of the two precipitates were compared. The precipitate at pH 3.8 contained predominantly proteins with some polysaccharides and lipopolysaccharides, and retained the ability to protect mice. The ethanol precipitate contained primarily polysaccharides with some protein and no lipopolysaccharide. Protein contamination of the ethanol precipitate was removed by treatment with trypsin. Mice were immunized with the purified ethanol-precipitated trypsinized polysaccharide. The purified polysaccharide was not protective in mice. However, this material could absorb some protective power from rabbit and cattle sera, which were obtained by
immunization of the animals with formalin-killed bacteria and had ability to completely protect mice following the passive immunization. Based on the results, they suggested that the polysaccharide was significant in immunity, although not exclusively so, because it could not remove all the mouse-protective power of the immune sera.

Kodama et al. (62) extracted a capsular material from type A P. multocida by treating the organisms at 56 C for 1 h with 2.5% NaCl solution. This saline extract induced protective immunity in turkeys and chickens but, when treated with cetylpyridinium chloride, the precipitated polysaccharide was not immunogenic. By continuing studies on the extract, Syuto and Matsumoto (98) demonstrated electronmicroscopically that the treatment of type A P. multocida cells with 2.5% NaCl solution removed the capsular material without disrupting the cells. They fractionated the saline extract by gel-filtration with a Sephadex G-200 column and obtained 4 different peaks. The protective ability of the 4 fractions was evaluated and detected only in the first peak. They further purified the protective antigen present in the first peak by absorbing it onto DEAE-cellulose and eluting with a linear gradient of NaCl solution. A single peak which induced protective immunity in turkeys was further characterized. They found that the purified protective antigen had a carbohydrate/protein ratio of 1.5 and formed a single precipitin line with a rabbit antiserum against the original saline extract in gel-diffusion and immunoelectrophoresis. Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis, the purified antigen showed 4
bands. By isoelectric focusing analysis, it showed two bands. Each band of the isoelectric focusing analysis was isolated and used to immunize turkeys. However, neither of the two components, alone or in combination, induced protection in turkeys. They could not explain why neither of the components induced immunity in turkeys.

MacLennan and Rondle (66) isolated lipopolysaccharides from 3 different serotypes of *P. multocida* by treatment of the bacteria with 45% phenol, followed by centrifugation at 100,000 x g for 4 h. The precipitate was poorly soluble in water but was used in gel-diffusion tests. They also prepared type specific antisera by immunizing rabbits with the heat killed bacteria of three serologically distinctive strains. Gel diffusion tests with homologous and heterologous type specific antisera indicated that the lipopolysaccharide showed at least one clear line of precipitation with homologous antisera but none with heterologous antisera. Treatment of the lipopolysaccharide at 100°C for 1 h did not affect the formation of the precipitation line. Therefore, they suggested that the type specific lipopolysaccharide antigen would be useful for typing.

Bain and Knox (6) treated their saline extract (61) with phenol-water to extract a lipopolysaccharide. The extracted lipopolysaccharide was found to be lethal for rabbits at a dosage of 500 ug when injected intravenously. Two week-old calves collapsed 2 h after receiving 300 and 700 ug respectively of the lipopolysaccharide but subsequently recovered. This lipopolysaccharide induced 20%
protection in mice against a homologous challenge. Antiserum, which
was obtained from rabbits immunized with the killed whole cells and
possessed abundant mouse-protecting antibody, could not passively
protect other rabbits against the injection of 500 µg of the
lipopolysaccharide.

Penn and Nagy (79) reported that a capsular antigen of types B
and E strains of \textit{P. multocida} was type specific while the endotoxins
of the two serotypes were not. Bacteria grown on the agar were washed
off with 2.5% NaCl solution followed by centrifugation at 3,500 x g.
The supernatant fluid was saved and the packed cells were treated with
phenol-water as described by Westphal and Jann (99). Antisera were
obtained from rabbits that were immunized with the formalin-killed
bacteria. In the immunodiffusion test, two major antigenic components
were found to be present in both the saline and phenol-water extracted
preparations. The immunoprecipitation lines were fused indicating the
extracts were antigenically similar. The saline extract of a non-
capsular variant lacked one of the precipitin lines, suggesting that
this line was due to a capsular antigen. One percent sodium
deoxycholate treatment of the saline extract altered the position of
the non-capsular immunoprecipitin line. Since sodium deoxycholate
breaks the endotoxin into smaller subunit, it was presumed that this
line is due to the endotoxin. By the use of rabbit antisera, the
extracts of both serotypes were compared by immunoelectrophoresis.
The capsular antigen of the one serotype reacted only with the
homologous antiserum, while the enotoxin reacted with both antisera.
Heddleston et al. (54) isolated a particulate antigen from two virulent and serologically distinctive strains (X-73 and P-1059) of type A *P. multocida*. The agar-grown bacteria suspended in formalized saline solution were incubated for 48 h at 4 C followed by centrifugation for 30 minutes at 12,000 x g. The supernatant fluid was filtered through a 400-mesh nylon cloth. The filtrate was centrifuged for 2 h at 105,000 x g. A gel-like pellet obtained by the centrifugation was washed and designated a particulate antigen. The particulate antigen possessed many of the properties ascribed to endotoxins. It was a high molecular weight, nitrogen-containing, phosphorylated lipopolysaccharide. Injection of 100 to 500 ug of the antigen intravenously into mice, rabbits or chickens produced severe effects on these animals such as depression and diarrhea; death followed. However, it protected almost 100% of the chickens and mice that were given 2 ug of the particulate antigen against challenges with the live organisms.

Rebers et al. (85) isolated a particulate antigen from an encapsulated strain of type B *P. multocida* by the method of Heddleston et al. (54). This antigen was also found to have properties similar to those of the particulate antigen (55) of a type A *P. multocida* strain. It was both toxic and protective in mice, rabbits and calves depending on dose, and similar to the endotoxin of *P. multocida* in chemical composition and toxicity.

Crutchley et al. (36) first observed an appreciable amount of toxic material, indistinguishable biologically from endotoxin, in the
culture supernatants of a wide variety of Gram-negative bacteria. Since this material contained primarily a lipopolysaccharide, it was referred to as free endotoxin. Rebers and Heddleston (84) compared the chemical and immunogenic properties of lipopolysaccharides (LPS) with free endotoxin isolated from a type A *P. multocida* strain. They prepared the LPS by the method of Westphal and Jann (99). The free endotoxin, which is secreted from or loosely bound to the surface of *P. multocida* organism, was obtained by washing the agar-grown bacteria with cold 0.85% NaCl solution containing formalin followed by centrifugation and gel filtration on a Sepharose 2B column. The free endotoxin induced active immunity in mice, but the LPS did not. Although the LPS and the free endotoxin both contained nitrogen, phosphorus, heptose and hexose, the free endotoxin had more nitrogen. Vigorous mixing of the free endotoxin or whole cell preparation with 50% phenol at room temperature and subsequent dialysis to remove phenol resulted in complete loss of ability to induce active immunity in mice. Since most polysaccharides are stable to phenol and most proteins are irreversibly denatured by it, it would appear that some form of protein is necessary for the induction of active immunity against *P. multocida* infection.

Brogden and Rebers (17) examined the serologic specificities of the Westphal-type LPS isolated from 16 serotypes of *P. multocida*. They compared the LPS with the Heddleston's heat-stable typing antigen in gel diffusion tests. They found that the LPS from each of the 16 serotypes reacted only with its homologous antiserum in 14 of the 16
serotypes. Therefore, they concluded that the LPS appeared to be one of major antigenic components responsible for the type specificity.

Rebers et al. (86) examined the serological and immunological properties of a purified LPS antigen isolated from an encapsulated strain of type A *P. multocida*. A crude LPS was obtained by the phenol-water extraction and contained nucleic acid, and capsular polysaccharides as well as LPS. The crude LPS was further purified by the treatment with RNase and DNase, followed by centrifugation at 165,000 x g for 3 h. The purified LPS was fractionated in a CsCl₂ density gradient centrifugation at 90,000 x g for 70 h and 3 visible bands were obtained. The middle band contained most of the LPS. The crude LPS and the middle band (1 to 10 ug) were used to immunize chickens to produce antisera. Passive administration of antiserum against the crude LPS protected 18% of the chickens. However, passive administration of antiserum against the middle band protected 86% of the chickens. Antisera obtained from the surviving chickens gave a single precipitation line in gel diffusion with the homologous Heddleston's serotype antigen (16), and no reaction was obtained with any of the other 15 serotype antigens. Therefore, they concluded that *P. multocida* LPS is an important serological marker in Heddleston's typing system and antibodies induced against the LPS contribute to the protection of chickens.

Bain (5) extracted a capsular protein-complex fraction from a type B *P. multocida* strain by treating the organisms with potassium thiocyanate (KSCN). To a dense suspension of bacteria in normal
saline there was added an equal volume of 1 M KSCN solution, followed by incubation at 37°C for 5 h. The bacterial suspension was centrifuged and the supernatant fluid was dialyzed against distilled water. This KSCN extract was found to be toxic for rabbits in a dose of 50 μg when injected intravenously. However, mice given 1 mg of the extract by the same route were not affected. For mice, the KSCN extract was found to be an important antigen conferring active immunity in small doses and removing the protective power from an antiserum that was obtained from rabbits immunized with heat-killed whole bacteria and possessing abundant mouse-protective antibody. Mukkur and Nilakantan (77) confirmed the immunogenicity of this material in cattle.

Gaunt et al. (44) reported that the KSCN extract from a serotype 3 (P-1059) *P. multocida* strain was immunogenic in chickens against a challenge infection of the homologous strain as well as one heterologous strain tested (serotype 1, X73). Gel-diffusion analysis revealed the presence of two components which were antigenically identical in KSCN extracts of both serotypes.

Mukkur (75) immunized mice with the KSCN extract of *P. hemolytica* serotype 1. These mice were found to resist a challenge infection of a type A *P. multocida* strain, thus demonstrating cross-protection between species. This finding was further supported by the finding that *P. multocida* organisms could be killed by incubation with guinea pig complement and an antiserum directed against the KSCN extract of *P. hemolytica*, and vice versa.
Mukkur (76) compared the immunizing efficiency of the KSCN extract of a type A *P. multocida* strain with a formalin-killed *P. multocida* bacterin in mice. All of the mice immunized with the KSCN extract survived a challenge dose of $1.6 \times 10^6$ cells. All of the mice immunized with the bacterin survived a challenge dose of 15 cells; the survival rate decreased with increasing challenge dose. With a challenge dose of $1.5 \times 10^3$ cells, only 10% of the immunized mice survived. When combination of KSCN extract and bacterin were used to immunize mice, the survival rate was no better than that of mice immunized with the bacterin alone. Ten times higher bactericidal titers were present in the sera of mice immunized with the extract compared with those given the bacterin. Three times higher titers were present in the sera of mice immunized with the extract compared with those given the bacterin plus the extract. Based on the results, they suspected that immunosuppression might conceivably be due to antigenic competition or partial denaturation of some vital protective antigen(s) as a result of formalin treatment.

Bhasin (10) tried to evaluate the immunogenicity and toxicity of a variety of antigens from a type A *P. multocida* strain such as, saline extract, free endotoxin, and phenol extracted endotoxin. The saline extract possessed significant immunogenicity (70% protection) and was non-toxic for mice. Free endotoxin in doses of 20 ug were highly immunogenic (100% protection) in mice without the development of signs of toxicity. Higher doses (40 to 160 ug) were toxic but also highly immunogenic. The phenol extracted endotoxin was highly toxic.
and non-immunogenic. An immunization trial with turkeys suggested that the free endotoxin was a better immunizing agent than heat-killed bacteria.

Srivastava et al. (97) compared the immunogenicity of the culture filtrate, cell walls and cytoplasmic components of a type A P. multocida strain, and tried to isolate a protective component(s) from the culture filtrate. The culture filtrate was prepared by centrifugation of bacterial cultures at 10,400 × g for 1 h followed by filtering the supernatant fluid through a 0.45 um membrane filter. The sediment obtained from the previous centrifugation was ruptured in a Ribi cell fractionator followed by centrifugation at 105,000 × g for 1 h. The sediment was designated as cell wall and the supernatant was filtered through a 0.45 um membrane filter. The filtrate was designated as cytoplasmic components. Cell walls induced more protection in mice than cytoplasm or culture filtrate. They fractionated the culture filtrate on a Sephadex G-50 column and obtained 4 fractions. The first fraction was found to contain a protective material responsible for the protection of mice and was more immunogenic than cell walls. They indicated that this material is different from the free endotoxin studied by Heddleston and Rebers (53), because it had very low levels of 2-keto-3-deoxy-octanate (KDO) and heptose which are known principal chemical components of the endotoxin. Srivastava and Foster (96) continued to study the first fraction obtained from column chromatography. They treated it with ether to yield a "glycolipid-like" material and with phenol to yield a
"lipopolysaccharide-like" material. They found that the ether-treated material was more protective for mice than the first fraction of the culture filtrate. However, the phenol-treated material was not immunogenic in mice and was toxic by rabbit skin tests.

Ganfield et al. (43) tried to purify a protective component(s) from the saline extract of a type A P. multocida strain. The starting material was obtained from the saline (0.85% NaCl containing 0.1% formalin) extract of P. multocida cells by differential centrifugation at 105,000 x g, and further purified by gel filtration on Sepharose 2B. Three fractions were obtained after gel filtration. The first fraction consisted of 10% of the starting material and was immunogenic in mice (80% protection). The second fraction consisted of 75 to 95% of the starting material and induced active immunity in mice and turkeys with the doses of 10 to 20 ug. Large doses were found to be lethal. The third fraction was much less effective in inducing a protective immune response.

Baba (4) examined the immunogenicity of a ribosomal fraction isolated from a type A P. multocida strain. Organisms grown in a liquid medium were harvested and washed by centrifugation. The packed cells were suspended in a tris-HCl buffer, pH 7.4, followed by the rupture in a French press. The ruptured cellular mass was centrifuged at 8,000 x g for 40 min to remove whole cells, cellular debris and cell walls. The supernatant fluid was further purified by differential centrifugation, zonal electrophoresis and gel filtration on a Sephadex G-200 column. The ribosomal fraction exhibited intense
protective antigenicity in mice and turkeys. Sodium deoxycholate treatment of the ribosomal fraction resulted in a 13% loss in immunological activity and ribonuclease treatment caused a 60% loss of the activity. Purified ribosomal protein or RNA were not immunogenic in mice.

Antigenic Analysis of Pasteurella multocida

Although current studies have demonstrated that \textit{P. multocida} organisms are a serologically and immunologically heterogeneous species, knowledge of the quantitative as well as qualitative nature of the antigenic components present in the various immunogenic preparations, their probable number and location, and identification of the protective or inhibitory component(s) is far from complete. There are only a few studies on the antigenic analysis of \textit{P. multocida} reported at present.

Prince and Smith (80, 81, 82) reported their series of antigenic studies on \textit{P. multocida}. They prepared a variety of antigens such as, capsular extract, mickle disintegrate, sonic disintegrate, cell wall, isoelectric precipitate at pH 3.8 and polysaccharide fraction. Antisera were prepared by immunizing rabbits with whole killed bacteria, disintegrated bacteria, capsular extract or cell wall. Using a standard electrophoresis system, up to 18 antigens of a strain of \textit{P. multocida} were identified. When they examined the cross-
reactivity \textit{P. multocida} antigens with a variety of Gram-negative bacteria, \textit{P. hemolytica} was the most cross-reactive bacteria with 5 cross-reactive antigens.

Baxi et al. (7) studied the antigenic structure of strains of 4 Carter's capsular types by immunodiffusion and immunoelectrophoretic techniques. They found that at least 10 different antigenic components from the sonicated type A bacteria, 6 from type B, 3 from type D and 7 from type E. However, the types could not be differentiated from one another by these techniques.

Mukkur (76) reported that there were three antigenic components in the KSCN extract of a type A \textit{P. multocida} strain by the use of immunoelectrophoretic techniques and antisera obtained from mice immunized with the KSCN extract.

Griffith (47) reported that different bacterial fractions isolated from the same type A organism shared some of antigenic component(s) with each other. At least one or more (up to 7) antigenic components were identified from each fraction by immunodiffusion and immunoelectrophoretic techniques.

Bhasin and LaPointe-Shaw (12, 13) examined the antigenic complexities of type A \textit{P. multocida} by the use of crossed-immunoelectrophoretic (CIE) techniques. They prepared a variety of antigens such as whole cell disintegrate, cell envelope (saline extract), KSCN extract, free endotoxin, and phenol-extracted lipopolysaccharide. They also prepared specific antisera against each antigen by immunizing rabbits with the antigens. The application of
CIE to the analysis of *P. multocida* antigens revealed antigenic complexity not found before. They identified at least 55 antigenic components in the cytoplasm, 19 in cell envelope, 5 in the capsular extract, 7 in the KSCN extract, 3 in the lipopolysaccharide, 3 in the heat-stable antigen and 5 in the free endotoxin.

McKinney and Rimler (74) examined the suitability of avian anti-*P. multocida* serum for application in CIE techniques to detect antigenic components of avian *P. multocida* strains. They found that 0.05 M sodium acetate buffer at pH 5.6 was the most suitable buffer, because avian antisera did not migrate at this pH, but migrated at pH 8.6 which is normally used in electrophoresis with mammalian sera.

**Immunization of Cattle against Pasteurella Infection.**

The most prevalent bacterial species found in the lungs of cattle with severe respiratory disease are *P. multocida* and *P. hemolytica* (25, 32, 33, 45, 57, 58, 72). Since numerous attempts to develop successful immunizing agents have failed, bacterial pneumonia due to *Pasteurella* organisms is still a serious problem in the cattle industry.

Palotay et al. (78) tested several biological agents in an attempt to prevent respiratory infection in newly weaned beef calves. Of these agents, three agents that significantly reduced mortality were (1) a commercial hemorrhagic septicemia bacterin, (2) a bivalent
bacterin prepared from a *P. multocida* and a *P. hemolytica* strains, and (3) a formalized bacterin prepared from chicken embryo-grown *P. hemolytica* which was isolated from cattle that died from acute pneumonia. However, there still remained 9.38% incidence of disease in the most effectively protected group when compared with 21.05% average for the control groups.

Hamdy et al. (48) examined the efficacy of 5 different commercial *Pasteurella* bacterins in young calves under field conditions. Although immunized calves showed as much as 500 times greater antibody titer than the controls, none of these bacterins effectively reduced the incidence of the disease.

Larson and Schell (64) compared the toxicity of *Pasteurella* bacterins with their potential protective value in calves undergoing the stress of weaning and shipping. Calves given the bacterin developed significantly higher antibody titers than the controls. However, the immunized calves developed fever and leukocytosis with neutrophilia, although these effects were transient and did not clinically alter their general state of health when compared with the non-vaccinated controls.

Bennett (9) examined the efficacy of commercial *Pasteurella* bacterins for yearling feedlot cattle. He found that the mortality rate, though not statistically significant, was increased in vaccinated calves.

Friend et al. (42) induced respiratory disease experimentally by the administration of the live *P. hemolytica* organisms in calves.
vaccinated with \textit{P. hemolytica} bacterin and non-vaccinated controls. All the calves became severely ill about 2 to 6 h after the challenge with the development of severe respiratory distress, abnormal lung sounds and coughing. Body temperature and total white blood cell counts tended to peak at 9 to 18 h with improvement or deterioration in clinical condition occurring subsequently. Calves with persistent signs of illness were killed at intervals, and the macroscopic and microscopic lesions of lungs were examined. The severity of the lung lesion were ranked. They found that the calves vaccinated with a \textit{P. hemolytica} bacterin developed more severe pneumonia than the non-vaccinated controls.

Markham and Wilkie (71) reported a detrimental effect of \textit{P. hemolytica} bacterin on bovine alveolar macrophages. They cultured the macrophages in Leighton tubes and added $^{125}$I-labeled \textit{P. hemolytica} with fetal calf serum or an immune serum that was obtained from calves immunized with \textit{P. hemolytica} bacterin. Tubes were then incubated at 37°C for 2 h, after which coverslips were removed, washed in normal saline to remove unphagocytized bacteria, and placed in a gamma counter. Coverslips were stained and the number of the macrophages and bacteria was counted. The uptake of $^{125}$I-\textit{P. hemolytica} by the macrophages was significantly enhanced in the presence of the immune serum. However, the ratio of macrophages and bacteria adhering to the coverslips was significantly decreased. This indicated that the macrophages probably died after the uptake of the bacteria or at least lost their ability to adhere on the coverslips. In a separate
experimentation, they prepared $^{51}$Cr-labeled macrophages and a culture supernatant of $P. \text{hemolytica}$. When the culture supernatant was added to the culture of the $^{51}$Cr-labeled macrophages, the release of $^{51}$Cr was significantly increased. Based on these results, they concluded that the immunization of cattle with $P. \text{hemolytica}$ bacterin may be detrimental to the macrophages of the host rather than protective. The detrimental components of $P. \text{hemolytica}$ on the bovine macrophages is probably a cytotoxin, which is released to the medium by $P. \text{hemolytica}$ during culture or released to the phagocytic vacuole of the macrophage after ingestion of the bacteria.

Due to the questionable efficacy and detrimental effects of Pasteurella bacterins in cattle, many new approaches for the preparation of an effective immunizing agent have been proposed. Chengappa and Carter (30) prepared live streptomycin-dependent (Str$^D$) mutants from a streptomycin-sensitive type A $P. \text{multocida}$ strain and a type 1 $P. \text{hemolytica}$ strain by treating the organisms with N-methyl-N-nitro-N-nitrosoguanidine as a mutagen. They found that the mutants were not pathogenic in mice while the wild types were pathogenic. In their continuing studies, Chengappa et al. (31) vaccinated rabbits with the type A Str$^D$ mutant either by intranasal or subcutaneous administration. Either method provided complete protection against homologous, wild type challenge as evidenced by absence of clinical signs or pathologic changes and failure to isolate the organism from mucous secretions or tissues from the vaccinated individuals.

Kucera et al. (63) developed a chemically altered type A $P.$
multocida vaccinal strain (76-63M) by treatment with acridinium salts. This strain was found to be protective for mice and hamsters not only against a homologous challenge exposure, but also against challenge exposure with a number of heterologous type A strains and a type B strain. This strain was also found to be protective against the type B challenge exposures in calves, as well as type A strains in swine and sheep.

Wong and Kucera (100) used the chemically altered 76-63M strain to vaccinate mice and hamsters to find the cross-protectivity between different capsular types of P. multocida strains. At least half of the mice and hamsters vaccinated survived against the challenge exposures with type A and B strains.

Cellular Immunity against Pasteurella multocida

It has been generally considered that immunity to P. multocida infection is primarily humoral in nature (34). Recently, it has been suggested that cell-mediated immunity of the host against P. multocida infection may be as important as humoral immunity (68, 69, 70). The cellular immunity to this organism can be measured by lymphocyte blastogenesis assay in some cases. There is evidence that this organism can inhibit the cellular immunity of host.

Maheswaran et al. (70) performed a lymphocyte blastogenesis assay using peripheral blood lymphocytes from turkeys that were immunized
with a bacterin or left as non-immunized controls. They found that a higher stimulation was obtained with immune lymphocytes than with non-immune lymphocytes.

Maheswaran and Thies (68) developed a whole blood lymphocyte stimulation assay to study cell-mediated immune responses in bovine pasteurellosis. Peripheral blood lymphocytes from cattle immunized with hemorrhagic septicemia strains (type B and E) of *P. multocida* exhibited higher stimulation indices when incubated with the antigens of homologous serotypes than the heterologous shipping fever serotype (type A), and vice versa. Based on the results, they suggested that lymphocyte stimulation assay is useful for the measurement of cellular immune response of cattle against pasteurellosis and for the differentiation of strains of *P. multocida* isolated from cases of the different diseases.

Maheswaran and Thies (69) examined the influence of encapsulation on phagocytosis of *P. multocida* by bovine neutrophils by using two encapsulated strains, NA77 (capsular type A) and C42 (capsular type B), and one unencapsulated strain (1173). When they measured the uptake of 
$[^{3}H]$thymidine-labeled bacteria in the presence of normal bovine serum, the encapsulated strains inhibited the phagocytic activity of bovine neutrophils while the unencapsulated strain did not. C42 (capsular type B) could be completely phagocytized by the neutrophils in the presence of hyperimmune anti-C42 serum. However, only 3.8% of the NA77 (capsular type A) bacteria were ingested by the neutrophils in the presence of hyperimmune anti-NA77 serum. When they
treated NA77 organisms with bovine testicular hyaluronidase, 90% of the decapsulated organisms were ingested. Therefore, they concluded that the factor associated with NA77 organism which inhibited the phagocytic activity of neutrophils was probably hyaluronic acid which is a major component of the capsule of type A P. multocida.
SECTION I.

EFFECT OF TYPE A PASTEURELLA MULTOCIDA FRACTIONS ON BOVINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS

Summary

The effect of various Pasteurella multocida fractions on bovine polymorphonuclear leukocyte (PMN) functions was examined in vitro by using two encapsulated strains, P-2383 and P-1062 (both Carter's capsular type A and of bovine origin). The ability of PMNs to ingest Staphylococcus aureus and iodinate protein was significantly inhibited in the presence of live cells, heat-killed whole cells, or saline extracted capsule but not in the presence of the decapsulated heat-killed cells. None of the fractions of the two strains inhibited nitroblue tetrazolium reduction by PMNs. The saline extract did not inhibit the binding of iodine to protein by a reaction involving xanthine, xanthine oxidase and horseradish peroxidase. The PMN inhibitory factor was further characterized as a heat-stable capsular material of greater than 300,000 molecular weight.

Introduction

*Pasteurella multocida* is a major etiological agent in bovine respiratory disease (3,6), with capsular type A strains most frequently isolated in North America. Some encapsulated strains of *P. multocida* are known to be highly pathogenic in experimental animals and the presence of the mucoid capsule is important for virulence (3,7). The capsule of type A strains is primarily composed of hyaluronic acid (4,5) which serves as a framework for the attachment of polysaccharides, proteins and lipids (3).

The importance of *Pasteurellae* in bovine respiratory disease has led to the development and use of various bacterial products for immunization. The efficacy and safety of most of these products still remains in question (2,13,17,19). There is evidence that the use of bacterins which are currently available may in fact be detrimental to the health of the animal (2,19).

Recent studies on type A *P. multocida* capsular materials indicated that a KSCN extract (12,20) and a saline extract (16,25) were protective against experimental challenge in mice, chickens and turkeys. The capsule of a type A *P. multocida* has also been demonstrated to inhibit the phagocytic activity of bovine neutrophils (18). Therefore, the capsule may contain not only a protective antigen but also a component which interferes with phagocytic cell function. It is not unusual for bacterial surface material to contain both a protective antigen and a virulence factor (8,9,14).
Phagocytosis of invading microorganisms by polymorphonuclear leukocytes (PMN) can be one of the major cellular defense mechanisms in protecting animals from microbial infection. Maheswaran and Thies (18) reported that an encapsulated type A P. multocida (NA 77) inhibited the phagocytic activity of neutrophils (PMNs). When they measured the uptake of [3H]thymidine-labeled bacteria by PMNs, only 3.8% of the encapsulated organisms were ingested. When the encapsulated bacteria were treated with bovine testicular hyaluronidase, however, 91.1% of the decapsulated organisms were ingested. They concluded that the factor which inhibited the phagocytic activity of PMNs was probably hyaluronic acid which is a major component of the capsule of type A P. multocida.

The purpose of this experiment was to further characterize an inhibitory factor present in type A P. multocida capsule, and determine its effects on specific aspects of PMN function.

Materials and Methods

Organisms

Two strains of Pasteurella multocida were used throughout this study, (P-2383 and P-1062). Strain P-2383 was isolated from a case of bovine pneumonia presented to the Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA. This isolate was a typical P. multocida strain; subsequent typing confirmed it to be a Carter's
capsular type A. Strain P-1062, also a type A and of bovine origin, is a challenge strain (IRP-198, National Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, IA). Each strain was inoculated into the yolk sac of 6-day-old embryonated chicken eggs. Following incubation at 37 C for 18 h, the yolk material was aseptically removed and frozen in aliquots at -70 C. These aliquots were used as inoculum for preparation of bacterial fractions used in this study.

**Bacterial fractions**

The preparation of bacterial fractions is illustrated at Fig. 1. Infected yolk material was thawed, inoculated on a 5% bovine blood agar plate and incubated at 37 C for 24 h. One colony was transferred to 5 ml of brain-heart-infusion (BHI) broth (Difco Laboratories, Detroit, MI) containing 0.5% yeast extract (Difco) and 5% sterile bovine serum (BHISY) and incubated at 37 C for 4 h. This culture was then added to 100 ml of BHISY and incubated an additional 4 h. Roux bottles containing dextrose-starch agar (Difco) were inoculated with 2.0 ml of BHISY culture and incubated at 37 C for 36 h. Cells were harvested by washing the agar surface with sterile phosphate-buffered saline solution (PBS, pH 7.2) and cell concentration was adjusted so that a 10-fold dilution had an optical density (O.D.) of 0.4 at 540 nm (approximately 1.0 x 10^10 cells/ml). The cells were washed three times in PBS and an aliquot was saved for the "live cell fraction" (LCF). The remaining cells were washed one more time in PBS. Cells
Fig. 1. Preparation of bacterial fractions.
packed by centrifugation were resuspended to the original volume in 2.5% (wt/vol) sodium chloride (NaCl) solution and placed in a water bath at 56 C for 1 h. After this saline extraction, an aliquot was saved for the preparation of "heat killed whole cell fraction". The remainder was centrifuged at 17,300 x g for 20 min at 4 C. The supernatant was saved and the pelleted cells were washed three times and resuspended with an equal volume of sterile PBS. This preparation was designated the "decapsulated heat-killed cell fraction" (DCF). The heat-killed whole cell and supernatant fractions were dialyzed against PBS for three days to remove excess salt and used as the "heat-killed whole cell" (KCF) and "saline extract" (SEF = 1.06 mg dry weight/ml for P-2383 and 0.91 mg/ml for P-1062) fractions. Aliquots of SEF were treated various ways including: (1) treatment with bovine testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO., 1 mg of hyaluronidase per mg dry weight of SEF) by incubating in a water bath at 37 C for 3 h (SEF-H); (2) autoclaving at 121 C for 15 min (SEF-A); (3) filtration with a 300,000 molecular weight cutoff filter (Amicon Corp. Lexington, MA). The filtrate was designated SEF-F (0.20 mg dry weight/ml for P-2383 and 0.25 mg/ml for P-1062) and utilized directly. The retentate was washed three times on the filtration membrane with PBS and designated as SEF-R (0.94 mg dry weight/ml for P-2383 and 0.80 mg/ml for P-1062). Control preparations of hyaluronic acid (Human umbilical cord, Sigma) and hyaluronidase (Sigma) at a concentration of 1 mg/ml in PBS were prepared. The SEF-R of strain P-2383 was treated with bovine testicular hyaluronidase (Sigma, 1 mg of hyaluronidase per
mg dry weight of SEF-R of strain P-2383) and then filtered with a 300,000 molecular weight cutoff filter (Amicon). The filtrate was designated SEF-RH-F (0.80 mg dry weight/ml for P-2383 and 0.62 mg/ml for P-1062) and utilized directly. The retentate was washed three times as previously described and designated SEF-RH-R (1.4 mg dry weight/ml for P-2383 and 0.98 mg/ml for P-1062).

**PMN preparation**

PMNs were isolated as previously described (23). Briefly, peripheral blood from healthy adult cattle was collected in acid-citrate-dextrose solution, centrifuged and the plasma and buffy coat layer were discarded. Erythrocytes in the packed cell fraction were lysed with distilled water and the remaining cells, which generally consisted of greater than 90% granulocytes, were washed and suspended in PBS to a concentration of 5.0 x 10⁷ PMNs per ml. The cells were held at room temperature and were used in all three PMN function tests.

**PMN function tests**

The procedures for evaluating PMN functions have been described in detail (23). All PMN function tests were conducted in duplicate and the average of duplicate values was used for calculation.

(1) *Staphylococcus aureus* ingestion. Heat-killed [¹²⁵I]iododeoxyuridine ([¹²⁵I]UdR, Amersham Corp., Arlington Heights, IL)-labeled *S. aureus* was used to evaluate ingestion by PMNs. The
test was conducted in 12 x 75 mm plastic tubes and the standard reaction mixture contained 0.1 ml of \([^{125}\text{I}]\text{UDR}\)-labeled \textit{S. aureus}, 0.05 ml of PMNs (2.5 x 10^6 PMNs, bacteria to PMN ratio = 60:1), 0.05 ml of a 1:10 dilution of bovine anti-\textit{S. aureus} serum and 0.3 ml of Earle's balanced salt solution (EBSS, Grand Island Biological Co., Grand Island, N.Y). To determine the effect of bacterial fractions on ingestion by PMNs, 0.05 ml of a \textit{P. multocida} fraction (5.0 x 10^8 cells or equivalent solution) or 0.05 ml of PBS as a control were added to the standard reaction mixture. The reaction was allowed to proceed for 10 min at 37 C and the extracellular \textit{S. aureus} was removed by lysostaphin (Sigma) treatment, the PMNs washed by centrifugation and the amount of PMN-associated radioactivity was determined. The results were expressed as the percentages of \([^{125}\text{I}]\text{UDR}\)-labeled \textit{S. aureus} that was ingested.

(2). \textit{Nitroblue tetrazolium (NBT) reduction}. This test was conducted in 15 x 100 mm silicon-coated glass tubes and the standard reaction mixture contained 0.2 ml of NBT solution (2 mg/ml), 5.0 x 10^6 PMNs, 0.1 ml of preopsonized zymosan preparation (10 mg/ml) and 0.6 ml of EBSS. To determine the effect of bacterial fractions on NBT reduction by PMNs, 0.1 ml of a \textit{P. multocida} fraction (1.0 x 10^9 cells or equivalent solution) or 0.1 ml of PBS as a control was added to the standard reaction mixture. After 10 min incubation at 37 C the reaction was stopped. The purple formazan formed by the reduction of NBT was extracted with pyridine and the optical density (O.D.) at 580 nm was determined. The results are expressed as O.D. per 5.0 x 10^6
PMNs per 10 min in 5.0 ml of pyridine.

(3). Iodination. The iodination test was conducted in 12 x 75 mm polystyrene snap-cap tubes (#2058, Falcon, Oxnard, CA). The standard reaction mixture contained 2.5 x 10⁶ PMNs, 0.05 uCi of ¹²⁵I (Carrier free, Amersham Corp., Arlington Heights, IL), 40 n mole of NaI, 0.05 ml opsonized zymosan (10 mg/ml) and 0.3 ml of EBSS. To determine the effect of bacterial fractions on iodination by PMNs, 0.05 ml of P. multocida fraction (5.0 x 10⁸ cells or equivalent solution) or 0.05 ml of PBS as control was added to the standard reaction mixture. The reaction was allowed to proceed for 20 min at 37 C and the amount of trichloroacetic acid (TCA)-precipitable radioactivity was determined. The results are expressed as nanomoles of NaI per 10⁷ PMNs per hour.

Data analysis of PMN function test

To determine the effect of bacterial fractions on each PMN function, the value obtained when a bacterial fraction was added to the PMNs was compared to the value obtained with control (PBS-treated) PMNs. An analysis of variance procedure was used to determine significance of the differences in PMN function. For the graphic presentation of the data, all treatment values were expressed as a percentage of the control.
Chemical analysis of bacterial fractions

Total carbohydrate was determined with a phenol-sulfuric acid procedure (11) using glucose as a standard. Hyaluronic acid was determined by the reaction of hexuronic acid with carbazole (Sigma) and sulfuric acid (18) using D-glucuronic acid (Sigma) as a standard. Protein content was determined colorimetrically from the reaction of protein with Serva blue G dye (Serva Fine Chemicals, Inc., Long Island, N.Y.) using bovine serum albumin (Sigma) as a standard (21).

Xanthine-xanthine oxidase-horseradish peroxidase mediated iodination

A chemical iodination procedure was formulated following the basic principle of PMN iodination (15, 22). Xanthine (Sigma) was used as a substrate and xanthine oxidase (Sigma) served as the enzyme for the production of superoxide anion. Horseradish peroxidase (Sigma) was used to catalyze the iodination reaction. The standard mixture contained 40 nmoles NaI, 0.05 uCi $^{125}$I, 0.3 ml of EBSS containing 0.1% bovine serum albumin, 0.5 mg xanthine, 0.5 unit of horseradish peroxidase and 0.05 ml of SEF or PBS as a control. The reaction was started by the addition of 0.02 unit of xanthine oxidase. The mixture was incubated and processed by the same procedures as used for PMN iodination. A blank containing all components except xanthine oxidase was run with each experiment. Results are expressed in counts per minute.
Results

Effect of *P. multocida* fractions on *S. aureus* ingestion

To determine the effect of *P. multocida* fractions on phagocytic activity, PMNs were added to a standard suspension of opsonized *S. aureus* in the presence or absence of *P. multocida* fractions. Control PMNs ingested 28.6 ± 2.3 (mean ± SEM) (n = 22) percent of the *S. aureus* in the reaction mixture. In the presence of LCF, KCF and SEF of the two strains of *P. multocida*, *S. aureus* ingestion was inhibited by 42 to 51% (Fig. 2). DCF had no effect on *S. aureus* ingestion by PMNs. To further characterize the inhibitory factor, SEF was treated with hyaluronidase, autoclaved or filtered through a 310,000 MW cutoff filter. Neither autoclaving nor treatment with hyaluronidase destroyed the inhibitory activity of SEF on *S. aureus* ingestion by PMNs (Fig. 3). SEF-R did inhibit *S. aureus* ingestion by PMNs by 41 to 60% but SEF-F had no effect on the PMN ability to ingest *S. aureus*. In a separate experiment to further characterize SEF-R of strain P-2383, the control PMNs ingested 36.1 ± 1.4 (mean ± SEM) (n = 8) percent of the *S. aureus* in the reaction mixture. SEF-RH-R inhibited *S. aureus* ingestion by PMNs by 59% but SEF-RH-F had no effect on the PMNs ability to ingest *S. aureus* (Fig. 7). *S. aureus* ingestion by PMNs was not inhibited by either hyaluronic acid or hyaluronidase. To determine if the inhibitory substance was binding to the *S. aureus* or the PMN, the *S. aureus* ingestion assay was performed using *S. aureus* and PMNs which had been separately treated with P-2383 SEF-RH-R by
Fig. 2. Effect of type A *P. multocida* fractions on *S. aureus* ingestion by bovine PMNs. Values represent mean (+ SD) percentages of the control value. Statistically significant differences from the control value are as indicated. **, p < 0.01. *, 0.01 < p < 0.05. n = 16 for the saline extract fraction (SEF) and n = 6 for all other fractions.
Fig. 3. Characterization of the inhibitory activity in the saline extracted fraction on *S. aureus* ingestion by bovine PMNs. Values represent mean (+ SD) percentages of the control value. Statistically significant differences from the control value are as indicated. **, p < 0.01; n = 6 for all fractions.
incubating for 20 min at 37 C in a shaking water bath, then washed 3 times with PBS. The results in Table 1 indicate that the inhibitory substance was removed by washing and did not bind to either the PMN or the S. aureus.

**Effect of P. multocida fractions on NBT reduction**

To study the effect of *P. multocida* fractions on oxidative metabolism of PMNs, the ability of PMNs to reduce NBT by the production of superoxide anion in the presence or absence of the bacterial fractions was determined. NBT reduction by control PMNs was $0.47 \pm 0.01$ (mean $\pm$ SEM) ($n = 15$) O.D. at 580 nm. None of the fractions of the two type A strains significantly ($p > 0.05$) affected NBT reduction by PMNs (Fig. 4).

**Effect of P. multocida fractions on iodination**

To study the effect of *P. multocida* fractions on the myeloperoxidase-$H_2O_2$-halide antibacterial system of the PMN the ability of PMNs to iodinate protein in the presence or absence of the bacterial fractions was determined. The value for iodination by control PMNs was $42.3 \pm 2.2$ (mean $\pm$ SEM) ($n = 22$) nmoles NaI per $10^7$ PMNs per hour. The ability of PMNs to iodinate protein in the presence of LCF, KCF and SEF of the two strains was inhibited 30 to 36%, but DCF had no effect on iodination by PMNs (Fig. 5). When SEF was processed for further characterization of the inhibitory factor as described for the *S. aureus* ingestion assay, neither autoclaving nor
Table 1. The effect of preincubation of PMNs and *S. aureus* with P-2383 SEF-RH-R on the ingestion of *S. aureus* by PMNs. Values represent the percents of radio-labeled *S. aureus* ingested by PMNs with various treatments.

<table>
<thead>
<tr>
<th>Pretreatment (followed by washing)</th>
<th>Bacterial fraction added to reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>29.8 ± 3.3a</td>
</tr>
<tr>
<td><em>S. aureus</em> preincubated with</td>
<td>27.1 ± 2.1</td>
</tr>
<tr>
<td>SEF-RH-R</td>
<td>17.6 ± 2.4</td>
</tr>
<tr>
<td>PMNs preincubated with</td>
<td></td>
</tr>
<tr>
<td>SEF-RH-R</td>
<td>31.8 ± 4.4</td>
</tr>
<tr>
<td>Both <em>S. aureus</em> and PMNs</td>
<td></td>
</tr>
<tr>
<td>preincubated with SEF-RH-R</td>
<td>29.8 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>23.4 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± standard error (n = 6).
Fig. 4. Effect of type A *P. multocida* fractions on NBT reduction by bovine PMNs stimulated with opsonized zymosan. Values represent mean (+ standard deviation) (*n* = 6) percentages of the control value. None of the differences are statistically significant (*p > 0.05*).
Fig. 5. Effect of type A P. multocida fractions on iodination by bovine PMNs stimulated with opsonized zymosan. Values represent mean percentages (± standard deviation) of the control value. Statistically significant differences from the control values are as indicated. **, p < 0.01. *, 0.01 < p < 0.05. n = 16 for SEF and n = 6 for all other fractions.
treatment with hyaluronidase destroyed the inhibitory activity of SEF on iodination by PMNs (Fig. 6). SEF-R inhibited iodination by PMNs by 35 to 49% and SEF-F did not. In a separate experiment to further characterize SEF-R of strain P-2383, the value for iodination by control PMNs was 37.8 ± 1.6 (mean ± SEM) (n = 8) nmoles NaI per 10^7 PMNs per hour. SEF-RH-R inhibited iodination by 70% and SEF-RH-F had no effect (Fig. 7). Iodination by PMNs was not inhibited by either hyaluronic acid or hyaluronidase.

**Titration of the inhibitory activity of the bacterial fraction**

P-2383 SEF was used to evaluate the concentration effect of the bacterial fraction on *S. aureus* ingestion and iodination by PMNs. The inhibitory activities were concentration dependent, but neither was completely inhibited even at the highest concentration of the bacterial fraction (Fig. 8).

**Chemical analysis**

The results of chemical analysis of the bacterial fractions are shown in Table 2.

**Effect of the saline extracted capsule on xanthine-xanthine oxidase-horseradish peroxidase mediated iodination**

To further study the effect of SEF on the iodination reaction a chemically mediated iodination reaction was used which did not involve PMNs. Xanthine and xanthine oxidase were used to generate superoxide
Fig. 6. Characterization of the inhibitory activity of the saline extract fraction on iodination by bovine PMNs. Values represent mean percentages (± SD) (n = 6) of the control value. Statistically significant differences from the control value are as indicated. **, p < 0.01. *, 0.01 < p < 0.05.
Fig. 7. Characterization of the inhibitory activity of the saline extract fraction of strain P-2383 on S. aureus ingestion and iodination by bovine PMNs. Values represent mean percentages (+ standard deviation) of the control value. Statistically significant differences from the control value are as indicated. **, *$, p < 0.01. *, 0.01 < p < 0.05. n = 8 for all fractions.
Fig. 8. The effect of various concentrations of P-2383 SEF on *S. aureus* ingestion and iodination by PMNs (n = 6).
Table 2. Chemical analysis of P-2383 bacterial fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration (mg dry weight/ml)</th>
<th>Carbohydrate (mg/ml)</th>
<th>Hyaluronic acid (mg/ml)</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEF</td>
<td>1.06</td>
<td>0.38</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>SEF-R</td>
<td>0.94</td>
<td>0.33</td>
<td>0.17</td>
<td>0.06</td>
</tr>
<tr>
<td>SEF-RH-R</td>
<td>1.40</td>
<td>0.35</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>SEF-RH-F</td>
<td>0.80</td>
<td>0.26</td>
<td>0.04</td>
<td>0.22</td>
</tr>
</tbody>
</table>
anion which spontaneously forms hydrogen peroxide. Horseradish peroxidase was used to catalyze the hydrogen peroxide-halide reaction. SEP did not inhibit the xanthine-xanthine oxidase-horseradish peroxidase iodination system (Table 3).

Discussion

Polymorphonuclear leukocytes are important in protecting animals from microbial infection by their phagocytic activities. According to Root and Cohen (22), two major microbicidal events occur inside the PMN when a microorganism is ingested: (1) the generation of highly toxic products of oxygen by the oxidative metabolism and (2) the enzymatic destruction and digestion of the microorganism by the lysosomal enzymes present in the intracellular granules which fuse with the phagocytic vacuoles. However, many pathogenic microorganisms have developed mechanisms to inhibit phagocytic cell activity to facilitate their survival in the host (9).

The ingestion of bacteria is the first step in the bactericidal activity of the PMN. The results of the S. aureus ingestion assay indicate that the capsule of type A P. multocida inhibits the ability of PMNs to ingest particles. When bacterial fractions containing whole organisms were used, the inhibition of ingestion of S. aureus may have been due to competition between the two bacterial species. However, when soluble bacterial fractions were used, competition for
Table 3. The effect of the saline extracted fraction (SEF) of type A *P. multocida* on xanthine-xanthine oxidase-horseradish peroxidase mediated iodination

<table>
<thead>
<tr>
<th>Bacterial fractions</th>
<th>Iodination value (cpm)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (no xanthine oxidase)</td>
<td>245 ± 18</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>1466 ± 129</td>
</tr>
<tr>
<td>SEF of strain P-2383</td>
<td>1453 ± 78</td>
</tr>
<tr>
<td>SEF of strain P-1062</td>
<td>1502 ± 89</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SEM of eight experiments. The differences from the control value are not statistically significant (p > 0.05).
ingestion should not have been a factor. In addition, the results in Table 1 indicate that the inhibitory activity was not retained after washing of pretreated PMNs or bacteria. Apparently, this substance has an effect on PMN metabolism which is reversible and is not mediated through binding to a high affinity receptor. The mechanism of action of this material is not known and is the subject of further investigation. Maheswaran and Thies (18) reported that removal of the capsule from type A P. multocida (the same capsular type used in this experimentation) with hyaluronidase enabled PMNs to ingest the decapsulated organisms. They speculated that the inhibitory substance was hyaluronic acid. The results reported here, however, indicate that hyaluronic acid per se is not responsible for the inhibition of PMN function. Hyaluronic acid isolated from human umbilical cord did not suppress PMN function. Thus, the repeating disaccharide (D-glucuronic acid and N-acetyl-D-glucosamine) backbone structure of hyaluronic acid, which would be common between the hyaluronic acids of human and bacterial origin was not responsible for the inhibition of PMN function. It is possible that the PMN inhibitory substance is structurally linked to the bacterial hyaluronic acid. The treatment of the capsular material with hyaluronidase did not destroy the inhibitory factor. The hyaluronidase treatment did destroy the mucoid viscous nature of the SEF. Even after hyaluronidase treatment there was still some glucuronic acid (a component of hyaluronic acid) which did not pass through the 300,000 MW cutoff membrane. Since hyaluronic acid of the P. multocida capsule serves as a framework for other
molecules (3), there may be a variety of chemical components attached to hyaluronic acid which would not be degraded by the hyaluronidase. These molecules may be large enough to retain associated glucuronic acid from passing through the 300,000 MW cutoff membrane. The hyaluronic acid fragments which did pass through the membrane (SEF-RH-F) did not inhibit PMN function.

Oxidative metabolism of the PMN is an important aspect of its bactericidal activity (22). When a PMN receives the proper stimulus, an oxidase enzyme on the surface of the plasma membrane or phagosomal membrane will catalyze the conversion of oxygen to superoxide anion. Superoxide anion spontaneously dismutates to hydrogen peroxide. NBT is directly reduced by the superoxide anion to an insoluble purple formazan (26). NBT reduction is therefore a measure of superoxide anion generation by the PMN. Since NBT reduction was not inhibited by whole bacteria or bacterial fractions (Fig. 4), type A _multocida_ apparently does not inhibit the production of superoxide anion by the PMN.

The iodination reaction is a measure of the ability of the PMN to convert inorganic iodide to a TCA-precipitable (protein-bound) form and occurs inside the phagocytic vacuole via the action of hydrogen peroxide and myeloperoxidase. This system has been found to exhibit a marked toxic activity toward bacteria, fungi and viruses (1,24). The iodination reaction by PMNs is dependent upon the generation of hydrogen peroxide, degranulation to release myeloperoxidase, the presence of iodine, the unimpaired ability of myeloperoxidase to
catalyze the reaction, and the presence of tyrosine to bind iodine. Hydrogen peroxide is formed spontaneously from superoxide anion. Since SEF did not inhibit NBT reduction, superoxide anion generation by PMNs is apparently not affected by the inhibitory factor. Since the xanthine-xanthine oxidase-horseradish peroxidase mediated iodination was not inhibited by SEF, the rate of formation of hydrogen peroxide from superoxide anion and the rate of hydrogen peroxide destruction were apparently not affected. In addition, the ability of the peroxidase enzyme to catalyze the reaction was apparently not impaired. It must be kept in mind that horseradish peroxidase and myeloperoxidase are different enzymes. It is possible that the inhibitory factor could inhibit myeloperoxidase directly without inhibiting horseradish peroxidase, but this does not seem likely.

This experimentation demonstrated that phagocytosis and protein iodination by PMNs were inhibited in the presence of whole *P. multocida* organisms and bacterial fractions. Since the removal of the capsule removed the inhibitory capability, the inhibitory activity apparently resides in the capsule or surface structure of the bacterial cell. The inhibitory factor is a heat-stable, saline (2.5%, wt/vol)-extractable capsular material of greater than 300,000 molecular weight. The inhibitory activity can not be attributed to hyaluronic acid, but it may be structurally associated with it.
Literature Cited


SECTION II.

IMMUNOGENICITY OF POTASSIUM THIOCYANATE EXTRACT OF TYPE A PASTEURELLA MULTOCIDA

Summary

Immunogenicity and cross-protectivity of potassium thiocyanate (KSCN) extracts from type A Pasteurella multocida (P. multocida) strains were evaluated in mice. Mice immunized with KSCN extracts from several P. multocida strains showed signs of depression for several hours after immunization but protected against challenge with the virulent homologous bacterium. Some of the mice died and many of the surviving mice were clinically ill up to 72 h after challenge exposure, but gradually recovered. However, there was no consistent reciprocal protection between different strains of a serotype indicating no correlation between serotype and cross-protection of mice. Antigenic analysis of KSCN extracts of type A P. multocida by crossed-immunoelectrophoresis techniques revealed at least 25 different antigenic components. When the antigenic contents of P-2383 and P-1062 KSCN extracts were compared, most antigenic components were common to both. Two antigenic components appeared to contain a
strain-specific antigenic determinant which could be identified only with the homologous system. One component present in each of the extracts gave a precipitation line similar to that associated with a crude lipopolysaccharide extracted from the organism. This component was isolated by sucrose density gradient centrifugation. The component isolated from P-2383 KSCN extract sedimented rapidly indicating a high molecular weight material which contained 12% carbohydrate and 27% protein. Immunization of mice with this component demonstrated resistance to challenge with the homologous strain and some degree of protection against certain heterologous strains.

Introduction

Capsular type A strains of Pasteurella multocida are known to be a major etiologic factor in important animal diseases such as fowl cholera in chickens and turkeys, and pneumonic pasteurellosis in cattle (11, 14).

The importance of the organism has led to the development and use of various bacterial preparations, either commercial or experimental, for immunization. Such bacterial preparations include killed bacterins (5, 6, 10, 18, 20, 24, 29), live attenuated or mutated vaccinal strains (7, 12, 13, 23, 25, 39) and isolated cellular fractions (2, 3, 4, 9, 16, 17, 22, 26, 27, 28, 31, 32, 33, 34, 35, 36, 37, 38).
Experimental findings indicated that animals immunized with many of these preparations were protected against challenge infection with the virulent homologous strain, and in some cases with strains of different serotypes. However, identification of the protective factor(s) resident in the various preparations was not determined and limited experimentation has been conducted on the immunogenic relationship between different strains of the same serotype. Due to the lack of scientific evidence on the nature of protection against *P. multocida* infection, the efficacy and safety of these preparations still remains in question.

Recently, potassium thiocyanate (KSCN) extracts of type A *P. multocida* have been reported to demonstrate immunogenicity in experimental animals not only against the homologous strain (3, 27, 28), but also against strains of different serotypes (17) and even against a different species, *P. hemolytica* (26). The objectives of this experimentation were to evaluate the immunogenicity and cross-reactivity of KSCN extracts from several different type A *P. multocida* strains in mice, and to identify the protective component(s) of the extracts on the basis of antigenic analysis.
Materials and Methods

Organisms

Six strains of *P. multocida* were used in this study: P-1062, P-2383, P-92481, P-03894, P-8103889 and P-813310. Strain P-1062, a capsular type A and somatic type 3, is a standard challenge strain of bovine origin (IRP-198, National Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, IA). The other five strains were isolated from cases of bovine pneumonia presented to the Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA. With one exception, these isolates were related on the basis of serotyping as summarized in Table 1. Each strain was inoculated into the yolk sac of 6-day old embryonated chicken eggs. Following incubation at 37 C for 18 h, the yolk material was aseptically removed and frozen in aliquots at -70 C. For the preparation of bacterial fractions, the infected yolk material was thawed, inoculated on a 5% bovine blood agar plate and incubated at 37 C for 24 h. One colony was transferred to 5 ml of brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) with 0.5% yeast extract (Difco) and 5% sterile bovine serum (BHISY), and incubated at 37 C for 4 h. This culture was then added to 95 ml of BHISY and incubated an additional 4 h. When the bacterium were grown in large volumes (5 liters) of liquid medium, 100 ml of BHISY culture were added to the medium and incubated an additional 18 h with a 5% CO₂ aeration. When organisms were cultivated on solid media, Roux bottles containing dextrose starch
agar (Difco) were inoculated with 2 ml of the BHISY culture and incubated at 37 C for 36 h.

Bacterial fractions

Bacterial fractions of *P. multocida* were prepared by several procedures including KSCN extract, whole cell, ribosomal protein and lipopolysaccharide.

KSCN extracts from all 6 strains were prepared by the method of Mukkur (27) with slight modifications. Briefly, *P. multocida* organisms grown at 37 C for 36 h in Roux bottles were harvested by suspending the bacteria in 0.5 M KSCN (Fisher Scientific, Fairlawn, NJ) containing 0.08 M NaCl. The bacterial suspension was incubated in a shaking water bath at 37 C for 5 h and centrifuged at 17,300 x g for 30 min. The supernatant fluid was filtered through a 0.9 um membrane filter (Gelman Instrument Co., Ann Arbor, MI). The filtrate was dialyzed against 0.32 M NaCl/0.01 M tris-HCl buffer (Sigma Chemical Co., St. Louis, MO) at 4 C for 5 days with the buffer changed twice a day. To reduce the viscosity of the preparation, bovine testicular hyaluronidase (Sigma, 1 mg of hyaluronidase per 10 mg dry weight of KSCN extract) was added and the mixture was incubated in a water bath at 37 C for 3 h.

Whole cell fraction was prepared from two strains (P-2383 and P-1062). Each strain was grown in BHISY at 37 C for 18 h and harvested by centrifugation at 8,000 x g for 30 min. Cells were washed with sterile phosphate-buffered saline (PBS) solution, pH 7.4, and
### Table 1. Serotypes of several bovine isolates of *P. multocida*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Somatic : Capsular</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1062</td>
<td>3 : A</td>
</tr>
<tr>
<td>P-2383</td>
<td>3 : A</td>
</tr>
<tr>
<td>P-813310</td>
<td>3 : A</td>
</tr>
<tr>
<td>P-8103889</td>
<td>3, 4 : A</td>
</tr>
<tr>
<td>P-92481</td>
<td>3, 4 : Untypable</td>
</tr>
<tr>
<td>P-03894</td>
<td>12 : Untypable</td>
</tr>
</tbody>
</table>
resuspended in 0.01 M Tris-HCl (Sigma) buffer containing $5 \times 10^{-3}$ M MgCl$_2$. Cells were disrupted in a Ribi cell fractionator (Ivan Sorvall, Inc., Newton, CT) and this preparation was used as the whole cell fraction (WC).

Ribosomal protein fraction was prepared from two strains (P-1062 and P-2383) by the method of Baba (2). Briefly, cellular debris were removed from the WC fraction by centrifugation at 27,000 x g for 1 h at 5 C. The supernatant fluid was centrifuged again at 65,000 x g for 1 h at 5 C. The pellet was discarded and the supernatant was passed through a 0.45 um membrane filter (Falcon Plastics, Los Angeles, CA). The filtrate was centrifuged at 108,000 x g for 3 h at 5 C, and the supernatant was termed the ribosomal protein fraction (RP).

Phenol-extracted endotoxin was prepared from two strains (P-2383 and P-1062) by a modification of the method of Westphal and Jann (38). Cells were grown in BHISY at 37 C for 18 h and harvested by centrifugation at 8,000 x g for 30 min. The cells were washed 3 times in sterile PBS and resuspended in sterile distilled water. The cells were mixed for 8 min in a Waring blender with 2 volumes of phenol at 25 C and centrifuged at 4,000 x g for 20 min. The aqueous phase was removed and the remainder re-extracted with a volume of distilled water equal to the volume of the original cell suspension. The aqueous phases were combined and dialyzed exhaustively against distilled water at 5 C.
Determination of lethal dose

Before the challenge experiments, the lethal dose (LD₅₀) of \textit{P. multocida} strains for mice was determined. Bacterial suspensions were adjusted spectrophotometrically and appropriately diluted (10 to \(10^4\) bacteria per 0.1 ml, as determined by direct plate counting). The LD₅₀ of P-1062, P-2383 and P-8103889 for mice was less than 10 bacteria while the LD₅₀ of P-813310 and P-92481 was less than \(10^0\) bacteria. However, the latter two strains did not kill all the mice at a dose of \(10^4\) bacteria. Strain P-03894 was not pathogenic for mice.

Mouse immunization and challenge

White Swiss mice (Sasco Inc., Omaha, NE) of one sex were utilized for immunization and challenge experimentation. At the time of immunization, the mice weighed 12 to 15 grams. All mice were immunized subcutaneously with 0.1 mg (protein concentration) of antigen and challenged intraperitoneally 3 weeks later with 0.1 ml of bacterial suspension. Mice were observed for one week after challenge and deaths recorded.

Production of antisera

Bacterial fractions (WC, RP and KSCN extract) of strains P-1062 and P-2383 were used for immunization of rabbits. Twenty-two New Zealand white female rabbits were randomly divided into two groups of 11 rabbits. Each group was further divided into three subgroups; 3,
5, and 3 rabbits for immunization with WC and RP fractions and KSCN extracts of the two strains respectively. Each bacterial fraction (5 mg of protein content per ml) was mixed with an equal volume of Freund's complete adjuvant for primary immunization, or with an equal volume of Freund's incomplete adjuvant for the subsequent administrations. Rabbits were immunized with one ml of the primary immunogen preparation subcutaneously in 6 different sites on the back. Animals received one ml of the booster immunogen preparation at monthly intervals. Sera were collected after the third booster injection, divided in aliquots and stored at -70 C.

**Immunoelectrophoresis**

Crossed immunoelectrophoresis (CIE), CIE with intermediate gel, tandem CIE and fused rocket immunoelectrophoresis were performed in an LKB multiphor system (LKB-Produkter AB, Bromma, Sweden) according to published procedures (1). Briefly, agarose prepared from commercial tablets (Bio-Rad Laboratories, Richmond, CA., 1%: 1 tablet per 5 ml of water) dissolved by heating in deionized water was used in both first and second dimensional electrophoresis. CIE in the first dimension was performed on a 10 cm x 10 cm glass plate. The plate was covered with 2 mm thick agarose gel. After gelling and cooling at room temperature, 4 antigen wells were punched out 2 cm apart in a line 3.5 cm from the cathodal end. For tandem CIE, a second well was punched out about 5 mm to the right and 5 mm below the first well. Antigens were applied to each well and electrophoresis was performed at 10 C
for 1.5 h at 200 volts in a water cooled electrophoresis chamber filled with tricine buffer (Bio-Rad, pH. 8.6). CIE and tandem CIE in the second dimension were performed on a 12.5 cm x 26 cm plastic plate (LKB). The actual space to be covered with agarose gel was limited to 10 cm x 24 cm by the use of brass bars. Agarose gel containing antiserum was prepared just before the end of the first dimensional electrophoresis. Four agarose tablets were dissolved in 18 ml of deionized distilled water by heating. The agarose gel was cooled to 48 C in a water bath while 2 ml of antiserum was warmed to the same temperature. Antiserum was added to the agarose gel and the mixture was used immediately. After first dimensional electrophoresis, four agarose strips (6 cm x 1.5 cm) containing antigen were cut and transferred to a plastic plate side by side so that a 1.5 cm x 24 cm agarose strip was made in a line 3.5 cm from the cathodal end. The remaining space on the plate was then covered with 2 mm thick agarose containing antisera at the anodal end and with plain agarose at the cathodal end. For CIE with intermediate gel, the antibody containing gel was divided into two parts. An intermediate gel containing one antiserum covered a width of 1 cm toward the anodal end and next to the antigen-containing agarose strip. The remaining space toward the anodal end was covered with agarose gel containing another antiserum. Electrophoresis in the second dimension was performed for 20 h at 60 volts. For fused rocket immunoelectrophoresis, a 10 cm x 10 cm glass plate was separated into two portions by use of a brass bar placed 5 cm from the cathodal end. The cathodal portion was cast with plain
agarose. After gelling, antigen wells were punched out evenly about 5 mm apart at alternate positions in two parallel lines across the plate. Antigen preparations (10 ul) were added to the wells and the plate were incubated for 1 h at room temperature in a humidified chamber. After incubation, the gel was trimmed so that the anodal end of the gel was about 0.5 cm from the wells. The remainder of the plate was covered with agarose gel containing antiserum and electrophoresis was performed for 20 h at 60 volts. After completion of the electrophoresis, the plate was washed in a PBS solution containing EDTA (Sigma) for 2 days, dried in an oven at 50 C, stained with 0.2% amido black 10B (Corning Medical and Scientific, Palo Alto, CA) for 5 min, washed with 5% acetic acid solution and dried in an oven.

Sucrose density gradient centrifugation

Thirteen ml of a sucrose gradient consisting of 5 to 40% (wt/vol) sucrose in PBS solution was prepared in a 20 ml cellulose nitrate tube (Beckman Instruments Inc., Palo Alto, CA). Five ml of KSCN extract treated with hyaluronidase were applied on top of the gradient and centrifuged at 87,000 x g for 18 h. After centrifugation, fractions were collected from the top to the bottom of the tube. The first fraction was collected in a volume of 5 ml and the remainder was collected in a series of 1 ml volumes. Each fraction was analyzed by fused rocket immunoelectrophoresis, dialyzed against tris-HCl buffer and stored at -20 C.
Chemical analysis

Bacterial fractions were assayed for basic chemical contents. Total carbohydrate was determined by the phenol-sulfuric acid procedure (15), using glucose (Sigma) as a standard. Protein content was determined colorimetrically from the reaction of protein with Serva blue G dye (Serva Fine Chemicals, Inc., Long Island, N. Y.), using bovine serum albumin (Sigma) as a standard (30). 2-keto-3-deoxyoctonate (KDO) content, a component of lipopolysaccharide-containing materials of Gram-negative bacteria, was determined colorimetrically from the reaction of KDO with sulfuric acid, periodic acid, sodium arsenite and thiobarbituric acid, using a commercial KDO preparation (Sigma) as a standard (21).

Results

Immunogenicity and cross-protectivity of KSCN extracts

To evaluate the immunogenic potential of KSCN extracts of P. multocida, mice were immunized with KSCN extracts of one non-virulent and five virulent P. multocida strains and challenged with the virulent strains. Mice immunized with the KSCN extract from virulent strains showed signs of depression for several hours after immunization but protected against challenge with the homologous strain (Table 2). Control mice as well as some immunized mice died during the period between 12 and 72 h after challenge exposure. Also,
Table 2. Immunogenicity in mice of KSCN extracts from several strains of *P. multocida*

<table>
<thead>
<tr>
<th>Challenge exposure</th>
<th>P-2383</th>
<th>P-1062</th>
<th>P-8103889</th>
<th>P-813310</th>
<th>P-92481</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.f.u.</td>
<td>160</td>
<td>130</td>
<td>230</td>
<td>120</td>
<td>150</td>
</tr>
</tbody>
</table>

Immunization with KSCN Extract of

<table>
<thead>
<tr>
<th></th>
<th>P-2383</th>
<th>P-1062</th>
<th>P-8103889</th>
<th>P-813310</th>
<th>P-92481</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-2383</td>
<td>9/10</td>
<td>9/10</td>
<td>0/10</td>
<td>4/10</td>
<td>10/10</td>
<td>0/9</td>
</tr>
<tr>
<td>P-1062</td>
<td>1/10</td>
<td>9/10</td>
<td>0/10</td>
<td>1/10</td>
<td>10/10</td>
<td>0/9</td>
</tr>
<tr>
<td>P-8103889</td>
<td>2/10</td>
<td>7/10</td>
<td>7/10</td>
<td>5/10</td>
<td>10/11</td>
<td>0/9</td>
</tr>
<tr>
<td>P-813310</td>
<td>1/9</td>
<td>2/10</td>
<td>0/10</td>
<td>9/10</td>
<td>10/10</td>
<td>0/9</td>
</tr>
<tr>
<td>P-92481</td>
<td>0/10</td>
<td>4/10</td>
<td>0/10</td>
<td>2/10</td>
<td>10/10</td>
<td>0/9</td>
</tr>
<tr>
<td>P-03894</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>8/10</td>
<td>8/10</td>
<td>0/9</td>
</tr>
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<td>Control</td>
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<td>0/9</td>
<td>0/9</td>
<td>3/10</td>
<td>5/10</td>
<td></td>
</tr>
</tbody>
</table>
many of the surviving mice were clinically ill up to 72 h after the challenge exposure, but gradually recovered. There was no consistent reciprocal protection between different strains of a serotype indicating no correlation between serotype and cross-protection of mice. For example, mice immunized with the KSCN extract of P-2383 were protected against the heterologous challenge exposure to P-1062 and P-92481. However, mice immunized with the KSCN extracts of P-1062 and P-92481 were not protected against the challenge with P-2383.

**Selection of rabbit antisera and antigenic analysis of the KSCN extract**

To analyze the antigenic content of KSCN extracts, rabbit anti-\textit{P. multocida} sera were prepared. In general, rabbits immunized with RP fraction produced better antisera for use in CIE than those immunized with WC fraction or KSCN extract (Fig. 1A, 1B, 1C). Since antisera were variable in quality, those sera which showed the most abundant and clearest precipitation lines on the CIE plates were selected. Although the antigenic content of the KSCN extracts was analyzed by immunoelectrophoretic procedure utilizing different combinations of antigens and antisera, two antiserum pools (one for 2383 and one for 1062) were prepared by mixing equal volumes of selected homologous antisera. To compare antigenic similarities and differences between KSCN extracts of P-2383 and P-1062, antigenic content of the KSCN extracts were analyzed by CIE, CIE with intermediate gel and tandem-CIE using homologous and heterologous antisera. Because the
Fig. 1. Crossed immunoelectrophoresis of P-2383 KSCN extract with selected rabbit P-2383 antisera. A: KSCN antiserum. B: RP antiserum. C: WC antiserum.
concentration of antigen and antibody utilized for immunoelectrophoresis influenced results, the procedure was standardized to 5 μl of antigen and a 1:10 concentration of the antisera. Optimal condition as indicated by the greatest number of precipitation lines in CIE was determined by varying the antigen concentration. Cross-reacting components were determined and numbered by the principles of electrophoresis (1) such as distance moved in CIE, line of identity in tandem-CIE, and presence or absence in CIE with intermediate gel. This approach demonstrated that at least 25 different antigenic components could be demonstrated in P-2383 KSCN extract with homologous antiserum (Fig. 2A), 23 components from P-2383 KSCN extract reacted with 1062 antiserum (Fig. 2B), 24 components from P-1062 KSCN extract with 1062 antiserum (Fig. 2C) and 21 components from P-1062 KSCN extract with 2383 antiserum (Fig. 2D). Most antigenic components were found to be common to both extracts. Some components, 8, 10 and 20, were not identified with the pooled antiserum pools as illustrated; these components were clearly identified with the selected individual antiserum. Two components, 11 and 12, appeared at the same positions in CIE in similar patterns in both extracts when homologous antisera were used, but were not detected with heterologous antisera. This indicated that these components of the two strains may be similar in their chemical structure but have a strain-specific antigenic determinant. It has been suggested that lipopolysaccharide-containing antigens would be effective immunogens against *P. multocida* infection (4, 16, 31, 32) as
Fig. 2. Crossed immunoelectrophoresis of the KSCN extracts of strain P-2383 and P-1062. A: P-2383 KSCN extract with 2383 antiserum pool. B: P-2383 KSCN extract with 1062 antiserum pool. C: P-1062 KSCN extract with 2383 antiserum pool. D: P-1062 KSCN extract with 1062 antiserum pool.
well as for serotyping organisms (8). *P. multocida* lipopolysaccharide was prepared and examined by CIE to verify the existence of a corresponding precipitation line(s) in the KSCN extracts. Component 1, present in both KSCN extracts, was appeared to be the corresponding precipitation line with the lipopolysaccharide. This component was purified for further charaterization.

Isolation of component 1 by the sucrose density gradient centrifugation

To purify component 1, the KSCN extract was centrifuged in a sucrose density gradient. Fused-rocket immunoelectrophoretic analysis of fractions collected following centrifugation revealed a distribution of components from top to bottom of the gradient (Fig. 3A). Most of the antigenic components of the P-2383 KSCN extract remained in the region of low sucrose concentration (less than 20%). A single component was observed in the bottom half of the gradient as indicated by CIE of pooled fractions 12 to 16 (Fig. 3B). The CIE analysis of this component was found to be component 1 of P-2383 KSCN extract (Fig. 1A). The bottom region of P-1062 KSCN extract in sucrose density gradient contained primarily one component with minor contamination (Fig. 3C). The isolated component of each KSCN extract will be referred to as P-2383-1 and P-1062-1, respectively.
Fig. 3. Immunoelectrophoresis of fractions by sucrose density gradient centrifugation. A: Fused-rocket immunoelectrophoresis of fractions of P-2383 KSCN extract and agarose gel contained 2383 antiserum pool. B: CIE of component 1 of P-2383 KSCN extract and agarose gel contained 2383 antiserum pool. C: CIE of component 1 of P-1062 KSCN extract and agarose gel contained 1062 antiserum pool.
Table 3. Immunogenicity of isolated components from the KSCN extracts in mice

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Experiment</th>
<th>P-2383</th>
<th>P-1062</th>
<th>P-8103889</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.f.u. (x 100)</td>
<td>11 13 1.6 10 11 1.3 2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Immunization with

|  | P-2383-1 | P-1062-1 | Control |
|  | 5/5 10/10 10/10 5/5 8/10 10/10 4/10 |
|  | 1/5 2/5 - 2/5 2/5 - - |
|  | 0/5 0/7 0/10 0/5 0/6 0/10 0/10 |
Immunogenicity of the isolated components

The immunogenicity of the isolated components in mice was examined in three separate experiments. Immunization of mice with P-2383-1 protected all of the mice against a homologous challenge with P-2383 that killed 100% of the control mice (Table 3). The mice immunized with P-2383-1 showed no clinical signs of sickness after immunization or challenge while the mice immunized with the KSCN extract did. Immunization of mice with component 1 also protected mice against heterologous challenge with P-1062 (92%) and to some extent against P-8103889 (46%). Immunization of mice with P-1062-1 protected only 40% of the mice against a homologous challenge and only 30% of the mice against a heterologous challenge with P-2383.

Chemical analysis of the isolated component

P-2383-1 contained approximately 12% (wt/wt) carbohydrate and 27% (wt/wt) protein while P-1062-1 contained 14% and 12% respectively. KDO was not detected in the two isolated components at the level of 5 mg dry weight per ml of the starting material.

Discussion

KSCN extracts of P. multocida have previously been reported to be effective immunogens against Pasteurella infection induced by homologous and heterologous strains (3, 17, 26, 27, 28). In
confirmation of previous findings, the results of mouse protection tests indicated that KSCN extracts were immunogenic. However, the results also indicated that KSCN extracts did not induce substantial cross-protection even between different strains of the same serotype. While there may be some correlation within a serotype of \textit{P. multocida} such as antigenic structure, host susceptibility and strain virulence (14), it does not necessarily mean that the serotyping antigens, capsular or somatic, are the same as the protective antigen(s). As indicated by the antigenic comparison of the two KSCN extracts by CIE techniques, most of the antigenic components were common to both extracts. The lack of cross-protection between different strains of a serotype is probably due to; (1) absence of an important common protective antigenic component(s), or (2) variation in some of the antigenic determinants associated with the protective antigenic component(s). Obviously, although the strains shared many common antigenic components, only one or a few of these are associated with protection in mice against the disease. Therefore, identification of the protective factor(s) resident in various preparations by antigenic analysis and chemical characterization would permit development of immunogens capable of protection against a variety of strains.

As indicated by clinical observation of mice following immunization and challenge exposure, the isolated component appeared to be a better immunogen than the crude extract. This may be a dose effect since the quantity of component 1 could not be related to the crude extract. This finding could also be an effect of purity of
immunizing preparation. In general, an immunogen containing a small number of antigenic components will induce a better immunogenic response than a highly complex antigenic mixture. This happening can be the result of antigenic competition or the presence of immunosuppressive factor(s) in the complex antigenic mixture. All of the antigenic components can produce antibodies in animals, however, only a few of them may induce protection against the microbial infection. It is possible that some of the antigenic components may have a detrimental effect on immunologic function rather than a protective effect. Evidence found at section I of this thesis indicated that a surface material of *P. multocida* inhibited antibactericidal functions of bovine neutrophil.

P-2383-1 demonstrated immunogenicity as indicated by complete or partial resistance of mice to challenge with various strains. Failure of complete protection against heterologous strains would indicate some variation in the protective antigen of this microorganism. This finding might also be explained by the marked virulence of these strains for mice. However, this variation was not evident from the challenge experiment in which the ID$_{50}$ of the organisms was determined. Chemical analysis of the two components demonstrated that P-2383-1 contained more protein than P-1062-1 and may be the reason P-2383-1 is a better immunogen than P-1062-1. This may reflect loss or alteration of crucial antigenic determinants of P-1062. This organism has been maintained on artificial medium in the laboratory for a long period of time and antigenic alteration would be expected.
The chemical nature of P-2383-1 was similar to the immunogenic complex described by Ganfield et al. (16). Their material was purified from a saline extract and was a high-molecular weight protein-polysaccharide complex contained 25 to 27% protein and 10.7% carbohydrate. Thus, the chemical composition was nearly identical to the extract prepared for this experimentation. Further comparison is difficult since Ganfield et al. were able to determine the presence of lipid but they did not determine presence or absence of KDO. KDO was not detected in P-2383-1 which might indicate that the fraction does not contain lipopolysaccharide. However, although KDO is a well-known component of lipopolysaccharide of Gram-negative organisms (21), it is not universally present in all lipopolysaccharide preparations. The observation that P-2383-1 is chemically similar to Ganfield's preparation and both are immunogenic would indicate that they are probably similar material even though they were isolated by different methods from different strains of P. multocida.

This experimentation demonstrated that KSCN extracts of P. multocida were immunogenic but had no consistent reciprocal protection between different strains of a serotype. The lack of cross-protection indicated that the various strains differed qualitatively or quantitatively in protective antigenic determinants. Evidence from CIE studies of P-2383 and P-1062 KSCN extracts would indicate that there are qualitative difference in component 1 that contribute to lack of cross-protection. More studies are needed to further characterize the protective antigenic component as well as the
protective antigenic determinant to verify the relationship between different strains of \textit{P. multocida}.

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GENERAL SUMMARY

The objective of this research project was to characterize the biological activities of fractions of type A Pasteurella multocida (P. multocida). By the use of a strain commonly utilized for production of bacterins (P-1062) and several clinical isolates (especially P-2383), the bacterial fractions were prepared. Examination of surface materials of type A P. multocida exhibited both inhibitory and protective properties.

Phagocytosis (ingestion of bacteria) and protein iodination (halogenation of ingested bacteria) by bovine polymorphonuclear leukocytes (PMNs) were inhibited in the presence of live cells, heat-killed whole cells, or saline-extracted capsule but not in the presence of the decapsulated heat-killed cells. None of bacterial fractions inhibited nitroblue tetrazolium reduction (oxidative metabolism) by the PMNs. PMN inhibitory factor was characterized as a heat-stable capsular material of greater than 300,000 molecular weight.

Potassium thiocyanate (KSCN) extracts of several P. multocida strains exhibited immunogenicity in mice against challenge with the virulent homologous bacterium. However, mice immunized with KSCN extracts were not completely protected against heterologous virulent strains of the same or similar serotypes indicating no correlation between serotypes and cross-protection of mice. Antigenic analysis of the KSCN extracts by crossed immunoelectrophoresis techniques revealed
at least 25 different antigenic components. A lipopolysaccharide-like component of the KSCN extracts was isolated by sucrose density gradient centrifugation. The component isolated from P-2383 extract sedimented rapidly indicating a high molecular weight material which contained 12% carbohydrate and 27% protein. Immunization of mice with this component demonstrated resistance to challenge with the homologous strain and some degree of protection against certain heterologous strains.
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