Biological activities of fractions

of type A Pasteurella multocida

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Hyoik Ryu

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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,

42, 71).

Recently, efforts have been made to find effective immunizing agents to prevent infection by P. 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), potasssium 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. multocida. Specific aims were: (1) to identify and characterize inhibitory factors for phagocytic cells present in the type A P. 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. multocida strains in mice and identify a protective component(s) in the KSCN extract on the basis of antigenic analysis.

LITERATURE REVIEW

Historical Background

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 staphyloccocal 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 varies 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 yieled 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 ug of the lipopolysaccharide.

Penn and Nagy (79) reported that a capsular antigen of types B and E strains of 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 centrifugaion 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 containig 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 $CsCl_2$

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 ug 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 1.6×10^6 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 1.5×10^3 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 immunosuppresion 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 andotoxin, 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-imunogenic. 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 immunogenicicty 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 x 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 x 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 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 P. multocida reported at present.

Prince and Smith (80, 81, 82) reported their series of antigenic studies on 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 P. multocida were identified. When they examined the cross-

reactivity P. multocida antigens with a variety of Gram-negative bacteria, 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 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 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 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 P.
hemolytica bacterin developed more severe pneumonia than the non-
vaccinated controls.

Markham and Wilkie (71) reported a detrimental effect of P.
hemolytica bacterin on bovine alveolar macrophages. They cultured the
macrophages in Leighton tubes and added ^{125}I-labeled P. hemolytica
with fetal calf serum or an immune serum that was obtained from calves
immunized with 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-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. 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. hemolytica bacterin may be detrimental to the macrophages of the host rather than protective. The detrimental components of P. hemolytica on the bovine macrophages is probably a cytotoxin, which is released to the medium by P. 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. multocida strain and a type 1 P. 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 respose 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 <u>PASTEURELLA</u> <u>MULTOCIDA</u> FRACTIONS ON BOVINE

POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS[1]

Summary

The effect of various <u>Pasteurella</u> <u>multocida</u> 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

<u>Staphylococcus</u> <u>aureus</u> 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 [^{3}H]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, 90% 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 (approximatly 1.0×10^{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

Agar-Grown *P. multocida* ──────────→ 10^{10} C.F.U./ ml PBS
(10X, 0.4 O.D. at 540 nm)   plate count

↓

Washed 3 times

↓

LCF ◄──────── Live Cells (packed)
        ↑
       PBS

↓

2.5% Saline,
56°C, 1Hr.

↓

Heat Killed Whole Cells ──── Dialysis in PBS ────►KCF

Centrifugation

↓

Pellet   Supernatant

Washed 3 Times   Dialysis in PBS
and Resuspended
in PBS   Saline Extract = SEF

↓

Decapsulated cells = DCF

Filter thru   Hyaluronidase
"Amicon" PM300 Filter   37°C, 3 Hr.
(Approx. 300K Cutoff)   Autoclaved
                                SEF-H   121°C, 15 Min.

                                                   SEF-A

Retentate = SEF-R   Filtrate = SEF-F

Hyaluronidase
37°C, 3Hr.

SEF-RH

Filter Thru
"Amicon" PM300 Filter
(approx. 300K Cutoff)
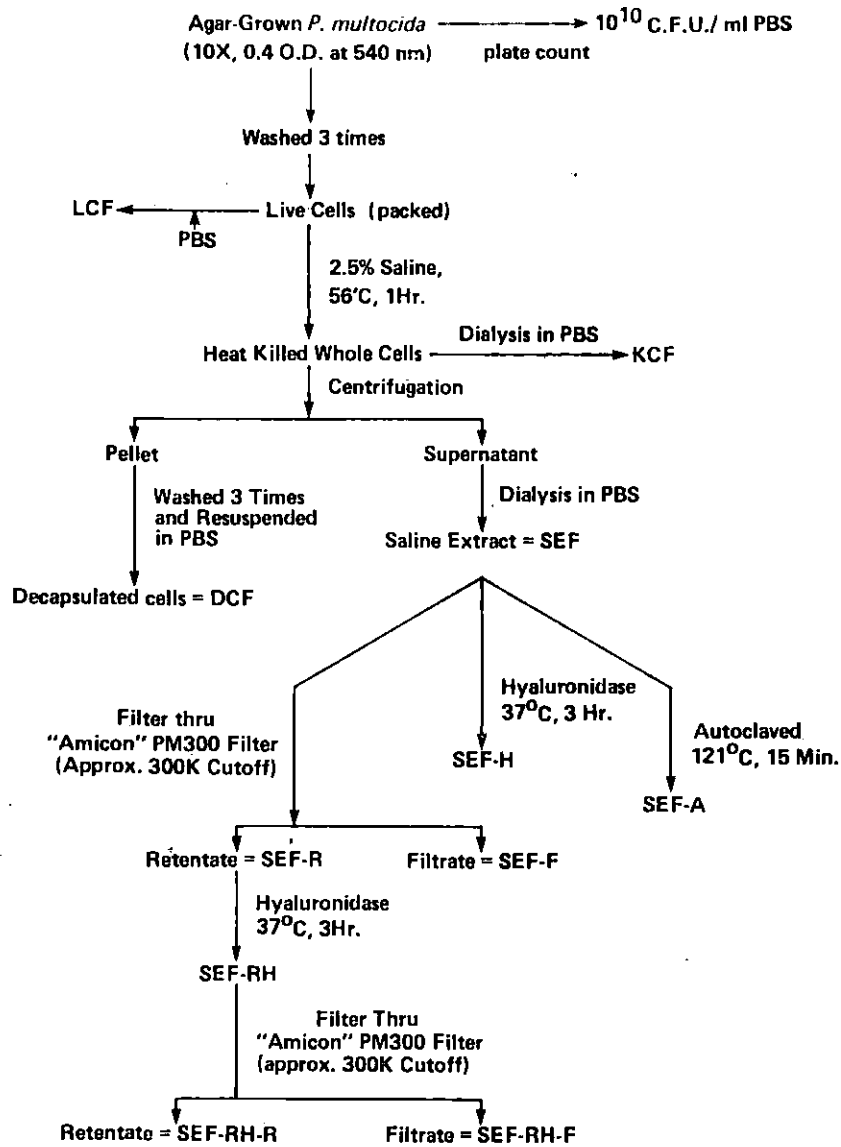
Retentate = SEF-RH-R   Filtrate = SEF-RH-F

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 5.0×10^7 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}I]UdR-labeled 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-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 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 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}I]UdR-labeled S. aureus that was ingested.

(2). 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 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 2.5×10^6 PMNs, 0.05 uCi of ^{125}I (Carrier free, Amersham Corp., Arlington Heights, IL), 40 nmole 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 (5.0×10^8 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 10^7 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 (10) 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 $\pm$ 2.3 (mean $\pm$ 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 300,000 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 $\pm$ 1.4 (mean $\pm$ 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

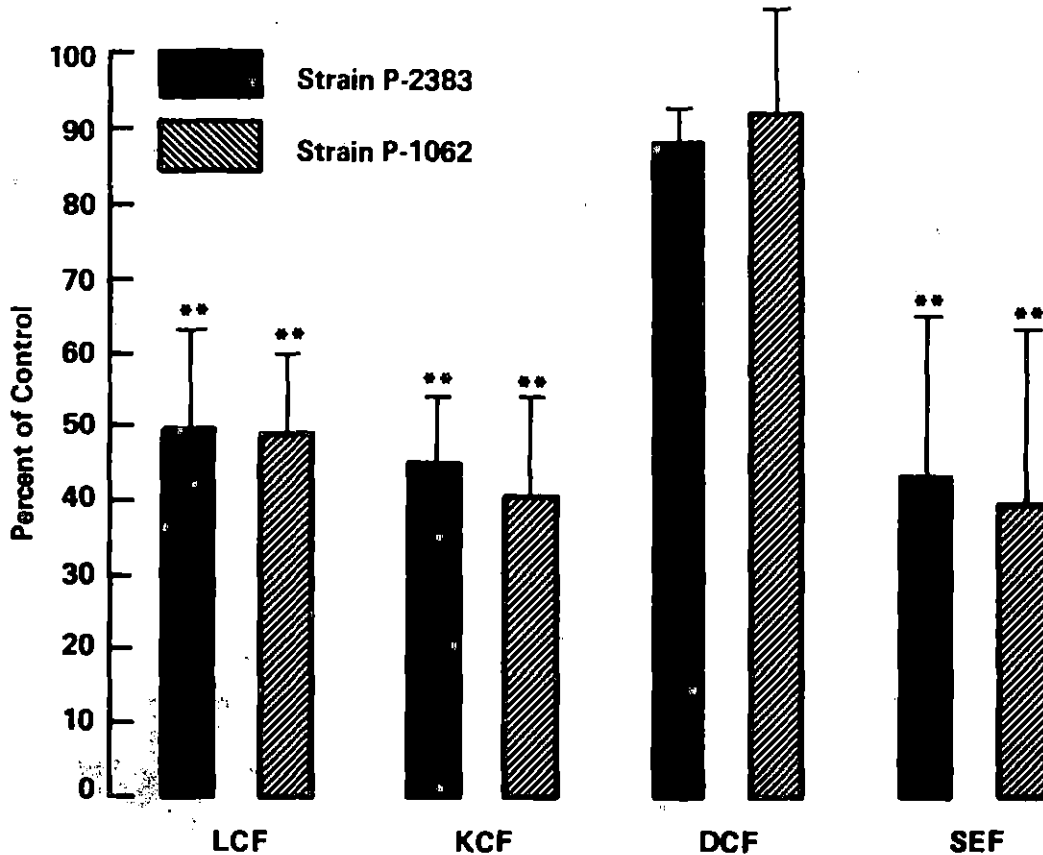and PMNs which had been separately treated with P-2383 SEF-RH-R by

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
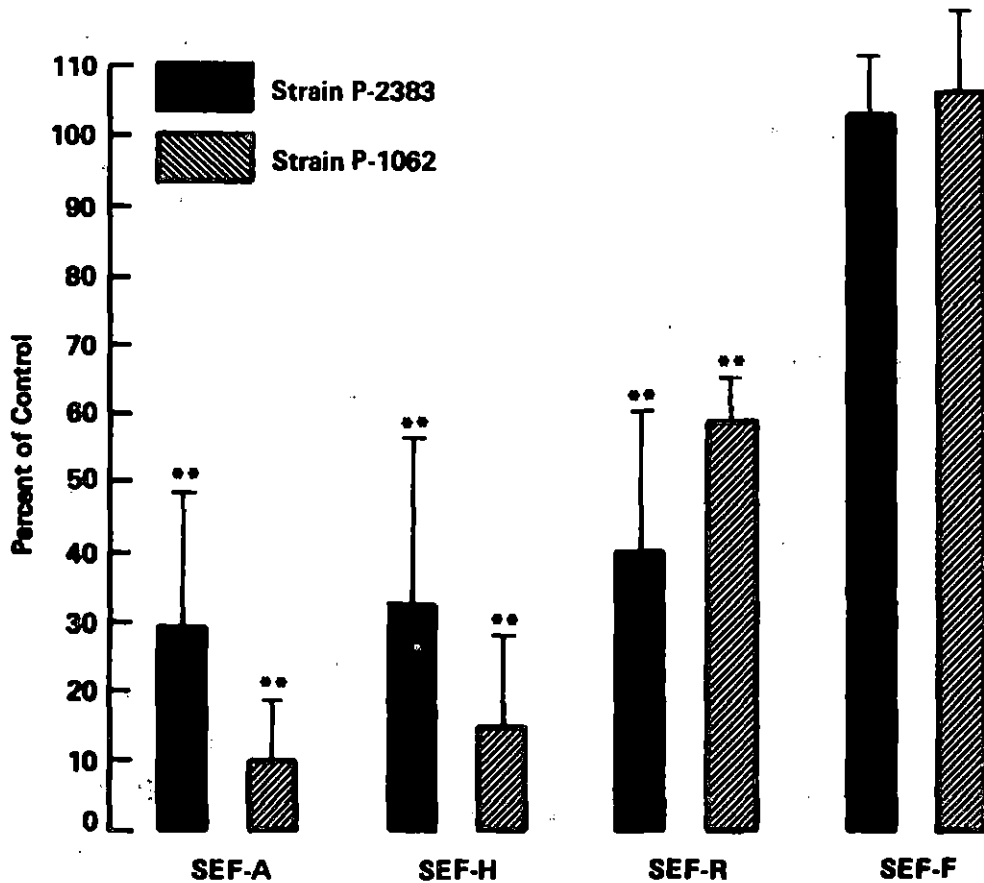
fraction (SEF) and n = 6 for all other fractions.

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

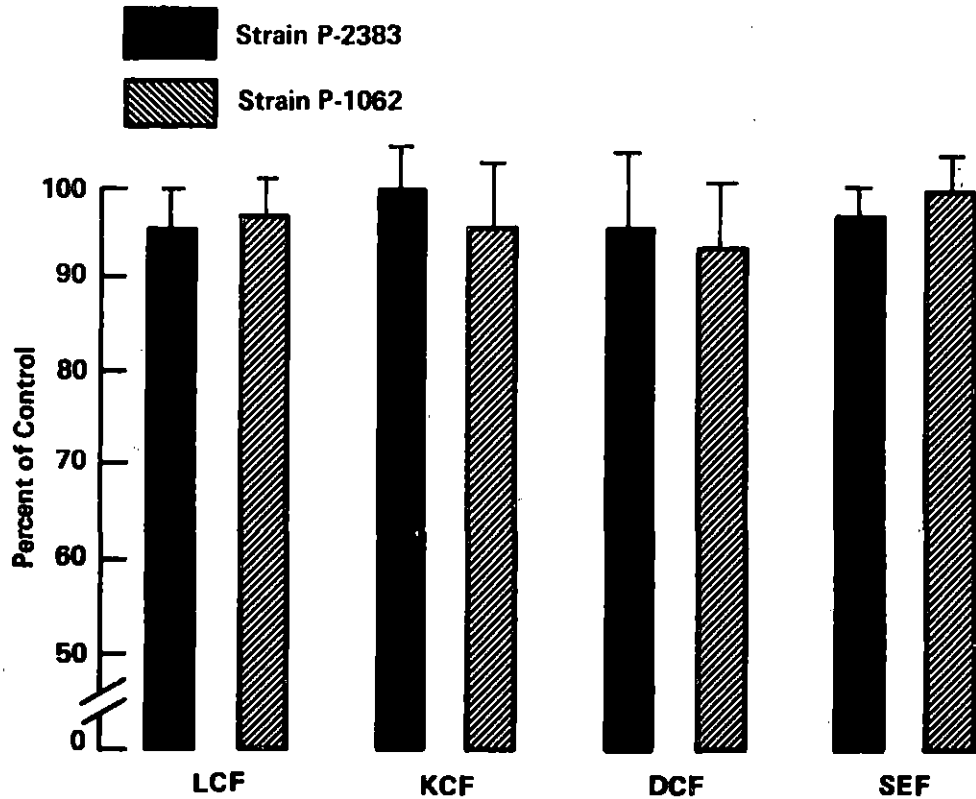| Pretreatment (followed by washing)	Bacterial fraction added to reaction mixture	
	None	SEF-RH-R
None	29.8 ± 3.3[a]	19.9 ± 1.8
S. aureus preincubated with		
SEF-RH-R	27.1 ± 2.1	17.6 ± 2.4
PMNs preincubated with		
SEF-RH-R	31.8 ± 4.4	21.2 ± 4.8
Both S. aureus and PMNs		
preincubated with SEF-RH-R	29.8 ± 3.1	23.4 ± 2.4

[a] Mean ± standard error (n = 6).

Fig. 4. Effect of type A <u>P. multocida</u> fractions on NBT reduction by
bovine PMNs stimulated with opsonized zymosan. Values
represent mean ($\pm$ standard deviation) (n = 6) percentages of
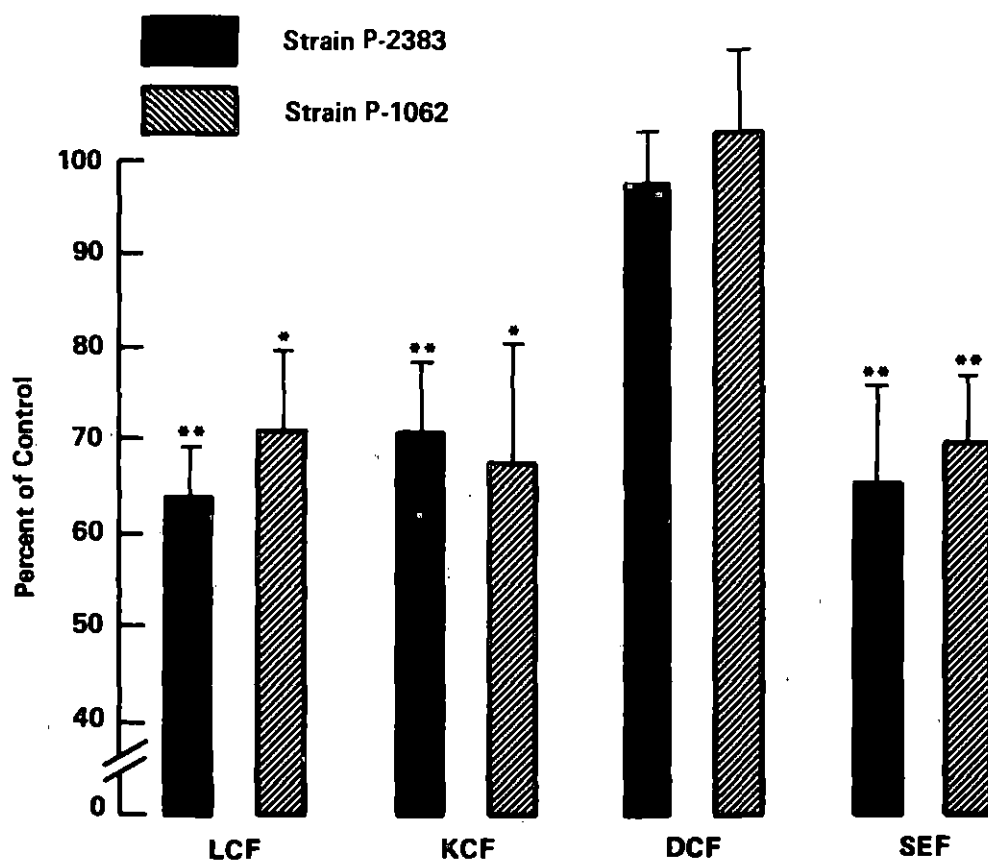the control value. None of the differences are statistically
significant (p > 0.05).

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 $\pm$ 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
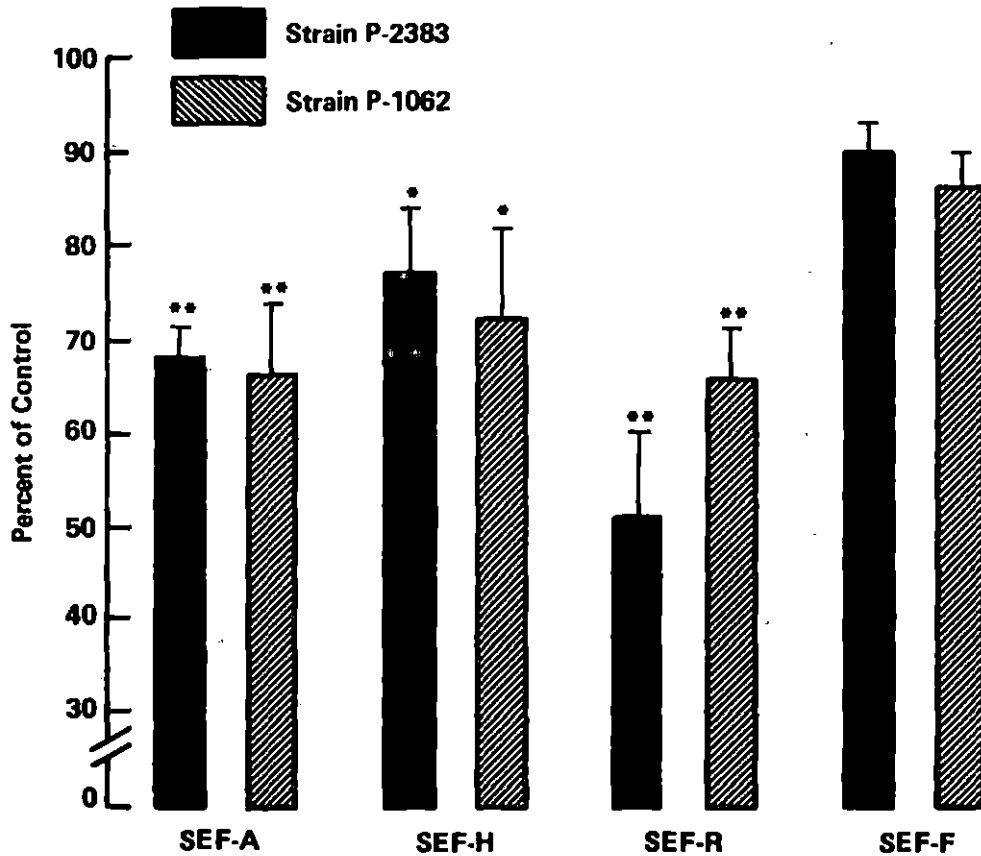PMNs. Xanthine and xanthine oxidase were used to generate superoxide

Fig. 6. Characterization of the inhibitory activity of the saline extract fraction on iodination by bovine PMNs. Values represent mean percentages ($\pm$ 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.
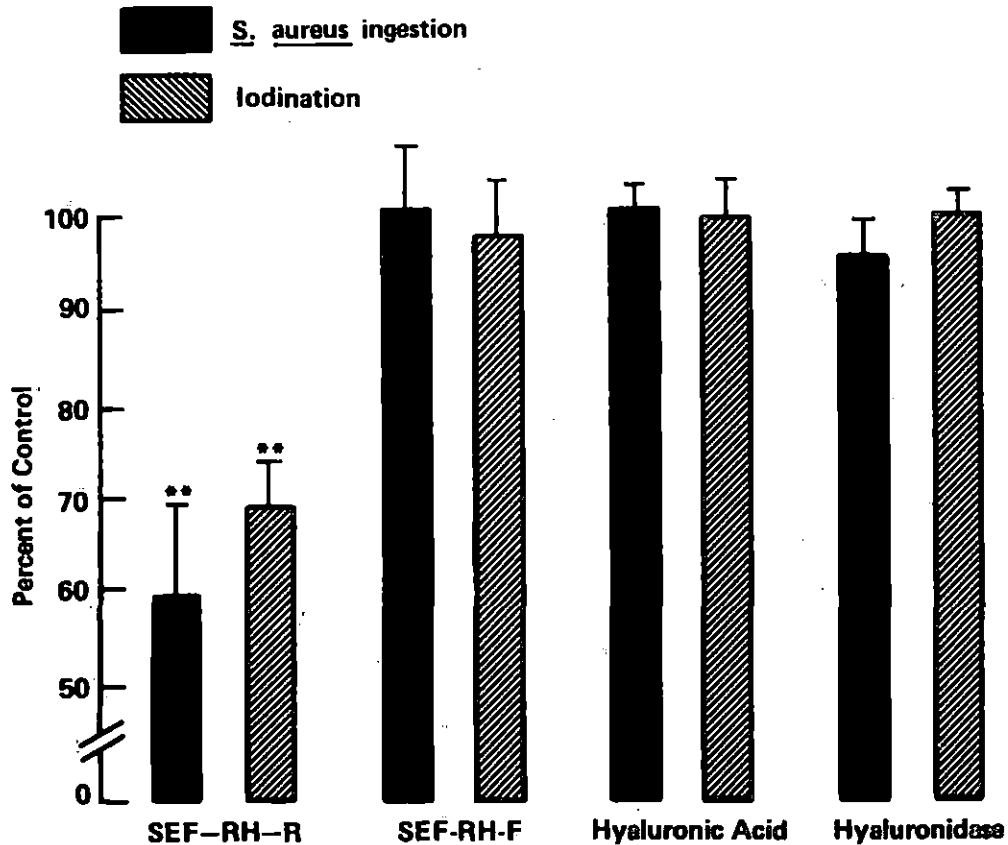
Fig. 7. Characterization of the inhibitory activity of the saline
         extract fraction of strain P-2383 on S. aureus ingestion and
         iodination by bovine PMNs. Values represent mean percentages
         (± standard deviation) of the control value. Statistically
         significant differences from the control value are as
         indicated. **, $p < 0.01$. *, $0.01 < p < 0.05$. n = 8 for all
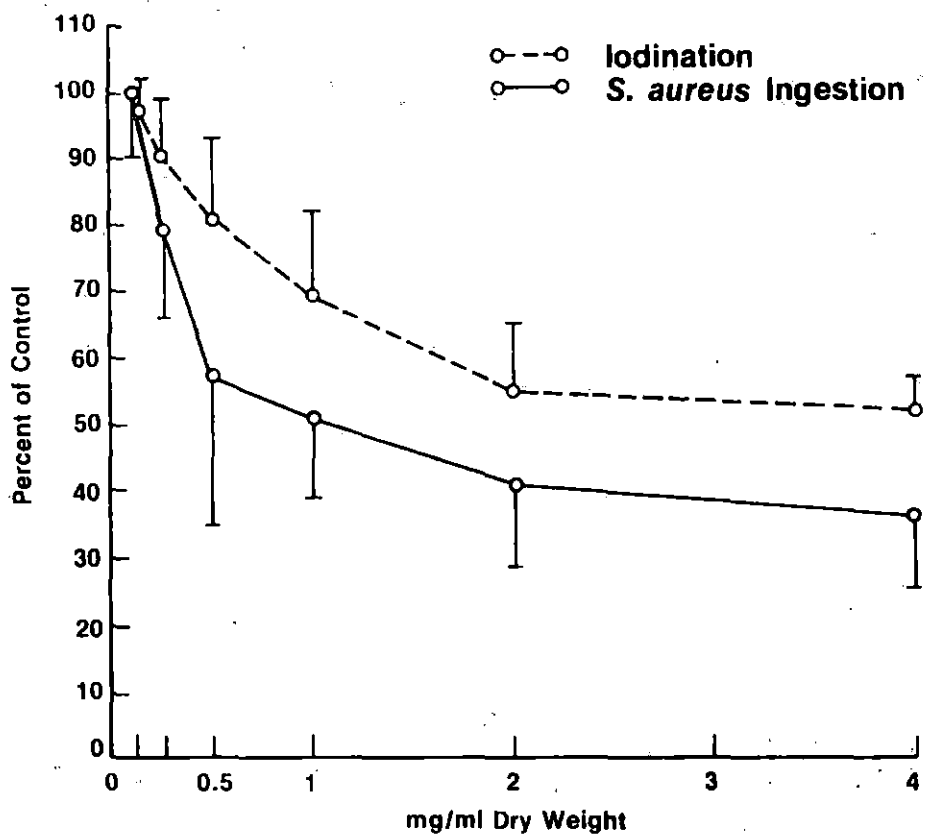   fractions.

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

Fraction	Concentration (mg dry weight/ml)	Carbohydrate (mg/ml)	Hyaluronic acid (mg/ml)	Protein (mg/ml)
SEF	1.06	0.38	0.17	0.07
SEF-R	0.94	0.33	0.17	0.06
SEF-RH-R	1.40	0.35	0.12	0.25
SEF-RH-F	0.80	0.26	0.04	0.22

anion which spontaneously forms hydrogen peroxide. Horseradish peroxidase was used to catalyze the hydrogen peroxide-halide reaction. SEF 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

Bacterial fractions	Iodination value (cpm)[a]
Blank (no xanthine oxidase)	245 ± 18
Control (PBS)	1466 ± 129
SEF of strain P-2383	1453 ± 78
SEF of strain P-1062	1502 ± 89

[a] Mean $\pm$ 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 P. 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.

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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). 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

<u>Organisms</u>

Six strains of <u>P. multocida</u> 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% CO_2 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 Scientic, 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 <u>P.</u> <u>multocida</u>

Strain	Serotype Somatic : Capsular
P-1062	3 : A
P-2383	3 : A
P-813310	3 : A
P-8103889	3, 4 : A
P-92481	3, 4 : Untypable
P-03894	12 : Untypable

resuspended in 0.01 M Tris-HCl (Sigma) buffer containing 5 x 10^{-3}

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 (LD_{50}) of 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 LD_{50} of P-1062, P-2383 and P-8103889 for mice was less than 10 bacteria while the LD_{50} of P-813310 and P-92481 was less than 100 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, tandom 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 tandom 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 eletrophoresis. 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 electrophoreisis, 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 colorimentrically 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-

containg 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

Challenge	(No. of survival/No. tested)				
exposure	P-2383	P-1062	P-8103889	P-813310	P-92481
c.f.u.	160	130	230	120	150
Immunization with					
KSCN Extract of					
P-2383	9/10	9/10	0/10	4/10	10/10
P-1062	1/10	9/10	0/10	1/10	10/10
P-8103889	2/10	7/10	7/10	5/10	10/11
P-813310	1/9	2/10	0/10	9/10	10/10
P-92481	0/10	4/10	0/10	2/10	10/10
P-03894	0/10	1/10	0/10	8/10	8/10
Control	0/9	0/9	0/9	3/10	5/10

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-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 tandom-
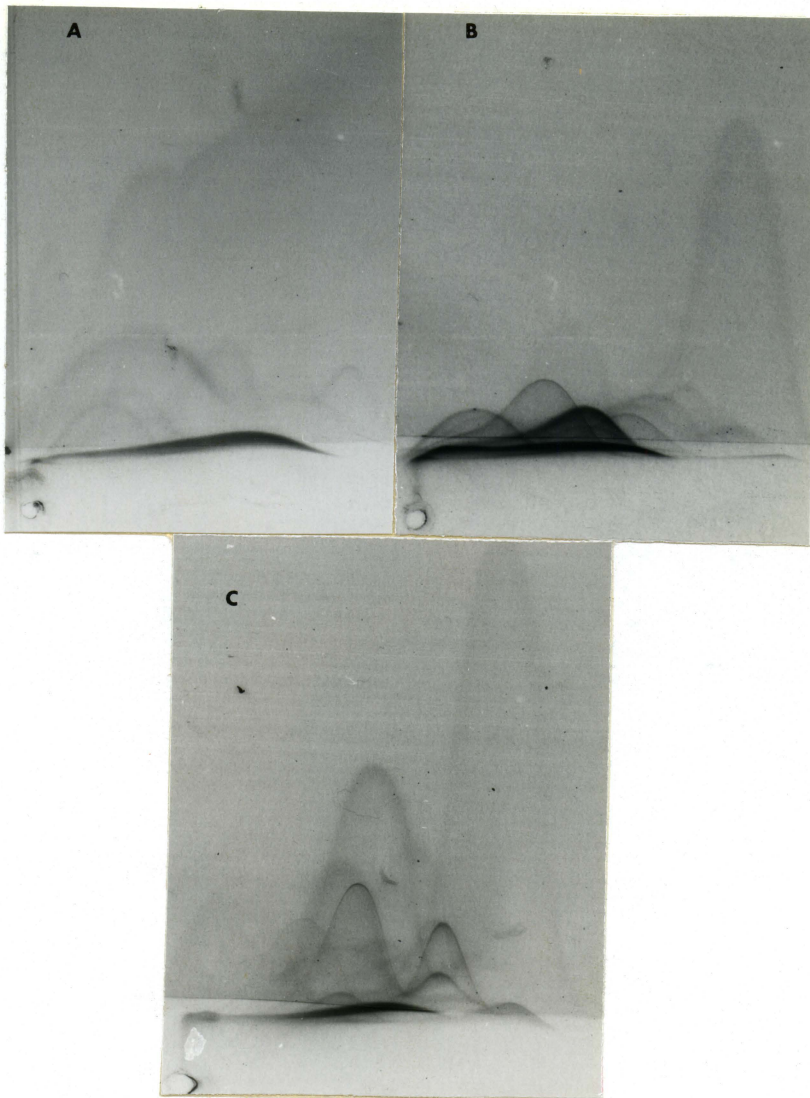CIE using homologous and heterologous antisera. Because the

Fig. 1. Crossed immunoelectrophoresis of P-2383 KSCN extact 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 ul 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 tandom-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
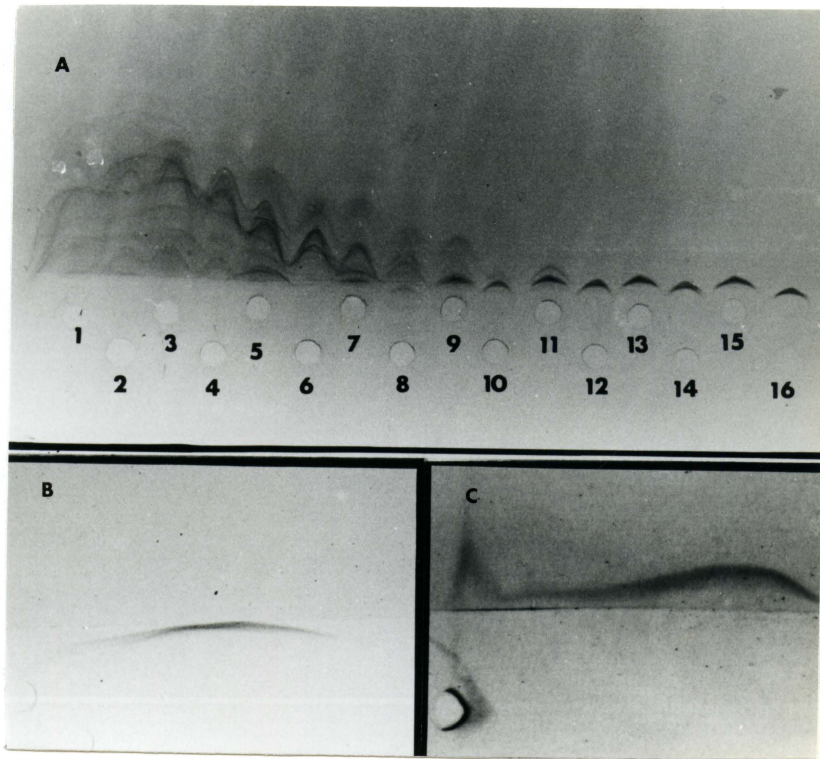
Fig. 2. Crossed immunoelectrophoresis of the KSCN extracts of strain P-2383 and P-1062. A: P-2383 KSCN extract with 2383 antiserum pool. B: P-2383 KSCN extract with 1062 antiserum pool. C: P-1062 KSCN extract with 2383 antiserum pool. D: P-1062 KSCN extract with 1062 antiserum pool.

well as for serotyping organisms (8). P. multocida lipopolysaccharide

was prepared and examined by CIE to verify the existance 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 lipopolysacharide. 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

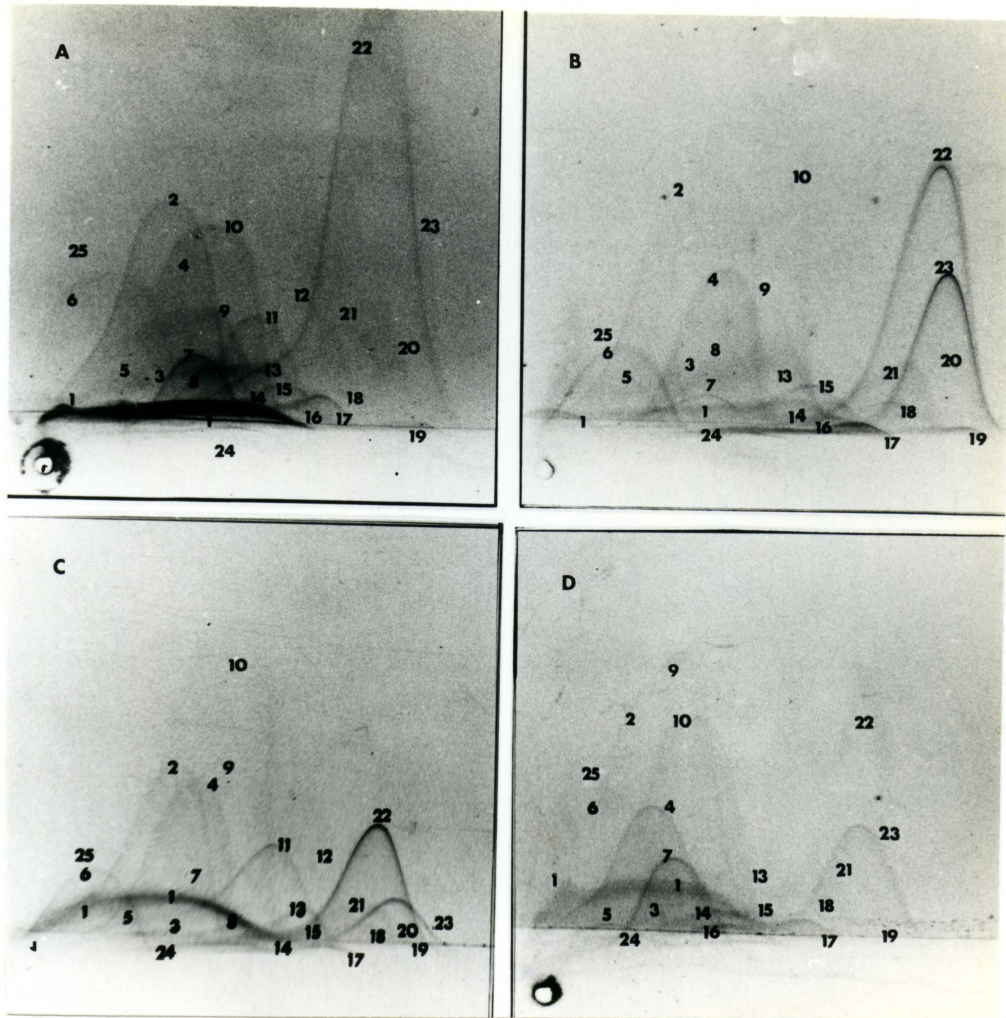will be referred to as P-2383-1 and P-1062-1, respectively.

Fig. 3. Immunoelectrophoresis of fractions by sucrose density
gradient centrifugation. A: Fused-rocket
immunoelectrophopresis 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 antiseum pool. C: CIE of component 1 of P-1062 KSCN
xtract and agarose gel contained 1062 antiserum pool.

Table 3. Immunogenicity of isolated components from the KSCN
extracts in mice

Challenge	(No. survival/ No. tested)						
	P-2383			P-1062			P-8103889
Experiment	1	2	3	1	2	3	3
c.f.u. (x 100)	11	13	1.6	10	11	1.3	2.3
Immunization with							
P-2383-1	5/5	10/10	10/10	5/5	8/10	10/10	4/10
P-1062-1	1/5	2/5	–	2/5	2/5	–	–
Control	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 (40%). 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 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 charaterization 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 fuctions 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 LD_{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 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 <u>Pasteurella</u> <u>multocida</u> (<u>P.</u>
<u>multocida</u>). 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 <u>P.</u> <u>multocida</u> 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 <u>P.</u> <u>multocida</u>
strains exhibited immunogenicity in mice against challenge with the
virulent homologous bacterium. However, mice immunized with KSCN
extracts were not completly 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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