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AN IMMUNOBIOLOGICAL STUDY OF PASTEURELLA MULTOCIDA

Iowa State University

PH.D.

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An immunobiological study of Pasteurella multocida

by

Kevin L. McKinney

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Immunobiology

Approved:

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GENERAL INTRODUCTION

Dissertation Format

The dissertation is presented in the alternate format which includes four manuscripts to be submitted to scientific journals for publication. The first manuscript will be submitted to the Journal of Immunological Methods, the second and third to the Canadian Journal of Microbiology and the fourth to the Journal of Avian Diseases. The manuscripts are presented in the form as generally required by the Department of Immunobiology for dissertations. References cited in each manuscript are included with that manuscript. The manuscripts are preceded by the dissertation format, general introduction and by the objectives of the research project. The manuscripts are then followed by a general discussion and conclusions (specific discussion and conclusion are included in each manuscript). Additional references cited refer to citations in the general introduction and general conclusions and are listed in the format consistent with the dissertation.

The Ph.D. candidate, Kevin L. McKinney, was the principal investigator and senior author for each of the manuscripts presented. Co-authors with direct and significant involvement are acknowledged in the title page of each manuscript.

Literature Review

This review will pertain to the articles that examine the nature of P. multocida antigens and the immunological responses, i.e.,

antibody production, phagocytosis and other cellular responses, to P. multocida. Additional information needed for the interpretation of the experimental data is provided in the individual sections of the dissertation. General reviews of fowl cholera have been written by Heddleston and Rhoades (1978), Collins (1977) and Namioka (1978).

P. multocida is the causative agent of fowl cholera and has been studied since the late 1700s. Pasteur isolated the organism from diseased chickens and then used this organism to perform his classic experiments using live attenuated P. multocida to induce immunity (Pasteur, 1880).

P. multocida, a gram negative rod, is found in pairs or chains. The organism may be capsulated and shows bipolar staining characteristics. The presence of a capsule on P. multocida was often correlated with virulence but not with the presence of immunogens¹. Heddleston, et al. (1964) found that virulent capsulated cultures (a fluorescent-iridescent colony type) would dissociate into a less virulent blue colony type and then into avirulent noncapsulated gray colony type, which still contained immunogenic antigen(s). The noncapsulated forms show reduced virulence (Heddleston and Rhoades, 1978).

Several factors are known to influence susceptibility to fowl cholera such as, the strain of P. multocida, age of the fowl (Hungerford, 1968) and species of fowl. The most virulent strain, X-73, serotype 1 (Heddleston's typing system) was originally isolated from a chicken and this serotype has been frequently isolated from migratory waterfowl. The most prevalent serotype in fowls is serotype 3 as exemplified by strain P-1059 which was originally isolated from a

¹Immunogen - this term denotes the ability of an injected substance to actively protect a host against virulent challenge.

turkey. As a rule, turkeys are more susceptible than chickens to all serotypes. Young turkeys (3 weeks and 9 weeks old) were more susceptible to P. multocida than were one-year-old turkeys (Bond and Olsen, 1974). Turkeys appear to be susceptible to both strains P-1059 and X-73.

Chickens on the other hand are more susceptible to strain X-73 than to P-1059. Challenge with as few as 100 organisms of X-73 is almost always fatal. The susceptibility of chickens to P-1059 varies with age for reasons unknown. For example, a nasopharynx challenge of approximately 10^6 P-1059 organisms killed 88% of 45-week-old chickens, while only 36% of 16-week-old chickens died in similar experiments (Heddleston, 1962).

Immunity against P. multocida can be induced by a variety of techniques. The degree of protection may be evaluated by the percentage of vaccinated animals protected against homologous challenge which killed more than 80% of the controls, by the degree of protection against heterologous challenge and by the duration of the acquired immunity. Several vaccines have been used that satisfy some of these criteria. An attenuated form of P. multocida was first used by Pasteur (1880) but was not widely accepted because of its inconsistency for use as a safe vaccine. Attenuated forms of P. multocida have been used in the drinking water (Bierer and Eleazer, 1968; Bierer and Scott, 1969) to induce immunity. Although a broad spectrum of protection is obtained, protection lasts only approximately 6 weeks. Bacterins that can induce cross-immunity can be made from either infected tissues or bacteria isolated from them (Heddleston and Rebers, 1972, 1974; Rebers and Heddleston, 1977). P. multocida P-1059 cultured in vivo in turkeys and separated from the blood can induce cross-protection against challenge with P.

multocida X-73 (Rimler, Rebers, and Rhoades, 1979). Generally, bacteria grown on agar induces homologous protection, while live vaccines or bacteria grown in vivo induces heterologous protection.

Bacterins of varying duration have been produced. Long-lasting protection can be obtained with formalin killed bacterins emulsified in Freund's incomplete adjuvant (Collins, 1977). Heddleston and Watko (1965) obtained serotype specific protection that lasted up to 39 weeks with an emulsified bacterin. Combinations of three serotypes incorporated into the same bacterin induced protection against each serotype (Heddleston, Gallagher, and Rebers, 1970). But, aqueous vaccines of killed capsulated or noncapsulated bacteria injected twice intramuscularly into chickens produced good immunity at 20 days. The oil emulsified equivalent produced immunity by 14 days but not at 7 days (Heddleston and Rebers, 1969).

P. multocida produces many antigens that could possibly stimulate immunity. The antigenic complexity of P. multocida has been demonstrated by Prince and Smith (1966). They identified 18 soluble antigens by using one-dimensional immunoelectrophoresis. Bhasin and Lapointe-Shaw (1980) demonstrated, by cross immunoelectrophoresis, that at least 55 cytoplasmic and 19 cell envelope antigens existed.

Crude extracts have been used to induce immunity, but the actual biochemical and antigenic make-up of these extracts have not been identified. The crude extracts consisted of proteins, polysaccharides, lipids or a mixture of these substances. Generally, the extracts are very heterogeneous in nature, making it hard to identify the components responsible for the induction of immunity (Heddleston and Rhoades,

1978; Collins, 1977; Namioka, 1978).

Some of the earlier attempts to characterize the immunogens of P. multocida have been crude. Pasteur (1880) described a soluble toxic substance in the culture filtrates of P. multocida. Later, Salmon (1880, 1881) also found toxic substances in the culture filtrates and concentration by boiling destroyed their immunogenicity. Priestley (1936) described both heat-labile and heat-stable antigen (56°C, 60 min) associated with P. multocida. Pirotsky (1938) reported the use of TCA (Boivin method) to isolate four different toxic glycolipids from both smooth and rough strains of P. multocida. The antiserum to the glycolipids passively protected mice against these toxins.

The isolation of an immunogenic capsular polysaccharide was reported by Carter (1952, 1955) and by Carter and Annau (1953). The polysaccharide was isolated by heating (56°C, 60 min) capsulated P. multocida and then precipitating the polysaccharide with three volumes of alcohol. The polysaccharide when isolated from fluorescent virulent type B (Carter's typing system) organisms were immunogenic for mice. But, polysaccharide isolated from mucoid strains (strains with hyaluronic acid) failed to immunize mice. Srivastava, et al. (1970) and Brown, et al. (1970) reported attempts to isolate and study immunogenic cell fractions and culture filtrates of P. multocida. Brown, et al. (1970) showed that P. multocida P-1059 culture filtrate was immunogenic in turkeys (77% survival) when compared to commercial bacterins (27% to 66% survival). Srivastava, et al. (1970) immunized mice with either cell wall, cytoplasm or culture filtrate, all of which induced varying levels of protection. The culture filtrate was passed through a Sephadex G-50

column and most of the immunogenicity was found in the high molecular weight fractions associated with the void volume. The immunogenic fraction developed a single immunodiffusion line as indicated by gel diffusion using rabbit antiserum to this fraction. This indicates that there was a minimum of one antigen found in this fraction. There is a definite possibility that the rabbit antiserum did not detect all of the antigens within the fraction, since the procedure is known to have limitations. Yaw and Kakavas (1957) reported that chickens and mice responded differently to the antigens from capsulated and noncapsulated strains. It would be unusual to expect that all antigens present in this fraction would induce an antibody response in any particular animal. Srivastava and Foster (1977) reported later that extraction of the immunogenic fraction with phenol destroyed immunogenicity, while extracted with ether maintained immunogenicity for mice.

Immunizing components were also isolated by ultracentrifugation of formalinized saline extracts of *P. multocida* (Heddleston, Rebers, and Ritchie, 1966; Rebers, Heddleston, and Rhoades, 1967; Ganfield, Rebers, and Heddleston, 1976). A particulate toxic antigen could be isolated from both avian and bovine isolates of *P. multocida*. Large amounts of antigens were toxic for mice, chicken embryos and cattle, but small amounts of this material consisting of protein, polysaccharide and lipid immunized turkeys, chickens, mice and rabbits against homologous challenge. This particulate antigen was further purified by gel filtration and isoelectrofocusing. The serological activity of the electrofocused purified antigens were found in a pH range of 3.0 to 4.0 with peak

serological activity at a pH of 3.7. The free-endotoxin (FET) isolated by ultracentrifugation has a lipopolysaccharide moiety similar to the phenol extracted lipopolysaccharide (LPS) (Rebers and Heddleston, 1974) which is associated with the heat-stable antigen used for Heddleston's serotyping system (Brodgen and Rebers, 1978).

Knox and Bain (1960) and Bain and Knox (1961) reported the isolation of lipopolysaccharide-protein complex by isoelectric precipitation. This complex was isolated from saline extracts of P. multocida by adjusting the pH of the extract solution to 3.8, resulting in the precipitation of this complex, the supernatant contained a separate "polysaccharide." Digestion of the "polysaccharide" with trypsin eliminated the immunogenicity of the supernatant polysaccharide. LPS extracted with phenol (Bain and Knox, 1961) from two related strains of P. multocida varied in their toxicity for mice. The phenol extracted LPS were weakly immunogenic in mice (20% survival).

Lipopolysaccharide extracted with phenol was also studied by Perreau and Petit (1963) and by Rebers and Heddleston (1974). The LPS would not directly immunize mice, but when absorbed onto RBC's induced antibodies in mice that would passively immunize mice (Perreau and Petit, 1963). Rebers and Heddleston (1974) showed that the LPS would not readily induce antibody nor active immunity, while the FET did. LPS extracted from whole bacteria or from FET with phenol were identical in gel diffusion and contained heptose, hexose and KDO, possibly indicating that the LPS isolated from whole cells is similar to the LPS associated with the FET.

Attempts have been made to develop a cross-protective vaccine

against heterologous P. multocida challenge. Cross-protection has been induced with live-attenuated P. multocida vaccines (Coates, Jensen, and Brown, 1977; Maheswaran, McDowell, and Pomeroy, 1973), formalinized infected tissue (Heddleston and Rebers, 1972, 1974) and recently P. multocida isolated from infected turkey blood (Rimler, Rebers, and Rhoades, 1979). One thing that these bacterins have in common is that the animals were exposed to P. multocida grown in vivo, possibly causing the expression of cross-protective antigens. Past reports demonstrate that in vitro grown cells killed with formalin or antigens isolated from these cells were not cross-protective (Heddleston, Gallagher, and Rebers, 1970). The actual identification of the cross-protective immunogens have not been reported to date.

The immunological responses necessary for protection is complex and may involve more than one specific response. The presence of antibody is necessary for protection, but whether this response is the only immunological response responsible for protection, is not clear. Fresh antiserum could not kill P. multocida in vitro (Bain, 1955; Yamaguchi and Baba, 1975). But, fowl antiserum can passively protect chickens and turkeys (Rebers, et al., 1975; Heddleston and Watko, 1965; Nathanson, Hofstad, and Jeska, 1980). Immunoglobulins (IgG) isolated from cross-protective serum transferred immunity to chickens (Rebers, et al., 1975). Antiserum absorbed with capsular material lost some of its passive protective activity (Penn and Nagey, 1976). The presence of antibody as determined by serological techniques has not correlated to the immune status of fowls (Heddleston and Watko, 1965; Alexander and Soltys, 1973). Vaccinated nonbursectomized turkeys were more resistant to i.m. challenge

than were vaccinated bursectomized turkeys (Nathanson, Hofstad, and Jeska, 1980). The data presented so far indicate that antibody plays an important role in protection, but still other immunological pathways may be important for survival.

T-cells may also play a role in protection against challenge. Yamaguchi and Baba (1975) showed that vaccinated bursectomized chickens and immunized chickens had higher survival rates than thymectomized chickens. They also demonstrated that there was production of a macrophage inhibiting factor by immune T-cells. Maheswaran, Thies, and Dua (1976) demonstrated that T-cells (peripheral blood lymphocytes) can be stimulated by P. multocida into blastogenesis, and that immunized T-cells respond faster than normal T-cells. T-cells and B-cells can have definite influences on the cellular responses of macrophages and monocytes by the production of various serum factors (antibody, MIF, etc.). If these factors could stimulate and increase phagocytosis, phagocytosis could be important in the protection of fowl against fowl cholera.

Phagocytosis of P. multocida has been suggested by various researchers. Bain (1955) speculated that phagocytosis occurs in the blood of immune animals. Collins (1973) showed that P. multocida in normal and incomplete immunized mice continued to grow uninhibited in blood, livers, or spleens, but in an immunized host the bacteria were eliminated. Peritoneal washings and organ examinations for mice challenged intraperitoneally (i.p.) showed that in normal mice the bacteria disseminated throughout the mice. In immunized mice the bacteria also spread, but growth was inhibited and finally eliminated after 24 hours. Peritoneal macrophages that were washed out of immunized and normal mice could not

kill the phagocytized P. multocida in vitro within 60 minutes. Bacteria that were opsonized were phagocytized better but were not killed after 60 minutes. The conclusion that Collins reached was that phagocytosis could be increased by the addition of specific immune serum, but the rate of inactivation of the bacteria was not greatly increased. The presence of opsonins did not inhibit early growth of the bacteria.

Yamaguchi and Baba (1975) support some of Collins' work, in that P. multocida (P-1059I) grew in normal peritoneal macrophages and that these macrophages died. The immune peritoneal macrophages inhibited intracellular growth of the bacteria, plus the immune macrophages survived better than the normal peritoneal macrophages. The lysate of the immune peritoneal macrophages had marked increases in the bactericidal activity as compared to normal peritoneal macrophages (using both enzyme activity levels and bactericidal activity of the lysate of the macrophages). These data indicate that if the macrophages are properly stimulated, they can kill P. multocida.

Further work by Woolcock and Collins (1976) showed that lesions in the feet of immunized mice when challenged by the way of the footpads contained vast numbers of polymorphonuclear macrophages and a few mononuclear macrophages. This supports the observation of Rhoades, Heddleston, and Rebers (1967) that in septicemic calves (caused by P. multocida) microscopic lesions contained leukocytes and macrophages in the edematous area. It seems from these data that macrophages were attracted to the sites of infection and that they could possibly contain the spread of infection.

Collins and Woolcock (1976) followed up on the possible role of

phagocytosis against infection for fowl cholera. One of the experiments involved bacteria opsonized with normal serum. The opsonized bacteria were injected i.p. and collected 2 minutes later by peritoneal washing and centrifuged so that extracellular bacteria could be separated from the macrophage associated bacteria. The data showed that 99% of the bacteria was extracellular. When antiserum was used to opsonize bacteria, only 5% of the bacteria were extracellular. This indicates that specific opsonins within the peritoneal cavity brought about the immediate phagocytosis of most all of the P. multocida.

The presence of a capsule associated with P. multocida could influence the ability of macrophages to phagocytize the bacteria. Maheswaran and Thies (1979) showed that some strains of capsulated P. multocida were not opsonized with heat-labile serum opsonins found in normal serum but that a noncapsulated strain was opsonized and was phagocytized. Hyperimmune antiserum increases phagocytosis of the capsular type B P. multocida, but has no effect on P. multocida with capsular type A. In the latter case, the capsule is known to contain hyaluronic acid and that the capsulated bacteria are not agglutinated with antiserum. In the case of the capsular type B, hyaluronic acid is not present and the bacteria are agglutinated with antiserum. McKinney and Rebers (1977) reported that noncapsulated P. multocida were readily opsonized and phagocytized by turkey monocytes but that the capsulated P. multocida were not. The antiphagocytic components were removed by washing the capsulated bacteria, thus increasing the rate of phagocytosis of the opsonized bacteria. Heat-stable serum components, presumably antibody, were

responsible for opsonization and were enhanced by heat-labile components (complement).

In conclusion, the immunological responses to P. multocida has not been completely defined. The production of antibody has a definite role to induce protection, but this probably is not the only protective response. Detailed immunological studies involving T-cells and their products have not been examined for their role in protection against P. multocida infection. The studies involving phagocytosis and humoral substances that mediate these responses have just begun. At this time, we know that heat-labile and heat-stable serum components (presumably complement and antibody) influence the phagocytosis of P. multocida. The correlation of antibody titer to immunity has not been demonstrated even though antibody is important in passive protection and phagocytosis. Most likely, there is a concerted effort of both cellular and humoral responses to provide immunity.

The protective antigens of P. multocida are not clearly identified. This is complicated by the multitude of potential antigens associated with P. multocida and the difficulty in separating these antigens into single entities. Problems have been encountered when trying to identify the antigens serologically, the particularities and the host's variations in their immunological responses influences the serological results. Thus, if one uses mammalian antiserum to identify the immunogens responsible for protection in fowls, the results could be misleading, especially if different antigens are responsible for inducing protection in mammals and fowls. The study of fowl cholera should be done using a homologous test system and in our case

it would necessitate the use of fowl antiserum to identify protective antigens for fowls.

Objectives

The objectives of this study were: (1) to develop an immunoelectrophoresis system employing avian antiserum to detect avian strain P. multocida antigens, (2) to develop a method for purifying P. multocida antigens, (3) to immunobiologically characterize these purified antigens as to their immunogenicity, antigenicity and toxicity, and (4) to describe a cellular response, chemotaxis, of fowl blood monocytes to P. multocida as stimulated by serum components.

IMMUNOELECTROPHORESIS EMPLOYING AVIAN ANTISERA FOR THE DETECTION AND
QUANTITATION OF PASTEURELLA MULTOCIDA ANTIGENS

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IMMUNOELECTROPHORESIS EMPLOYING AVIAN ANTISERA FOR THE DETECTION AND
QUANTITATION OF PASTEURELLA MULTOCIDA ANTIGENS

Abstract

Immunoelectrophoresis with various buffer systems at high and low pH was examined for suitability to detect and quantitate Pasteurella multocida antigens with turkey or chicken anti-P. multocida sera. Counterimmunoelectrophoresis was used to develop a buffer system for one dimensional-, two dimensional-, and rocket-immunoelectrophoresis. The effects of pH, buffer, and molarity on resolution of immunoprecipitates were determined; 0.05 M sodium acetate-acetic acid buffer at pH 5.6 was the most suitable buffer. This buffer could be used in counter-immunoelectrophoresis with turkey or chicken sera to detect minute amounts of P. multocida protein antigens (4.3 ng/test) or lipopolysaccharide (3.12 µg/test). One dimensional-immunoelectrophoresis with the acetate buffer system required treatment of the gels with a 17% NaCl solution to induce immunoprecipitation of P. multocida lipopolysaccharide. Other techniques using the acetate buffer system did not require the high salt treatment. In two dimensional-immunoelectrophoresis, antisera migrated into the second dimension at pH 8.6, but did not migrate at pH 5.6. Rocket-immunoelectrophoresis with the acetate buffer system was effective for quantitating P. multocida antigens.

Introduction

Techniques that are used routinely in clinical and research laboratories for qualitative and quantitative immunoelectrophoresis generally employ mammalian antisera. Reports that describe the use of

avian antisera for immunoelectrophoresis techniques are sparse. The lack of such reports most probably reflects the difficulty of inducing immunoprecipitates of avian immunoglobulin with antigen. In contrast to mammalian immunoglobulin, high salt (1.0 to 2.0 M NaCl) is required to induce or optimize immunoprecipitation of some avian immunoglobulins with antigen (Gallagher and Voss, 1969a; Benedict, et al., 1963). High salt content in a gel is prohibitive to electrophoresis because it adversely influences migration and resolution.

Rocket-, countercurrent-, one dimensional-, and two dimensional-immunoelectrophoresis are usually done at pH 8.6, a pH near the pI range of mammalian immunoglobulins (Briles and Davie, 1975; Awdeh, et al., 1968). Our attempts to use chicken or turkey antisera with the same techniques at pH 8.6 have not been successful in many instances. Gallagher and Voss (1969b, 1970) showed that immunoprecipitation by chicken immunoglobulins at low pH was similar to that observed with high salt. They also showed that the binding constants of chicken 7S anti-DNP at pH 8.0 and pH 5.0 were similar. The pI's of avian immunoglobulins, particularly chicken immunoglobulins, are reported to be pH 6.6, 5.6, or 5.2 (Gallagher and Voss, 1970; Tenenhouse and Deutsch, 1966).

Herein, we describe immunoelectrophoresis techniques at low pH (pH near the pI of avian immunoglobulin) that improved the detection and quantitation of P. multocida antigens by chicken or turkey antisera.

Materials and Methods

Bacterial antigens

Lipopolysaccharide (LPS) of nonencapsulated Pasteurella multocida strain P-1059 (serotype 3) was prepared as described previously (Rimler and Brown, 1980). Potassium thiocyanate (KSCN) extract antigens were prepared from encapsulated P. multocida strain X-73 (serotype 1).

To prepare KSCN extract antigens, a 16-h culture of P. multocida grown on dextrose starch agar at 37°C was harvested and washed twice in phosphate buffered saline (PBS) by centrifugation. The washed bacteria (250 g wet weight) were extracted with 1.2 L of 0.50 M KSCN-0.08 M NaCl solution (pH 6.3) containing 0.1% neomycin and 0.1% sodium azide. After extraction at 37°C overnight on a rotary shaker, the extracted bacteria were pelleted by centrifugation at 12,000 x g for 3 h. The supernatant fluid was removed by aspiration, and the bacterial pellet was re-extracted for 3 h. The re-extracted bacteria were pelleted by centrifugation and the supernatant fluids from the 1st and 2nd extractions (crude KSCN extract) were combined. The bacterial pellet was discarded.

The crude KSCN extract was centrifuged at 105,000 x g for 2 h. The supernatant fluid was centrifuged two additional times at 105,000 x g, dialyzed against a 1.0% glycine solution, and designated 40s antigens. The pellet was washed twice with 0.1 M KSCN solution by centrifugation at 105,000 x g, resuspended in 20 ml 1.0% glycine solution, and designated 40p antigens.

Antisera and γ -globulins

Antisera against LPS were made in adult, male, white Leghorn chickens. The chickens were inoculated intravenously (IV) with 50 μ g of LPS in 1 ml of PBS at weekly intervals for 4 weeks. Blood sera were collected 1 week after the final inoculation.

Antisera against P. multocida were made in adult, female, Small Beltsville white turkeys or adult, female, New Hampshire red chickens. To prepare antisera, turkeys and chickens were inoculated IV with 1 ml of 0.3% formalinized physiological saline suspension of strain X-73 adjusted to 10 x a number 1 McFarland nephelometer standard. The turkeys and chickens were inoculated at weekly intervals for 6 weeks. One week after the last inoculation, the turkeys and chickens were exposed IV to 1000 live X-73 P. multocida. At 2 and 4 weeks after the initial exposure, the turkeys and chickens were exposed IV to 10^8 live X-73 P. multocida. Blood sera collected 5 days after each vaccination with formalin-killed P. multocida were pooled and designated anti-formalinized P. multocida. Blood sera collected 1 week after the second and third exposures to live P. multocida were pooled and designated anti-complete P. multocida. All sera were preserved with thimerosal to a final concentration of 0.01%.

A portion of the pooled turkey anti-complete P. multocida serum was used to prepare a γ -globulin fraction. The γ -globulins were precipitated from the serum with Na_2SO_4 as described by Benedict (1967), dialyzed against 1.0% glycine solution, and adjusted with 1.0% glycine solution to the original serum volume.

Electrophoretic system and staining

All electrophoresis techniques were done with a LKB Multiphor system (LKB-Produkter AB, Bromma, Sweden). Staining of gel plates was with a solution of 0.5% Coomassie blue in ethanol:acetic acid:distilled water (9:2:9).

Counterimmunoelectrophoresis

Counterimmunoelectrophoresis (CIE) was used to evaluate the influence of pH, buffer, and buffer molarity on ability to improve resolution of immunoprecipitates of avian sera and antigens.

Test buffers were: 0.09 M diethylbarbiturate-Tris (DBT; LKB application note No. 249); maleic acid-Tris (MAT; Gomori, 1955); sodium citrate - citric acid (CC; Gomori, 1955); sodium acetate-acetic acid (AA; Gomori, 1955); 0.05 M maleic acid - 0.05 M 2-(N-morpholino)ethanesulphonic acid (MES). All buffers were adjusted to 0.05 M unless stated otherwise.

CIE gel plates were made as follows: Twelve ml of a 1.0% solution of agarose (SeaKem HEE0, Marine Colloids, Rockland, Maine, USA) in test buffer were pipetted onto 8.7 x 10.5 cm GelBond films (Marine Colloids). Opposing antigen and antibody wells (4 mm in diameter) were cut in the gel 12 mm apart (center to center). Adjacent wells were 8 mm apart. Appropriate wells were filled with 10 μ L of antisera or antigen.

CIE plates were positioned so that antiserum wells were toward the anode buffer chamber, antigen wells toward the cathode buffer chamber. Each buffer chamber contained 1 L of test buffer.

Runs were made at 6V/cm for 45 min at 10°C. Constant temperature was maintained via the cooling plate. CIE gel plates were evaluated immediately after the run and after staining.

One dimensional-immunoelectrophoresis

One dimensional-immunoelectrophoresis (ODIE) was done with a 0.05 M AA buffer system (pH 5.6) and a 0.09 M DBT buffer system (pH 8.6). Gel plates (8.7 x 10.5 cm) were made with 1.0% agarose (SeaKem LE) in AA or DBT buffer. Electrophoresis of antigens was done at 8V/cm for 1.5 h at 20°C. After the run, troughs were cut in the gel using a LKB template, and the troughs were filled with about 0.15 ml of antisera or γ -globulin preparation. Gel plates were incubated at 37°C for 24 h and then examined.

To test the effect of high salt on resolution, a duplicate set of gel plates was soaked in AA or DBT buffer containing 17% NaCl for 15 min after the electrophoresis and diffusion of antisera from the trough (about 3 to 5 h). After soaking of the duplicate plates, they were briefly rinsed with AA or DBT buffer (to prevent salt crystallization) and incubated further at 37°C for 24 h at which time they were examined.

Two dimensional-immunoelectrophoresis

Two dimensional-immunoelectrophoresis (TDIE) was done with a 0.05 M AA buffer system (pH 5.6) or a 0.09 M DBT buffer system (pH 8.6). Gel plates were made by pouring 10 ml of 1.0% agarose (SeaKem LE) solution onto 8.7 x 10.5 cm glass slides. Electrophoresis of antigens in the first dimension was done at 8V/cm for 1.5 h at 10°C. After the run,

a 1.5 x 10.5 cm strip that contained the electrophoresed antigens was cut and removed from the glass slide, placed onto an 8.7 x 10.5 cm film of GelBond, and 10 ml of AA or DBT buffered 1.0% agarose (SeaKem LE) containing 0.075 ml of γ -globulins was poured onto the gel free surface of the film. The antigen strip was placed toward the cathode, and electrophoresis in the second dimension was done at 8V/cm for 4 h at 10°C.

After the run, the plates were examined, washed in AA or DBT buffer, dried, and stained.

Rocket-immunoelectrophoresis

Rocket-immunoelectrophoresis (RIE) was done with a 0.05 M AA buffer system (pH 5.6) or a 0.09 M DBT buffer system (pH 8.6).

To prepare RIE gel plates, 10 ml of 1.0% agarose (SeaKem LE) in AA or DBT buffer containing various concentrations of antiserum was poured onto 8.7 x 10.5 cm GelBond film. Wells (4 mm in diameter) were cut in the gel at 8 mm intervals. Ten μ l of various concentrations of 40p antigens were added to the wells and electrophoresis was done at 8V/cm for 4 h at 10°C. The gel plates were washed in AA or DBT buffer, dried, stained, and examined for immunoprecipitates.

Results

Influence of pH on counterimmunoelectrophoresis

Initially, two test buffer systems were studied to evaluate the influence of pH on the resolution of immunoprecipitates of avian sera. The test buffer systems were 0.09 M DBT at pH 8.6, a standard electrophoresis buffer system for mammalian sera, and 0.05 M MAT at pH 5.2,

5.6, 6.2, and 6.6. Figure 1 compares the resolution of immunoprecipitates of turkey and chicken antisera with LPS, crude KSCN, 40s, or 40p antigens in the different test buffer systems.

As shown by Figure 1, more precipitates formed at pH 5.2 and pH 5.6 than at pH 6.2, 6.6 or 8.6. Precipitates at pH 5.2 and 5.6 were more intense than those at the other pH values. A reaction between chicken antisera and LPS was not observed at pH greater than 5.6 and the more intense precipitin lines formed at pH 5.2 in that antigen-antibody system.

Smearing of immunoprecipitates was evident at pH 5.2 and 5.6. Most of the smearing occurred with the systems employing complex antigen mixtures at pH 5.2.

Controls showed that some nonspecific precipitation occurred with the turkey antisera at low pH. This nonspecific precipitation was not sufficient to interfere with formation of immunoprecipitin lines.

Figure 2 compares the precipitin reactions from CIE of 2 chicken antisera with the complex antigen mixtures (40s, 40p, and crude KSCN extract) using 0.09 M DBT and 0.05 M MAT buffer systems.

Optimum immunoprecipitation for both antisera was at pH 5.2 with the MAT buffer system. Few or no immunoprecipitates were formed above pH 5.6 for both antisera. Some nonspecific precipitation occurred at pH 5.2 and 5.6 with chicken anti-complete *P. multocida* sera, but this nonspecific precipitation did not interfere with formation of immunoprecipitin lines.

Fig. 1. Effects of pH in counterimmunoelectrophoresis for the detection of Pasteurella multocida antigens with turkey and chicken anti-sera.

- A. Maleic acid-Tris buffer pH 5.2.
- B. Maleic acid-Tris buffer pH 5.6.
- C. Maleic acid-Tris buffer pH 6.2.
- D. Maleic acid-Tris buffer pH 6.6.
- E. Diethylbarbiturate-Tris buffer pH 8.6.
- 1. Turkey anti-complete P. multocida serum.
- 2. Chicken anti-LPS serum.
- . Test buffer control.
- a. 40p antigens.
- b. Crude KSCN extract antigens.
- c. 40s antigens.
- d. LPS.

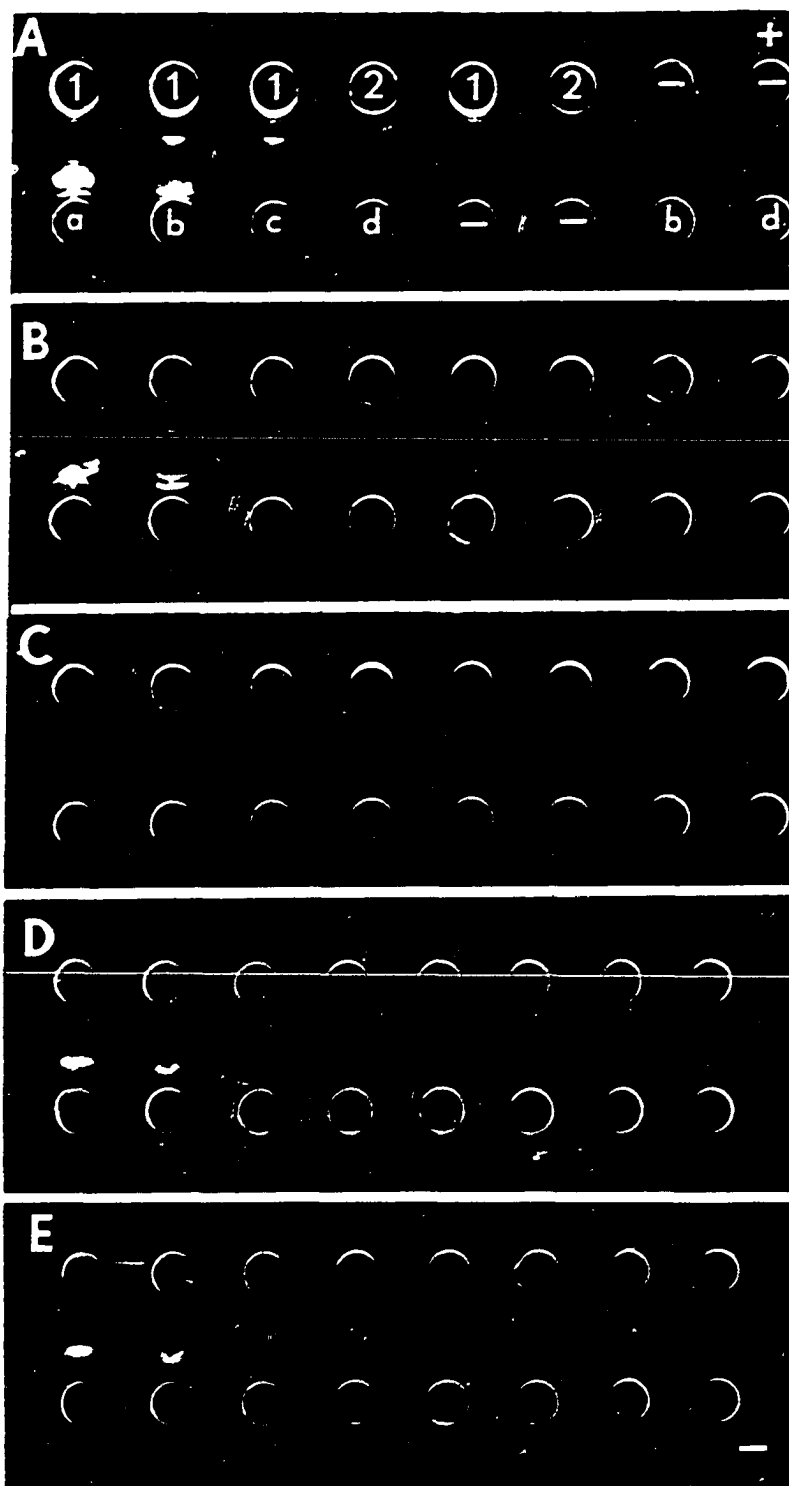


Fig. 2. Effects of pH in counterimmunoelectrophoresis for the detection of Pasteurella multocida antigens with chicken antisera.

- A. Maleic acid-Tris pH 5.2.
- B. Maleic acid-Tris pH 5.6.
- C. Maleic acid-Tris pH 6.2.
- D. Maleic acid-Tris pH 6.6.
- E. Diethylbarbiturate-Tris pH 8.6.
- 1. Chicken anti-formalinized P. multocida serum.
- 2. Chicken anti-complete P. multocida serum.
 - a. 40p antigens.
 - b. Crude KSCN extract antigens.
 - c. 40s antigens.
- . Test buffer controls.

Influence of buffer system on counterimmunoelectrophoresis

After the initial finding that CIE at pH 5.2 and 5.6 using MAT buffer improved resolution of immunoprecipitates, several buffers (MAT, CC, AA, MES) at 0.05 M concentrations were tested to determine whether type of buffer might further improve resolution and provide flexibility for utilization in different electrophoretic techniques.

Figure 3 compares the turkey and chicken antisera immunoprecipitates after CIE in the different test buffer systems. The resolution of immunoprecipitates with AA buffer was equivalent to or better than the other test buffer systems used for CIE of both turkey and chicken antisera against P. multocida antigens. Some smearing and nonspecific precipitation occurred around the antisera wells with the AA buffer system but this did not affect interpretation of the results. Precipitation around the wells diminished when γ -globulin was used instead of whole antisera.

Influence of buffer molarity on counterimmunoelectrophoresis

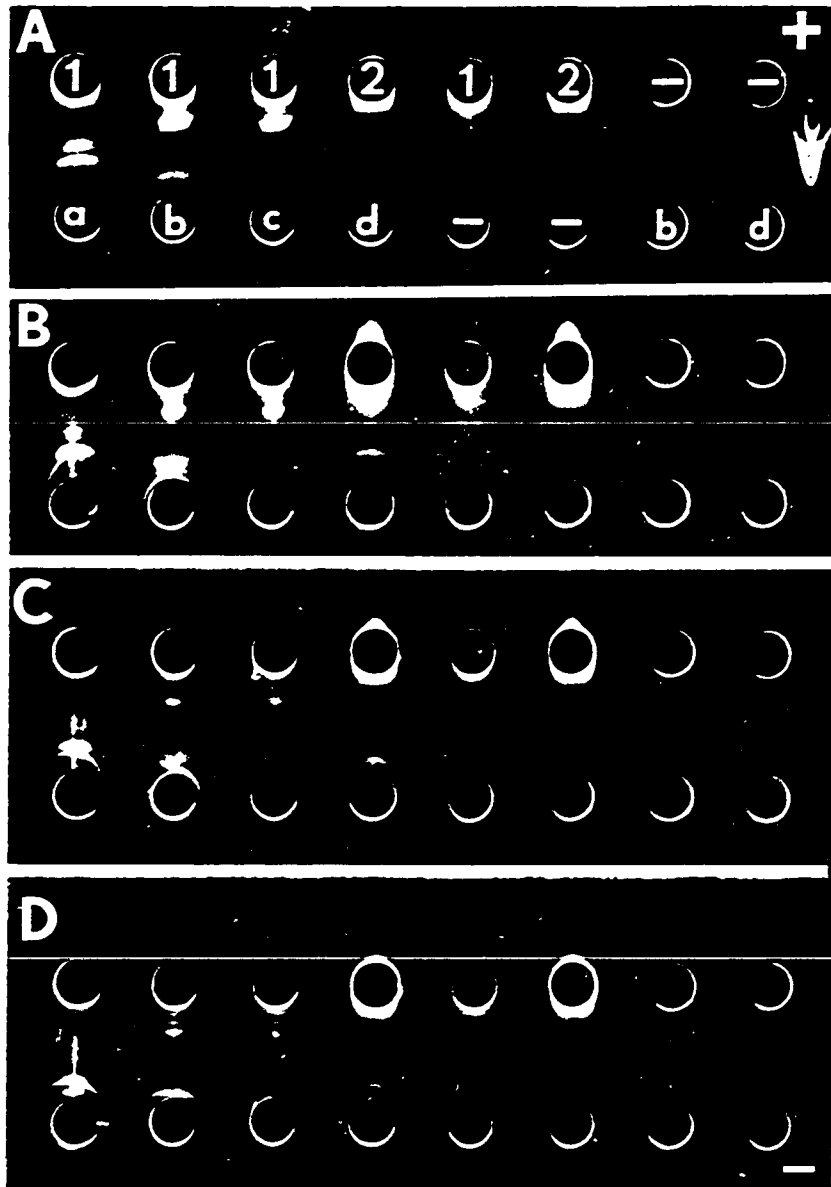
Attention was focused on the use of AA buffer for CIE and other immunoelectrophoresis techniques because it is inexpensive, easy to prepare, and produced good results. CIE studies were done to determine the optimal buffer molarity. The AA buffer (pH 5.6) at 0.025, 0.05, 0.075 and 0.1 M levels was tested. Comparisons of the effects of buffer molarity on CIE of turkey and chicken antisera are shown in Figure 4. Optimum resolution of immunoprecipitin lines was at the 0.05 M concentration. The lines at that concentration were sharper and more intense than those at other molarities.

Fig. 3. Effects of different test buffer systems on resolution of immunoprecipitates in counterimmunoelectrophoresis.

- A. 0.05 M sodium citrate-citric acid buffer pH 5.6.
- B. 0.05 M sodium acetate-acetic acid buffer pH 5.6.
- C. 0.05 M Maleic acid-MES buffer pH 5.6.
- D. 0.05 M Maleic acid-Tris buffer pH 5.6.
- 1. Turkey anti-complete P. multocida γ -globulins.
- 2. Chicken anti-LPS serum.
- 3. Chicken anti-formalinized P. multocida serum.
- 4. Chicken anti-complete P. multocida serum.
 - a. 40p antigens.
 - b. Crude KSCN extract antigens.
 - c. 40s antigens.
- . Test buffer control.

Fig. 4. The effects of sodium acetate-acetic acid (AA) buffer molarity in counterimmunoelectrophoresis for resolution of immunoprecipitates.

- A. 0.025 M sodium acetate-acetic acid buffer pH 5.6.
- B. 0.05 M sodium acetate-acetic acid buffer pH 5.6.
- C. 0.075 M sodium acetate-acetic acid buffer pH 5.6.
- D. 0.10 M sodium acetate-acetic acid buffer pH 5.6.
- 1. Turkey anti-complete P. multocida serum.
- 2. Chicken anti-LPS serum.
- . Test buffer control.
- a. 40p antigens.
- b. Crude KSCN extract antigens.
- c. 40s antigens.
- d. LPS.



A visual precipitin endpoint titration with CIE was used to further examine the suitability of the 0.05 M AA buffer system. Serial two-fold dilutions of LPS or 40p antigens were made and reacted against turkey and chicken antisera. Figure 5 shows that 3.12 μ g amounts of LPS per sample well could be detected with chicken anti-LPS sera, and 4.3 ng amounts of protein of 40p antigens per sample well could be detected with turkey anti-complete Pasteurella-antisera.

In an attempt to further enhance resolution of CIE immunoprecipitin lines, calcium lactate (0.001 M final concentration) or methylcarboxyphenolboronic acid (0.003 M final concentration) (Anhalt, et al., 1978) was added to the 0.05 M AA buffered agarose. A discontinuous CIE system was run with 0.05 M AA buffer. Resolution was decreased with use of calcium lactate, and methylcarboxyphenolboronic acid produced continuous intense smearing between antigen and antibody wells so that immunoprecipitin lines could not be discerned.

One dimensional-immunoelectrophoresis

Separation of crude KSCN, 40s, and 40p antigens and resolution of their immunoprecipitates was better in the AA buffer system than in the DBT system (Figure 6). Particularly noticeable was the improved resolution of the immunoprecipitates of the 40s antigens (low molecular weight antigens).

Treatment of the gels with 17% NaCl in AA buffer improved immunoprecipitate resolution of all antigens regardless of the buffer system used. Precipitation of LPS with chicken antisera did not occur unless gels were treated with 17% NaCl.

Fig. 5. Precipitin endpoint titration of Pasteurella multocida LPS or 40p antigens by counterimmunoelectrophoresis.

Gel A. Row a contains chicken anti-LPS serum. Bottom row contains doubling dilutions of LPS starting with 2.5 mg/ml.

Gel B. Row b contains turkey anti-complete P. multocida serum. Bottom row contains doubling dilutions of 40p antigens starting with 0.27 mg/ml. Arrow indicates endpoint.

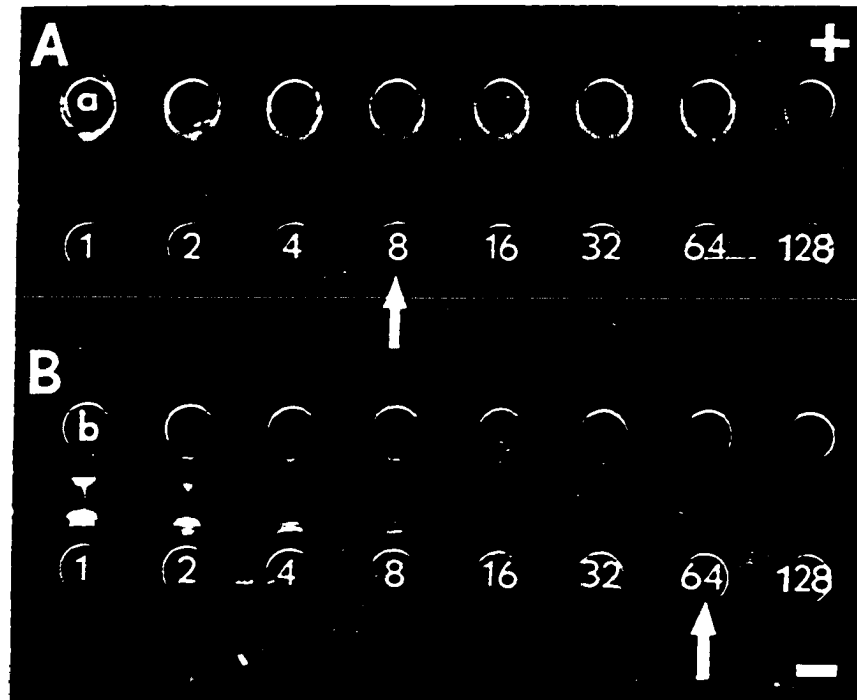


Fig. 6. Influence of pH and 17% NaCl treatment on the resolution of immunoprecipitates of Pasteurella multocida antigens with fowl antiserum.

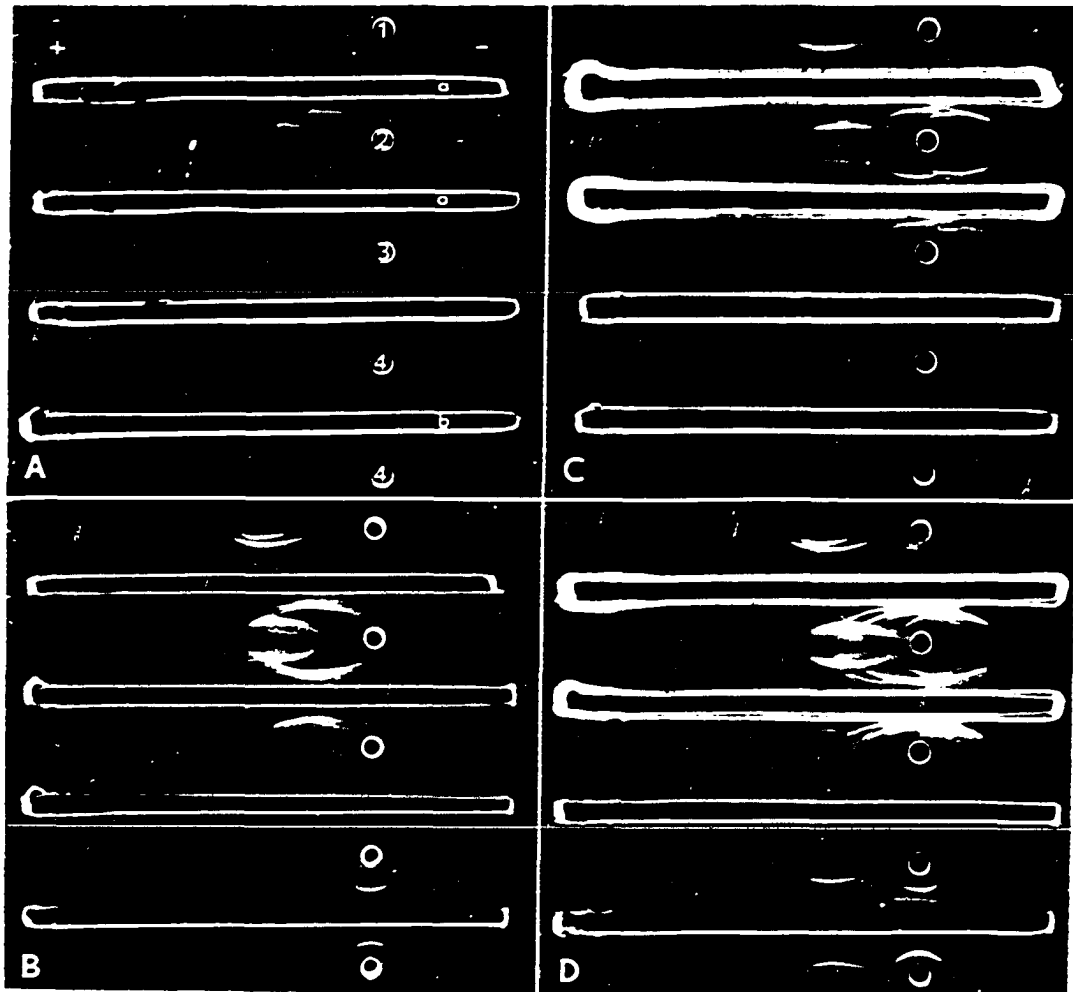
Plate A - Diethylbarbiturate-Tris buffer pH 8.6 without 17% NaCl treatment (see text).

Plate B - Diethylbarbiturate-Tris buffer pH 8.6 with 17% NaCl treatment.

Plate C - 0.05 M sodium acetate-acetic acid buffer pH 5.6 without 17% NaCl treatment.

Plate D - 0.05 M sodium acetate-acetic acid buffer pH 5.6 with 17% NaCl treatment.

1. 40p antigens.
2. Crude KSCN extracted antigens.
3. 40s antigens.
4. LPS.
 - a. Turkey anti-complete *P. multocida* γ -globulins.
 - b. Chicken anti-LPS serum.



Two dimensional-immunoelectrophoresis

Figure 7 compares the results of TDIE of 40p antigens and turkey antisera in AA and DBT buffer systems. Resolution of immunoprecipitates was improved by TDIE in the AA buffer system. Immunoprecipitate patterns in the DBT buffer system were well advanced into the second dimension indicating that the γ -globulin migrated in the same direction as the antigens. No migration occurred in the AA buffer system.

Rocket-immunoelectrophoresis

Two buffer systems (AA or DBT) were used to quantitate 40p antigens with turkey γ -globulin by RIE. Best results were obtained with the AA buffer system. Figure 8 shows the results with the AA buffer system. With this system, the linear correlation of peak height versus concentration of the standard was 0.9997. With the DBT buffer system, the rockets migrated with the current and measurement of peak height was difficult.

Discussion

Chicken or turkey antisera are used routinely in serologic tests (i.e., gel diffusion precipitin, fluorescent antibody, agglutination) to determine the immunological state of the animals or to quantitate antigens. Analytical immunoelectrophoresis techniques have not been used for those purposes. The lack of use of avian antisera in analytical immunoelectrophoresis is probably due to the 1.0-2.0 M NaCl concentration required for optimum immunoprecipitation in gels at pH 7.0 or above. Buffers with ionic strengths between 0.025 and 0.05 are usually used in immunoelectrophoresis. To effect immunoprecipitation with avian antibodies

Fig. 7. Two dimensional immunoelectrophoresis (at pH 5.6 or pH 8.6) of 40p antigens using turkey anti-complete Pasteurella multocida γ -globulins for electrophoresis in the second dimension.

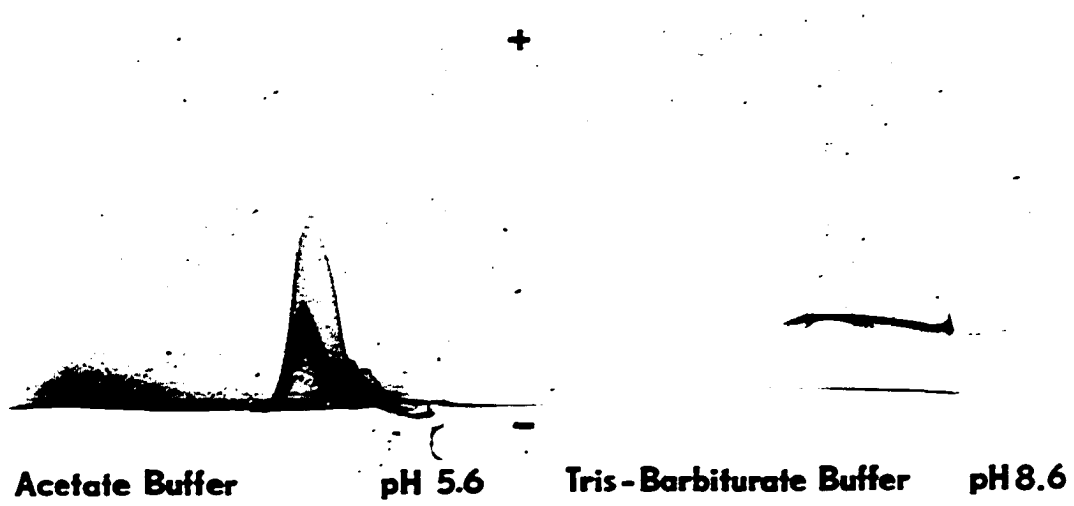
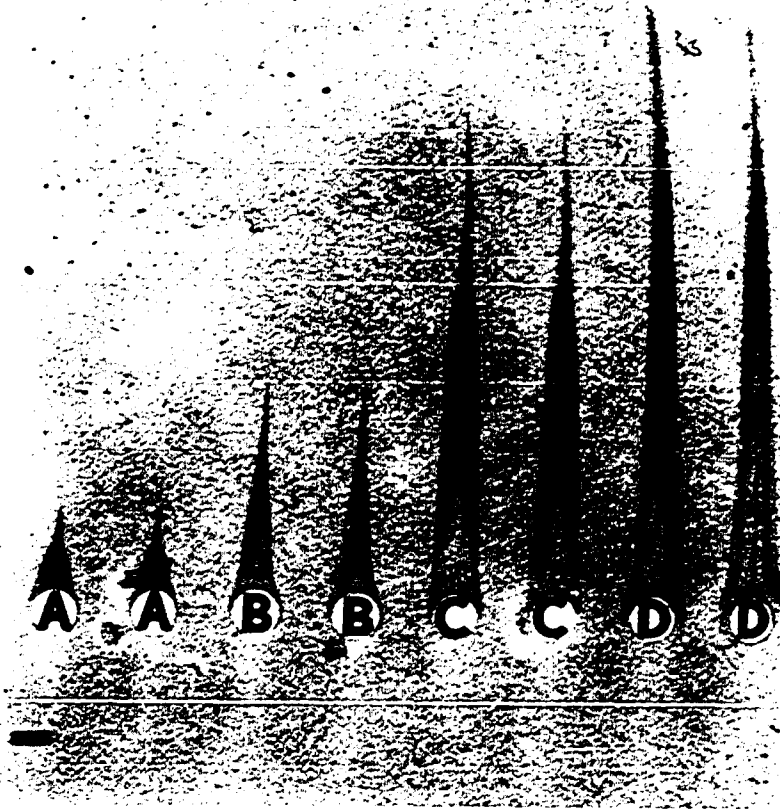


Fig. 8. Rocket immunoelectrophoresis (at pH 5.6) of 40p antigens into turkey anti-complete P. multocida γ -globulins.

- A. Well contains 0.34 μ g of 40p protein.
- B. Well contains 0.68 μ g of 40p protein.
- C. Well contains 1.37 μ g of 40p protein.
- D. Well contains 2.73 μ g of 40p protein.

+



by adding 1.0 to 2.0 M NaCl to the buffer system, thereby increasing the ionic strength, would result in a loss in separation or mobility. Compensating for that loss by increasing the applied current results in a rise in temperature which can produce the deleterious effects of change in density, decrease in viscosity, and increase of diffusion in the electrophoresis medium (Weime, 1965).

Because immunoprecipitation of chicken immunoglobulins at low pH is similar to immunoprecipitation in a high salt system, we investigated different immunoelectrophoresis techniques in the pH ranges 5.2-6.6.

Preliminary experiments and control experiments in the present study showed that chicken or turkey antibodies migrated in the same direction as the antigens when electrophoresis was done at pH 8.6. The conditions for agarose electrophoresis should preferably be such that antibodies do not migrate during the electrophoresis (Weeke, 1973). Electrophoresis at pH 5.6, a pH near the pI of chicken antisera satisfied the conditions of enhanced immunoprecipitation in a system not containing high salt and nonmigration of antibodies during the electrophoresis.

A 0.05 M AA buffer at pH 5.6 produced optimum resolution for each of the electrophoresis techniques examined. Nonspecific precipitation that was evident around antisera wells at that pH did not influence immunoprecipitation or interpretation of the results. The nonspecific precipitation was diminished by use of a γ -globulin preparation instead of whole antisera. The cause of the nonspecific precipitation was not investigated further.

Smearing of immunoprecipitates that occurred in CIE when complex

antigen mixtures were used presented no problems for interpretation of results; the smearing was diminished or not evident when less complex antigen mixtures or antigen specific antisera were used. Calcium lactate and carboxyphenolboronic acid which have been reported to enhance formation and resolution of CIE immunoprecipitates (Anhalt, et al., 1978) were not suitable for CIE with the AA buffer system because they diminished the reaction or produced an intense smearing so that immunoprecipitin lines could not be discerned.

The 17% NaCl treatment that enhanced immunoprecipitation with OCIE did not improve resolution of immunoprecipitates with CIE, TDIE, and RIE. At present we offer no explanation for the enhancement by the high salt treatment.

Acid buffers are not uncommon for use in electrophoresis, and AA buffer at pH 5.3 has been used to resolve streptococcal antigens which could not be distinguished at a more neutral pH (Crowle, 1973). Immunoelectrophoresis with the AA or other buffer systems employing avian antisera might prove useful for serological analyses of viral, mammalian γ -globulins and other bacterial antigens.

References

- Anhalt, J. P., G. E. Kenny, and M. W. Rytel. 1978. Detection of microbial antigens by counterimmunoelectrophoresis. *Cumitech* 8: 1-11.
- Awdeh, Z. L., A. R. Williamson, and B. A. Askonas. 1968. Isoelectric focusing in polyacrylamide gel and its application to immunoglobulins. *Nature* 219: 66-67.
- Benedict, A. A. 1967. *Methods in Immunology and Immunochemistry*. Vol. 1: pp. 229-237 in C. A. Williams and M. W. Chase (eds). Academic Press, Inc., New York.
- Benedict, A. A., H. T. Hersh, and C. Larson. 1963. The temporal synthesis of chicken antibodies: The effect of salt on the precipitin reaction. *J. Immunol.* 91: 795-802.
- Briles, D. E., and J. M. Davie. 1975. Detection of autoradiography and hemolysis of antigen-coated erythrocytes. *J. Immunol. Methods* 8: 363-372.
- Crowle, A. J. 1973. *Immunodiffusion*. Academic Press, Inc., New York. 307 pp.
- Gallagher, J. S., and E. W. Voss. 1969a. Molecular weight of a purified chicken antibody. *Immunochemistry* 6: 199-206.
- Gallagher, J. S., and E. W. Voss. 1969b. Binding properties of purified chicken antibody. *Immunochemistry* 6: 573-585.
- Gallagher, J. S., and E. W. Voss. 1970. Immune precipitation of purified chicken antibody at low pH. *Immunochemistry* 7: 771-785.
- Gomori, G. 1955. *Methods in Enzymology*. Vol. 1: p. 138 in S. P. Colowick and N. O. Kaplan (eds.). Academic Press, Inc., New York.

- Orlans, E., M. E. Rose, and J. R. Marrack. 1961. Fowl antibody. I. Some physical and immunochemical properties. *Immunology* 4: 262-277.
- Rimler, R. B., and W. E. Brown. 1980. Pasteurella multocida: Plasma lysozyme in bacteremic and lipopolysaccharide-exposed turkeys. *J. Inf. Dis.* (in press).
- Tenenhouse, R. S., and H. F. Deutsch. 1966. Some physical-chemical properties of chicken gamma-globulins and their pepsin and papain digestion products. *Immunochemistry* 3: 11-20.
- Weeke, B. 1973. A manual of quantitative immunoelectrophoresis; methods and applications. p. 16 in N. H. Axelson, J. Kroft and B. Weeke (eds.). Universitetsforlaget, Oslo.
- Weime, R. J. 1965. Agar Gel Electrophoresis. Elsevier, Amsterdam. p. 10.

PURIFICATION OF PASTEURELLA MULTOCIDA ANTIGENS BY
ULTRACENTRIFUGATION AND ISOELECTROFOCUSING

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PURIFICATION OF PASTEURELLA MULTOCIDA ANTIGENS BY
ULTRACENTRIFUGATION AND ISOELECTROFOCUSING

Abstract

A procedure was developed to purify potassium thiocyanate extracted Pasteurella multocida X-73I antigens. The crude extract was centrifuged at 105,000 x g separating the antigens into a particulate (40p) fraction and a soluble (40s) fraction consisting of proteins and polysaccharides.

The ultracentrifuged antigens were resolved further by preparative isoelectrofocusing. The 40p antigens were found in a pH range of 3.0 to 6.0 with distinctive proteins focusing at pH's of 3.5, 3.6, and 3.8. The electrofocused 40p antigens were antigenically similar but different from the 40s antigens. The 40s antigens were found within a pH range of 4.4-9.0. Upon refocusing in a narrower pH range, these antigens could be separated and unique antigenic activities could be identified. Specific antigens from defined pH ranges were pooled and further examined as to M.W., pI, immunoelectrophoretic patterns, and analytical electrofocusing patterns.

Introduction

Pasteurella multocida, the causative agent of fowl cholera and haemorrhagic septicaemia, has been shown to be antigenically complex (Prince and Smith, 1966). Various extraction procedures have been used to obtain crude extracts of somatic or capsular antigens (Bain, 1955; Heddleston, et al., 1972; Namioka and Murata, 1961). Purification of these antigens have been attempted by ultracentrifugation (Heddleston, et al., 1966), gel filtration (Ganfield, et al., 1976;

Srivastava and Foster, 1977), or by isoelectric precipitation (Bain, 1955; Knox and Bain, 1960). Free-endotoxin and lipopolysaccharide antigens have been isolated in semi-pure form (Rebers, et al., 1967; Ganfield, et al., 1976). However, between 19 to 55 antigens have been reported to be present in crude extracts of P. multocida (Prince and Smith, 1966; Bhasin and LaPointe-Shaw, 1980).

We were interested in separating and identifying potential antigens of P. multocida. To obtain these antigens, we chose to extract P. multocida with potassium thiocyanate, a chaotropic agent. This agent is capable of disrupting hydrophobic bonding near the surface membrane (Hughes, 1976). Although several researchers (Bain, 1955; Mukker, 1968 and 1979; Mukker and Nilakantan, 1972) have already used potassium thiocyanate to extract antigens from P. multocida as a vaccine against homologous challenge, Gaunt, et al. (1978) reported that this extract induced protection against heterologous challenge, but these workers did not attempt to separate and purify the immunogen.

In this study we will describe the purification and partial characterization of the antigens present in the crude KSCN extract of P. multocida. The purification procedure involves ultracentrifugation and preparative isoelectric focusing in a granulated gel. Purification was monitored by gel diffusion, immunoelectrophoresis, and by analytical electrofocusing.

Materials and Methods

Bacteria

Encapsulated Pasteurella multocida X-73I (Heddleston's serotype 1) was obtained from the National Animal Disease Center, Ames, Iowa, and grown on Dextrose Starch Agar (McKinney and Rimler, 1980).

Preparation of KSCN extract and ultracentrifuged P. multocida antigens

Extraction of P. multocida antigens with KSCN and fractionation by ultracentrifugation was performed according to the methods of McKinney and Rimler (1980). The ultracentrifugation fractions 40s and 40p were dialyzed against 1% glycine-0.01% sodium azide pH 6.3 and stored at 4°C.

Preparative isoelectric focusing of P. multocida antigens in a granular flatbed

Preparative electrofocusing was performed according to the LKB application note #198 using LKB Multiphor 2117 system. Briefly, samples (25 to 50 mg protein) in 1% glycine were mixed with a solution of ampholines to attain a concentration of 1.75% ampholines. Four grams of Ultradex was slowly stirred into 100 ml of the antigen-ampholine solution and then slowly poured into the LKB tray for isoelectrofocusing. The slurry was air dried to 65% of the original weight. Electrofocusing was performed at a constant power of 8 watts with the maximum voltage of 850 volts for 18 hours at 4°C. Immediately after electrofocusing, a zymogram using Whatman No. 1 filter paper was made of the flatbed. The zymogram was stained with Coomassie Brilliant Blue R-250 (0.5%). The flatbed was sectioned into fractions with LKB's 30 slot grid. The pH of every third fraction was determined using a

microcombination pH probe (Microelectrodes, Inc., Londonberry, NH).

The antigens were eluted from the fractions with 1% glycine-0.01% sodium azide pH 6.3 or 0.1 M KSCN or distilled H₂O. The glycine solution eluted higher percentages of antigens from the fraction than did distilled water or 0.1 M KSCN and thus was used for the elutant throughout this report. When refocusing was done, fractions within a specific pH range were pooled and refocused in a pH range slightly larger than itself, e.g., fractions within a pH range of 4-5 were focused in a pH range of 3.5 to 5.5. The separation of the antigens were monitored by gel diffusion and analytical isoelectrofocusing.

Analytical electrofocusing in polyacrylamide gel

Analytical electrofocusing in polyacrylamide gel was performed using LKB Multiphor 2117 system with a LKB 2103 power supply. Polyacrylamide gels of a pH range of approximately 3.5 to 9.5 were prepared according to LKB application note #250. The focused antigens were stained with Coomassie Brilliant Blue R-250 (0.5%) or by Alcian Blue (0.5% Alcian Blue in 3% acetic acid for 24 hours and destained with 3% acetic acid).

Molecular weight determination by SDS-PAGE

Molecular weights of the extracted antigens were determined using preformed gradient gels 4% to 30% acrylamide concentrations (Pharmacia Fine Chemicals, Sweden). The electrophoresis buffer consisted of 0.1% SDS-0.05 M Imidazole buffer at a pH of 7.0 (10 g SDS/10 L and 34 g imidazole/10 L). To prevent smearing, ampholines were removed by using a Minicon B-15 concentrator. The samples were concentrated and reconsti-

tuted with 0.1 M glycine. This process was repeated 2 more times. Samples were solubilized with 1.0% SDS/0.01 M imidazole/1.0% mercaptoethanol/10% sucrose at 56°C for 30 min.

SDS-PAGE was carried out for 3 hours at 100 volts and 100 mA using Ortec 4100 pulse constant power supply and an Ortec 4200 electrophoresis tank (Ortec, Inc., Tennessee) and molecular weight markers (BDH Chemical Ltd., England) of a weight range of 14,300 to 85,800 were used. The SDS-PAGE gel was then stained with Coomassie Brilliant Blue R-250 (0.05% in 25% isopropylalcohol/10% acetic acid) for 24 hours and then destained with 0.01% Coomassie Brilliant Blue R-250 in 10% isopropyl alcohol/10% acetic acid for 24 hours and finally destained with 10% isopropyl alcohol/10% acetic acid.

Anti-P. multocida serum and gammaglobulin preparation

Turkey anti-P. multocida serum and turkey gammaglobulins were prepared as described in McKinney and Rimler (1980). Antisera were collected from turkeys that received formalin killed P. multocida and multiple challenges of live P. multocida.

One dimensional immunoelectrophoresis (ODIE)

One dimensional immunoelectrophoresis was performed as described in McKinney and Rimler (1980) using an electrophoresis buffer of 0.05 M sodium acetate at a pH of 5.6.

Gel diffusion

Gel diffusion was performed as described in McKinney and Rimler (1980) using a Veronal buffer pH 7.6 with 1.5 M NaCl. Turkey anti-

P. multocida sera or turkey anti-P. multocida gammaglobulins were used to detect the extracted antigens.

Results

Immunoelectrophoretic precipitant patterns of the crude KSCN extract and ultracentrifugation fractions

The separation of KSCN extracted antigens by ultracentrifugation as indicated by ODIE is shown in Figure 1. The crude extract was antigenically complex with at least eight major components as detected by turkey anti-P. multocida serum. Fractionation of the crude extract by ultracentrifugation produced two separate groups of antigens. The pelleted material, 40p, formed two double arcs, one at the application well, another anodic to the well. The supernatant material, 40s, consisted of at least five lines which migrated differently than the 40p antigens. Both the 40p and 40s fractions contained protein and carbohydrates. The 40p contained detectable amounts of KDO indicative of LPS. No KDO was found in the 40s fraction.

Preparative electrofocusing of 40p antigens

After electrofocusing of the 40p antigens in a pH range of 2.9 to 10.3, a band with a pI of 3.6 was observed (Figure 2 left zymogram). Most of the 40p antigenic activity in gel diffusion (not shown) was found in a pH range of 3 to 6. In order to improve the resolution, the 40p antigens were refocused in a pH range of 3 to 6 (Figure 2, right zymogram). Three different bands with pI's of 3.5, 3.6, and 3.8 were observed. All of the electrofocused fractions collected from this flat-

Fig. 1. Immuno-electrophoretic patterns of antigens from the crude KSCN extract of P. multocida X-73I. Troughs contained turkey anti-P. multocida gammaglobulins: 40s-ultracentrifuged supernatant antigens; 40p-ultracentrifuged pelleted antigens; crude-KSCN extract of P. multocida.

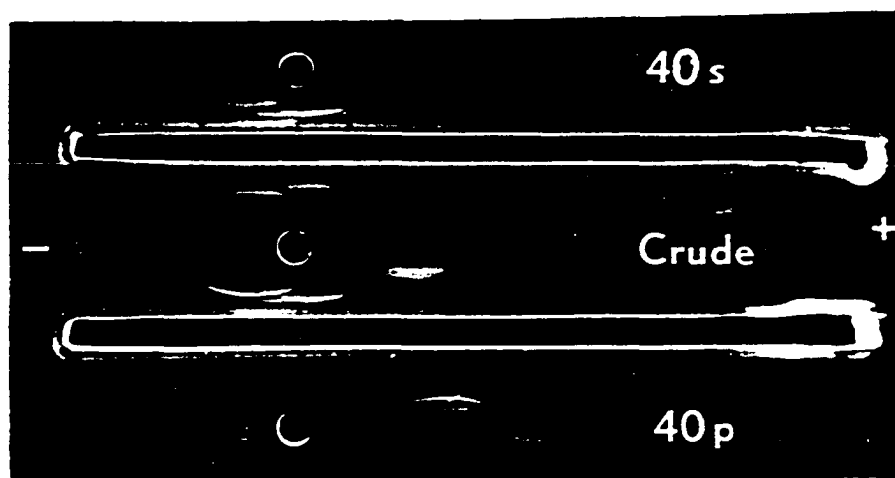
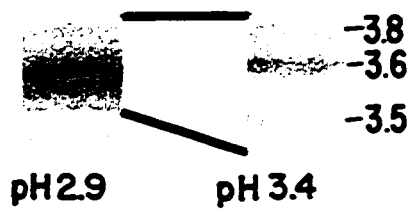


Fig. 2. Comparison of granulated flatbed isoelectrofocusing profiles of 40p *P. multocida* X-73I antigens in a broad pH range (2.9 to 10.3) or a narrow pH range (3.4 to 6.3).

Profile of isoelectric focused *P. multocida*
40 p antigen

pH 10.3

pH 6.3



bed gave lines of identity in gel diffusion (Figure 3). The intensity of the precipitin lines varied with concentration of the antigens found in the fractions. The protein bands with close but different pI's (fractions 25-29) formed lines of identity. Analytical electrofocusing could not be used to examine the 40p antigens because of the large molecular size of these antigens.

Preparative electrofocusing of 40s antigens

The 40s fraction was electrofocused in a pH range of 3.8 to 9.0 (Figure 4, 40s). The 40s fraction proteins had pIs that ranged from 4 to 9. Electrofocused fractions were examined by analytical electrofocusing (Figure 5) to determine the degree of homogeneity. The electrofocused fractions contained multiple proteins (Figure 5, gel A) and polysaccharides (Figure 5, gel B). The highest concentrations of polysaccharides were found in the fractions closest to the anode.

Improved resolution was achieved by refocusing electrofocused fractions within specific pH ranges in a pH range slightly larger than itself. Because protein bands were further apart, contaminating proteins could be separated from the proteins within the targeted pH ranges (Figure 4). When the refocused fractions were checked for homogeneity by analytical electrofocusing (Figure 6) separation was enhanced. The electrofocused fractions from specific antigen groups (2, 3, 4 and 5) now contain three or less proteins as compared to six or more found in the original electrofocusing fractions (see Figure 5). Also, when the refocused antigens were examined for antigenic homogeneity by gel diffusion (Figure 7), the fractions contained one (i.e., Figure 7, row a,

Fig. 3. Gel diffusion patterns of fractions collected from preparative electrofocusing of 40p P. multocida X-73I antigens. Well numbers correspond to fraction numbers from preparative electrofocusing. Well No. 1 - pH 6.3; Well No. 30 - pH 3.4; Well s - turkey anti-P. multocida serum.

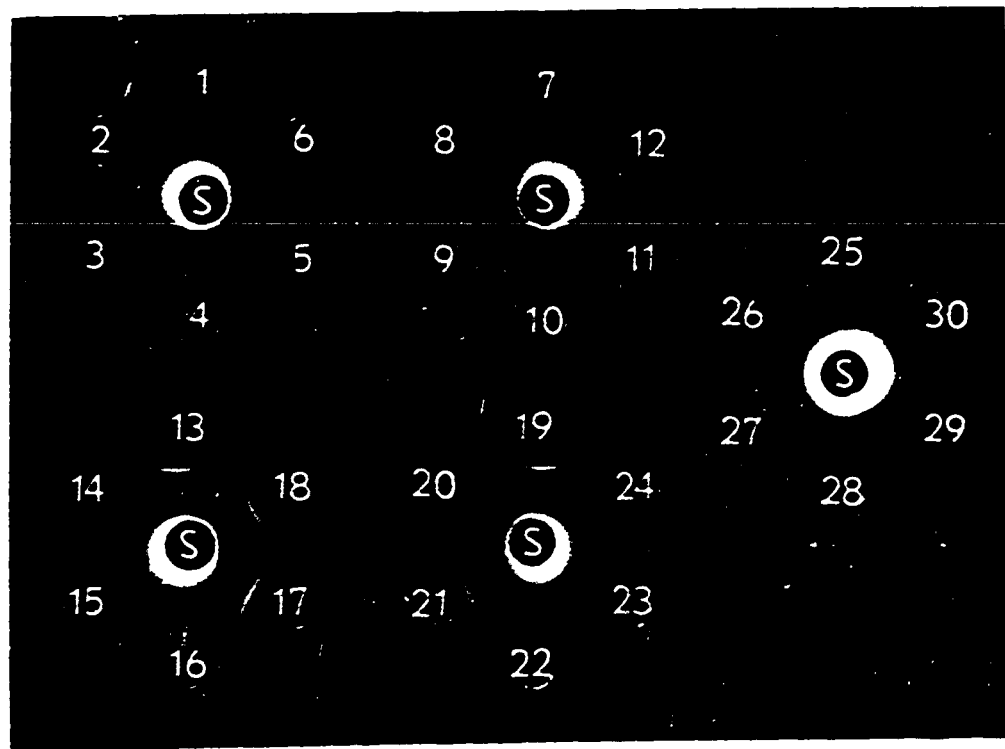


Fig. 4. Isoelectric profiles of *P. multocida* X-73I 40s antigens in broad and narrow pH ranges in preparative isoelectrofocusing. The initial electrofocusing of the 40s antigens is exemplified by the middle strip with a pH range of 3.8 to 9.0. The brackets indicate the fractions pooled from the initial electrofocusing (40s) which were then refocused in a narrow pH range. The brackets in the narrow pH ranges indicate the fractions pH ranges used to form the specific antigenic groups. Group 1 antigens were not refocused but were collected from the initial electrofocusing within a pH range of 8.8 to 9.0.

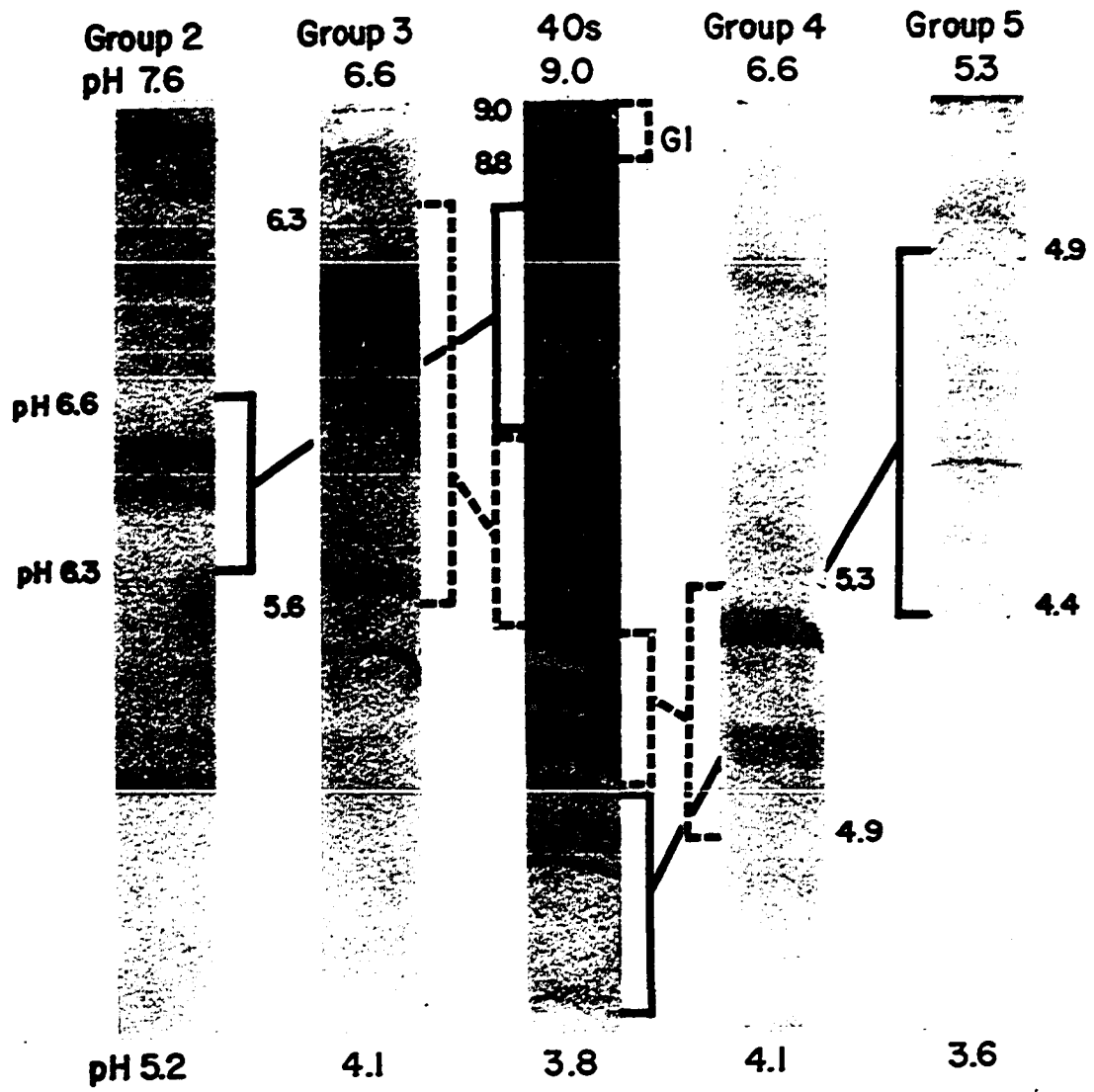


Fig. 5. Analytical polyacrylamide gel electrofocusing examination of fractions collected from a *P. multocida* X-73I 40s antigen preparative isoelectrofocusing gel with a pH range of 3.8 to 9.0. (Gel A) was stained with Coomassie Brilliant Blue R-250 for protein. (Gel B) was stained with Alcian Blue for acidic polysaccharides.

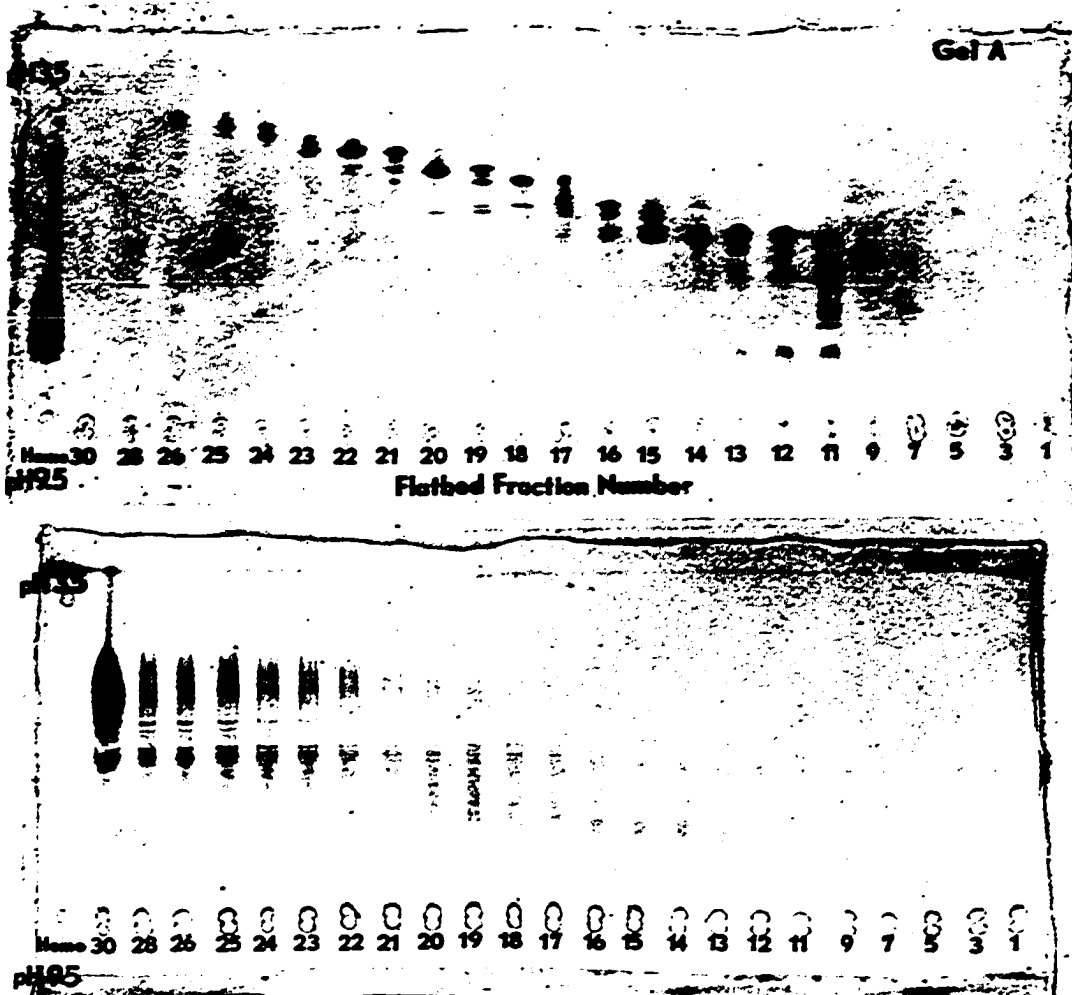
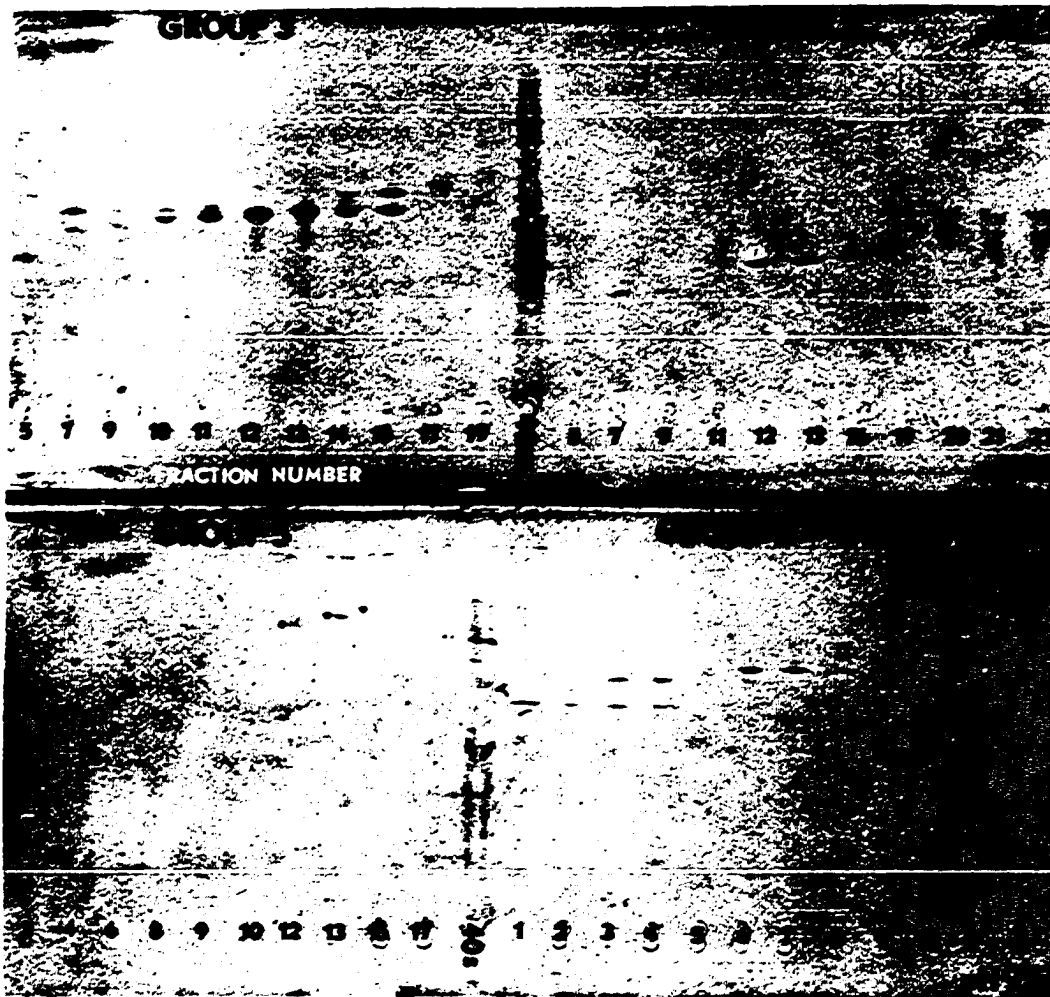


Fig. 6. Analytical electrofocusing in polyacrylamide gel of fractions collected from electrofocusing flatbeds of P. multocida X-73I antigens. Gels were stained with Coomassie Brilliant Blue R-250. Dots indicate faintly stained proteins. Group 2 - antigens pI range 6.3 to 6.6; Group 3 - antigens pI range 5.6 to 6.3; Group 4 - antigens pI range 4.9 to 5.3; Group 5 - antigens pI range 4.4 to 4.9.



fraction 8) or two (Figure 7, row b, fraction 7) antigens. Sometimes a series of fractions would have common antigenic determinants as indicated by lines of identity (Figure 7, row b, fractions 3-6).

Examination of specifically pooled electrofocused purified KSCN extracted *P. multocida* antigens

In light of the demonstrated complexity of the KSCN extracted antigens, the purified antigens were pooled to form identifiable groups for future use in immunogenicity trials. The antigens were grouped according to their pIs. The 40s antigens were divided into five groups: group 1, pI range of 8.8 to 9.0; group 2, pI range of 6.3 to 6.6; group 3, pI range 5.6 to 6.3; group 4, pI range 4.9 to 5.3; and group 5, 4.4 to 4.9. The 40p antigens were divided into two groups with group 6 having a pI range of 3.5 to 3.9 and group 7 with a pI range of 5.5 to 6.0.

The antigen groups were examined by analytical electrofocusing and stained for proteins and carbohydrates (Figure 8). Each group has a distinctive electrofocusing pattern. Group 1 migrated towards the cathode and appeared as a weakly stained band (Figure 8, left gel, 2g1); groups 2, 3 and 4 (Figure 8, left gel, 2g2, 2g3, and 2g4) have distinct patterns with groups 3 and 4 sharing one common protein. Group 5 antigens stain weakly with Coomassie Brilliant Blue R-250 (but were stainable in the crude extract (K_3)). The groups 6 and 7 did not migrate through the polyacrylamide gel.

Polysaccharides were observed in some of the pooled groups (Figure 8, right gel). Polysaccharides were evident in groups 4 (2g4), 5 (2g5), 6 (2g6), and 7 (2g7); some of which were common to all of the groups.

Fig. 7. Gel diffusion examination of electrofocused fractions collected from preparative isoelectrofocusing 40s P. multocida antigens. Fractions were placed in the outer wells with well number (1) being closest to the cathode and well number (30) being closest to the anode. Turkey anti-P. multocida gammaglobulin was placed in the center wells. Row (A) — preparative isoelectrofocusing fractions of Group 2 antigens. Row (B) — preparative isoelectrofocusing fractions of Group 3 antigens. Row (C) — preparative isoelectrofocusing fractions of Group 4 antigens. Row (D) — preparative isoelectrofocusing fractions of Group 5 antigens.

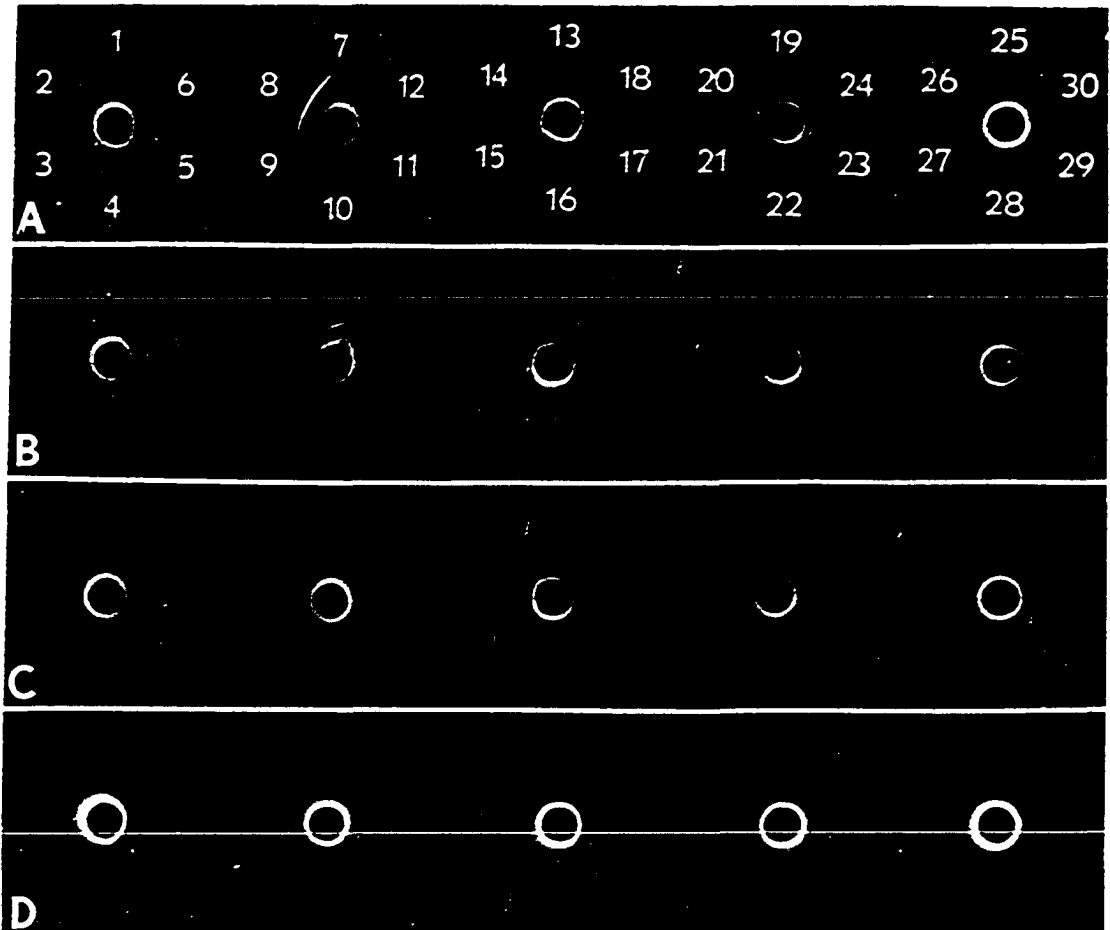
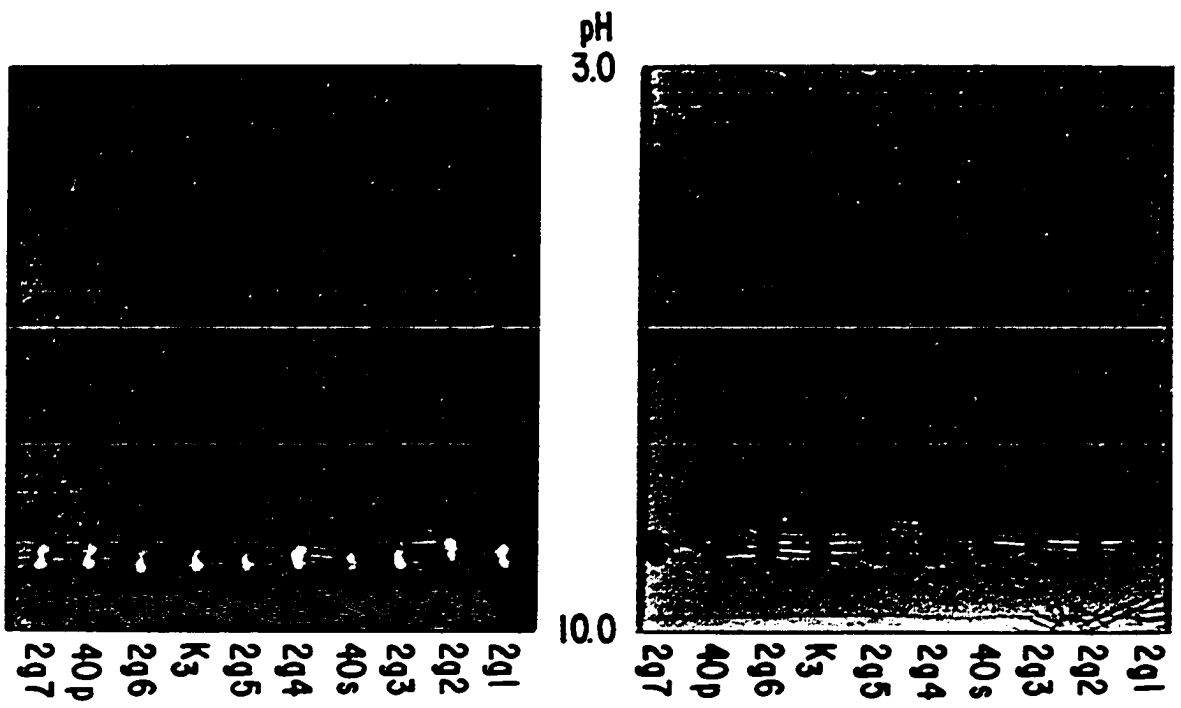


Fig. 8. Examination of pooled electrofocused KSCN extracted P. multocida X-73I antigens by analytical electrofocusing in polyacrylamide gel. Gel on the left was stained with Coomassie Brilliant Blue R-250, and the gel on the right was stained with Alcian Blue. (Groups 2g1 through 2g7) were purified by isoelectric focusing. (40p) and (40s) were ultracentrifuged products of the crude extract. (K₃) is the crude KSCN extract of P. multocida X-73I.



Next, the antigen groups were examined for antigenic similarities by gel diffusion (Figure 9). Groups 2, 3, and 4 formed lines of non-identity, but groups 1 and 5 formed weak lines of identity with groups 2 and 4, respectfully. Groups 6 and 7 formed lines of identity which differ from the 40s antigens.

The pooled antigen groups were also examined by immunoelectrophoresis for variations (Figure 10). Groups 1 and 5 did not form immunoprecipitin lines. Groups 2, 3, and 4 formed distinctive immunoelectrophoretic patterns. Groups 6 and 7 were similar but differed from the 40s antigen groups.

The pooled groups were examined for similarities and differences of molecular weights as determined by SDS-PAGE (Figure 11). Generally the groups shared proteins of similar molecular weights. The molecular weights as determined by SDS-PAGE of the groups ranged from approximately 14,000 to 83,000. Major proteins found associated with the 40s antigens had molecular weights of 60,000 to 83,000 and 34,000 to 38,000. Major 40p antigens had molecular weights of 33,000 to 47,900. The SDS-PAGE patterns associated with the purified 40s and 40p groups varied between another but were still heterogenous as to molecular weights of the antigens found in these groups.

Discussion

Researchers have successfully used KSCN extracted antigens of P. multocida to immunize against homologous challenge and possibly against heterologous challenge. But, these crude extracts are very heterogenous

Fig. 9. Gel diffusion patterns of purified P. multocida X-73I antigens. (KSCN) - crude KSCN extract. (40p) - ultracentrifuged pelleted antigens. (40s) - ultracentrifuged supernatant antigens. (G1 through G5) - purified antigen groups from 40s antigens. (G6 and G7) - purified antigen groups from 40p antigens. The center wells were filled with turkey anti-P. multocida serum.

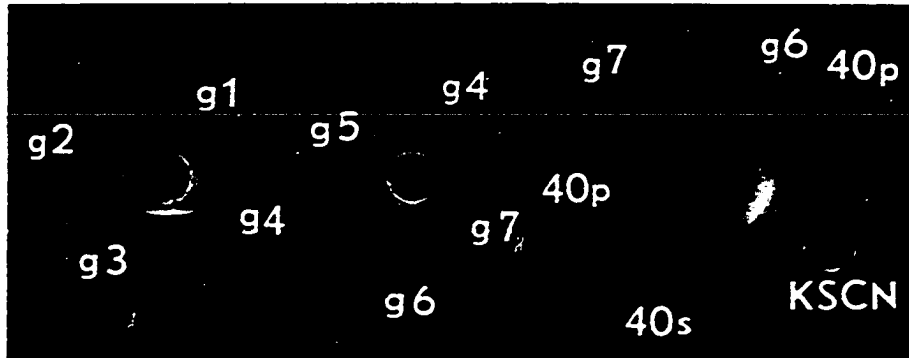


Fig. 10. Immunelectrophoresis of purified P. multocida X-73I antigens. Groups (G1 through G5) are the purified antigen groups from 40s antigens. Groups (G6 and G7) are the purified antigen groups from 40p antigens. (40p) is the ultracentrifuged pelleted antigens. The troughs were filled with turkey anti-P. multocida gammaglobulins.

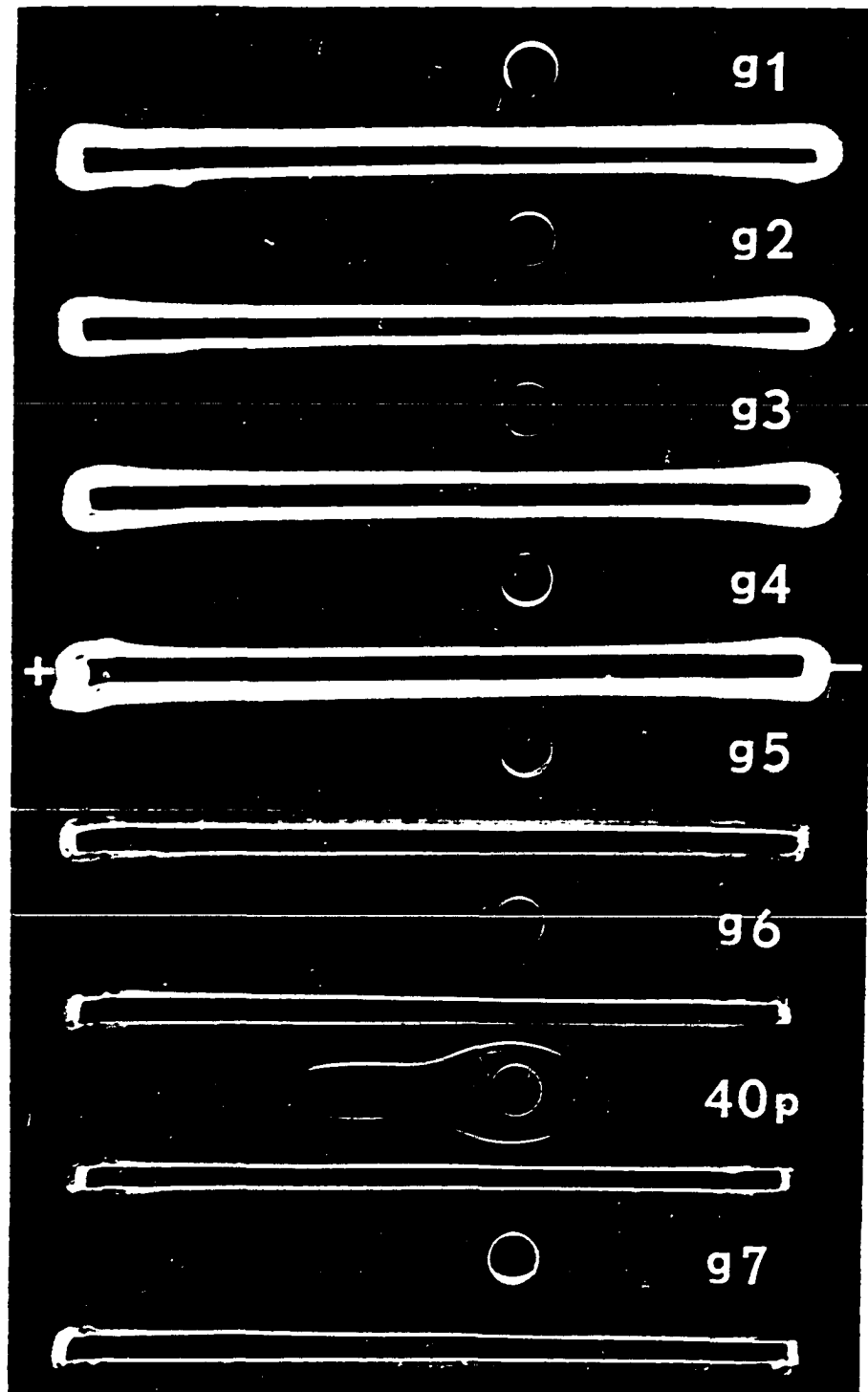
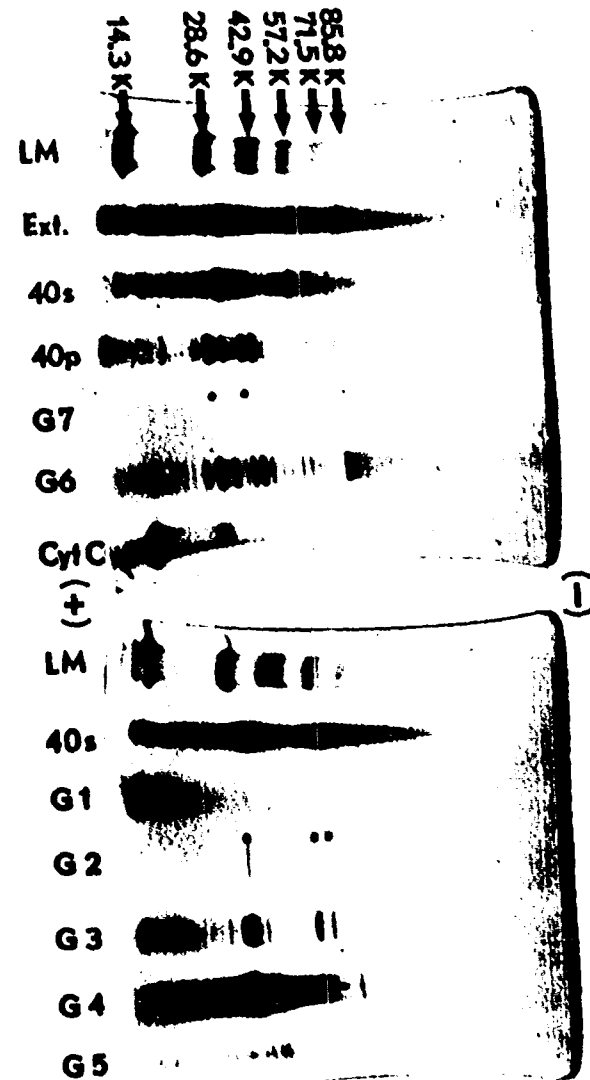


Fig. 11. Comparison of purified KSCN extracted P. multocida X-73I antigens by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gradient polyacrylamide gels (4% to 30%) were used. (LM) - molecular weight markers; (Ext.) - crude KSCN extract; (40s) - ultracentrifuged supernatant antigens; (40p) - ultracentrifuged pelleted antigens; (G1 through G5) - were isolated from the 40s antigens; (G6 and G7) - were isolated from the 40p antigens; (Cty. C) cytochrome C.



(Figures 1, 2, and 3). Ultracentrifugation and preparative isoelectrofocusing were used to separate and purify the extracted antigens into single entities in order to identify potential protective antigens.

Due to the nature of the extracted antigens, some problems arose with preparative isoelectrofocusing. High concentrations of acidic polysaccharides associated with capsulated *P. multocida* found in the 40s fraction caused the gel to crack. Cracking occurred close to the anode where high concentrations of polysaccharides were found (Figure 5). The cracking, if not checked, would stop the current and thus electrofocusing. This problem was eliminated by either decreasing the amount of 40s antigen focused or by using a thicker gel. The heterogeneity of the 40s antigens and the similarity of their pIs made it necessary to refocus the antigens to achieve purity. Most of the antigens could be isolated as single entities but some antigens (with very close pIs) could not use this method. Possibly, by electrofocusing of the antigens in very narrow pH range (one pH unit) could total resolution be achieved.

Although isoelectrofocusing can resolve some of the 40s antigens into single entities, this was not true for the 40p antigens. The free-endotoxin isolated by Ganfield, Rebers, and Heddleston (1976) from saline extracts *P. multocida* by ultracentrifugation had a pI range of 3 to 4. Bain (1955) and Knox and Bain (1960) isolated endotoxins with specific pI values of 3.5 and 3.8. Endotoxins were protein-lipopolysaccharide complexes. The KSCN extracted 40p antigens had a similar pI range from 3 to 6 with specific pIs of 3.5, 3.6, and 3.8 as indicated by Coomassie Blue stained zymograms. Similarly, the 40p antigens we

isolated also were protein-lipopolysaccharide complexes as indicated by protein staining and the presence of KDO. In spite of the differences in pI values that we found, 40p antigens were all antigenically similar. The antigenic determinant of the endotoxins is associated with the lipopolysaccharide moiety (Rebers and Heddleston, 1974). The heterogeneity of the pIs could be caused as a result of breaking and reformation of aggregates during the extraction procedure. Another possibility is that the lipopolysaccharide was in different stages of synthesis and as a result had different acidities (Palva and Mäkelä, 1980; Goldman and Leive, 1980). Finally, the amount of protein associated with the lipopolysaccharide could vary causing the pIs to vary producing a distribution of antigenically similar complexes.

The broad pI range of the 40p antigens may result from the binding of ampholines to the antigens (Wallevik, 1973). This artifact could occur when ampholines bind to the phosphate groups of the 40p antigens (Drysdale and Righetti, 1972; Galante, et al., 1976) or possibly to the lipid moiety of the 40p antigens (Phillips, et al., 1976).

The purified antigens were grouped according to specific pI ranges in order to examine their immunogenicity. This was due to the complexity of the 40s and 40p antigens (approximately 24 protein antigens). The groups derived from the 40p fraction were identical in respect to gel diffusion, immunoelectrophoresis and analytical electrofocusing but differed in their SDS-PAGE patterns. Group 7 was more (Figure 11) complex than group 6. The groups derived from the 40s fraction were antigenically different and had different mol. wt distributions

between the groups. Preparative isoelectrofocusing to purify P. multocida antigens and the antigen groups described in this report could be used to identify immunogens associated with P. multocida.

References

- Bain, R. V. S. 1955. Studies on haemorrhagic septicaemia of cattle.
- IV. A preliminary examination of the antigens of Pasteurella multocida type 1. Brit. Vet. J. 111: 492-498.
- Bhasin, J. L., and L. Lapointe-Shaw. 1980. Antigenic analysis of Pasteurella multocida serotype 1 by cross immunoelectrophoresis: Characterization of cytoplasmic and cell envelope associated antigens. Can J. Microbiol. 26: 676-689.
- Drysdale, J. W., and P. G. Righetti. 1972. Fractionation of nucleic acids by isoelectric focusing. Biochemistry 1: 4044-4052.
- Galante, E., T. Caravaggio, and P. G. Righetti. 1976. Binding of ampholine to transfer RNA. Biochimica et Biophysica Acta 442: 309-315.
- Ganfield, D. J., P. A. Rebers, and K. L. Heddleston. 1976. Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from Pasteurella multocida. Infect. Immun. 14: 990-999.
- Gaunt, G., R. Moffat, and T. K. S. Mukker. 1978. Fowl cholera: Immunization of chickens with potassium thiocyanate (KSCN) extract of Pasteurella multocida serotype 3. Avian Dis. 21: 543-548.
- Goldman, R. C., and L. Leive. 1980. Heterogeneity of antigen-side-chain length in lipopolysaccharide from Escherichia coli 0111 and

- Salmonella typhimorium LT2. Eur. J. Biochem. 107: 145-153.
- Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1972. Fowl cholera: Gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. Avian Dis. 16: 925-936.
- Heddleston, K. L., P. A. Rebers, and A. E. Ritchie. 1966. Immunizing and toxic properties of particulate antigens from two immunogenic types of Pasteurella multocida of avian origin. J. Immunol. 96: 124-133.
- Hughes, R. C. 1976. Membrane glycoproteins: A review of structure and function. Butterworths, Boston.
- Knox, K. W., and R. V. S. Bain. 1960. The antigens of Pasteurella multocida type 1. 1. Capsular polysaccharides. Immunology 3: 352-362.
- McKinney, K. L., and R. B. Rimler. 1980. Immunoelectrophoresis employing avian antisera for the detection and quantitation of Pasteurella multocida antigens. J. Immunol. Methods (prepared for submission).
- Mukker, T. K. S. 1968. Immunological and physiologic responses of calves inoculated with potassium thiocyanate extract of Pasteurella multocida type A. Am. J. Vet. Res. 39: 1269-1273.
- Mukker, T. K. S. 1979. Immunogenicity of a chaotropically extracted protective antigen(s) of Pasteurella multocida type A (bovine origin) against experimental pasteurellosis in mice. J. Gen. Microbiol. 113: 37-43.
- Mukker, T. K. S., and P. R. Nilakantan. 1972. Preliminary characterization of the crude capsular extracts of Pasteurella multocida var bovine type 1 in phase 1. Cornell Vet. 42: 289-296.

- Namioka, S., and M. Murata. 1961. Serological studies on Pasteurella multocida. II. Characteristics of somatic (O) antigen of the organism. Cornell Vet. 51: 498-507.
- Palva, E. T., and P. H. Mäkelä. 1980. Lipopolysaccharide heterogeneity in Salmonella typhimurium analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Eur. J. Biochem. 107: 137-143.
- Phillips, M., R. E. Patterson, J. M. Patterson, and S. S. Stone. 1976. Comparison of methods for separating proteins using bisalbuminemia as a model. Prep. Biochem. 6: 265-283.
- Prince, G. H., and J. E. Smith. 1966. Antigenic studies of Pasteurella multocida using immunodiffusion techniques. I. Identification and nomenclature of the soluble antigens of bovine haemorrhagic septicaemia strain. J. Comp. Pathol. 76: 303-314.
- Rebers, P. A., K. L. Heddleston, and K. R. Rhoades. 1967. Isolation from Pasteurella multocida of a lipopolysaccharide antigen with immunizing and toxic properties. J. Bacteriol. 93: 7-14.
- Rebers, P. A., and K. L. Heddleston. 1974. Immunologic comparison of Westphal-type lipopolysaccharides and free-endotoxins from an encapsulated and a noncapsulated avian strain of Pasteurella multocida. Am. J. Vet. Res. 35: 555-560.
- Srivastava, K. K., and J. W. Foster. 1977. Characterization of an immunogenic fraction of Pasteurella multocida culture filtrates. Can. J. Microbiol. 23: 197-201.
- Wallevik, K. 1973. Isoelectric focusing of bovine serum albumin: Influence of binding of carrier ampholytes. Biochimica et Biophysica Acta 322: 75-87.

TOXICITY AND IMMUNOGENICITY OF POTASSIUM THIOCYANATE (KSCN)
 EXTRACTED AND ELECTROFOCUSED
PASTEURELLA MULTOCIDA X-731 ANTIGENS

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TOXICITY AND IMMUNOGENICITY OF POTASSIUM THIOCYANATE (KSCN)
EXTRACTED AND ELECTROFOCUSED PASTEURELLA MULTOCIDA X-73I ANTIGENS

Abstract

Potassium thiocyanate extracted Pasteurella multocida antigens that were separated into soluble (40s) and particulate (40p) fractions by ultracentrifugation and then fractionated by isoelectrofocusing and grouped into specific pI ranges were examined for immunogenicity and toxicity.

The crude extract, the 40p fraction and the electrofocused 40p antigens were highly immunogenic in chickens and mice against homologous challenge and induced detectable serological responses in more of the animals as measured by gel diffusion and counterimmunoelectrophoresis than did the 40s antigens. The LD₅₀ of these immunogens for chicken embryos ranged from 0.22 µg to 6.8 µg of protein.

The 40s fraction and some of the five electrofocused purified 40s antigen groups protected some of the chickens and none of the mice against homologous challenge. Immunogenicity increased when an adjuvant was used. The 40s fraction and not the purified 40s antigens were toxic for chicken embryos (LD₅₀ 2.5 µg to 5.8 µg of protein). The 40s bacterins induced fewer serological responses than the 40p bacterins.

The KSCN extracted antigens did not induce protection in mice or chickens against heterologous challenge.

The data indicated that there are more than one immunogen associated with P. multocida capable of inducing protection in chickens. The immunological response of chickens and mice to these antigens differed.

Introduction

Pasteurella multocida is antigenically complex (Prince and Smith, 1966; McKinney and Rebers, 1980; Bhasin and Lapointe-Shaw, 1980). Crude extracts of the organism consisting of capsular, somatic or cytoplasmic antigens can be immunogenic. Potassium thiocyanate (KSCN) has been used to extract P. multocida immunogens (Bain, 1955; Mukker, 1968, 1979; Mukker and Nilakantan, 1972) and it has been suggested that this extract could induce protection against challenge with homologous or heterologous serotypes (Gaunt, et al., 1978).

Ultracentrifugation and preparative isoelectric focusing can be used to separate and purify P. multocida antigens extracted with KSCN (McKinney and Rebers, 1980). The extracted antigens were divided into two fractions after ultracentrifugation, particulate antigens (40p) and soluble antigens (40s). Preparative isoelectric focusing was used to separate the extracted antigens into seven groups according to their pI ranges. The groups were heterogeneous in nature and serological differences were found between groups.

The purpose of this paper was to describe the immunogenic and toxic properties of the purified P. multocida antigens.

Materials and Methods

Cultures

Encapsulated P. multocida strains X-73I (Heddlestone's serotype 1) and strain P-1059I (Heddlestone's serotype 3) used were obtained from the National Animal Disease Center, Ames, Iowa.

Animals

New Hampshire Red chickens were from the closed flock at the National Animal Disease Center, Ames, Iowa; CF1 female mice were purchased from Charles River Laboratories (Wilmington, Mass.).

Antiserum preparation

Turkey anti-complete P. multocida serum was prepared as described by McKinney and Rimler (1980).

Sera samples, from vaccinated chickens, to be used for analysis by gel diffusion and counterimmunoelectrophoresis analysis were collected 17 days after the first vaccination. All sera were preserved with 1:10,000 merthiolate and stored at 4°C.

Extraction and purification of P. multocida X-73I antigens

The KSCN extract of P. multocida and the 40p and 40s antigens were prepared as described by McKinney and Rimler (1980). The 40p and 40s antigens were separated into specific electrofocused groups by preparative isoelectric focusing in a granular flatbed as described by McKinney and Rebers (1980). The electrofocused P. multocida antigens were grouped, as to pI ranges, as follows: from 40s material, group 1, pI 8.8 to 9.0; group 2, pI 6.3 to 6.6; group 3, pI 5.6 to 6.3; group 4, pI 4.9 to 5.3; and group 5, pI 4.4 to 4.9; from the 40p antigens, group 6, pI 3.5 to 3.9; and group 7, pI 5.5 to 6.0. Serological and analytical data for these specific groups are described by McKinney and Rebers (1980).

Standardization of bacterins

Bacterins were standardized to contain a specific protein concentration of 15 µg or 20 µg per dose. Protein was determined by the method of Bradford (1976) using Bio-Rad Protein assay reagents (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used for the protein standard. Corrections due to interference by ampholines and glycine were made as follows: glycine levels in the test samples were equilibrated by dialysis against multiple changes of 0.1 M glycine (ph 6.3). Ampholines were reduced in the samples by concentration and resuspension (2 times) in a Minicon B-15 concentrator (Amicon Corp., Lexington, Mass.) using 0.1 M glycine as a diluent. This method lowered the concentration of ampholines so that there was no interference with the protein assay. Correction blanks for the spectrophotometric reading consisted of a 2% solution of ampholines within a pH range equivalent to the pI range of the samples. The correction blanks were treated the same as the samples.

Bacterin preparation, immunization and challenge of chickens and mice

Bacterins of P. multocida antigens were preserved with 0.01% sodium azide.

Aqueous bacterins of formalin killed P. multocida X-73I were prepared as described by Heddleston and Rebers (1969); the bacterin contained 10^{10} cells per ml.

Adjuvant bacterins consisted of 1 volume of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich.) mixed with 1 volume of aqueous bacterin.

Aqueous bacterins were inoculated intramuscularly (i.m.) into chickens and intraperitoneally (i.p.) into mice on day 1 and day 12. The adjuvant bacterins were inoculated subcutaneously (s.c.) into the necks of the chickens at day 1. Random blood samplings of chickens in each vaccine group were made at day 17. The immunity of chickens and mice was challenged on day 21 with either P. multocida X-73I or P. multocida P-1059I. Chickens were challenged i.m. and mice i.p.

Chickens that became lame or developed sternal bursitis were considered morbid and were included in the mortality counts. All challenge experiments were terminated 14 days postchallenge.

Toxicity of P. multocida antigens for chicken embryos

Ten-day-old White Leghorn chicken embryos were used in toxicity studies. They were inoculated by the chorioallantoic membrane route as described by Horsfall and Tamm (1965). Control solutions of 1% glycine-0.01% sodium azide or 2% ampholines were also examined because of their presence in the various samples that were being tested. Embryos were examined 24 hours after inoculation. Dead embryos were cultured for bacterial contamination. Embryos dying from bacterial contamination were not included in the results. LD₅₀ of the test samples were determined by probit analysis¹.

Serological methods

Gel diffusion (g.d.) was performed using a buffer of 1.5 M NaCl, 0.01 M CaCl₂, 0.015 M barbital, 0.02 M sodium barbital at a pH of

¹Appreciation is extended to Dr. G. D. Booth for the statistical analysis.

7.6 with a 1% SeaKem LE agarose support. The g.d. plates were incubated at 37°C, after application of antiserum and antigen, then observed the next day.

Counterimmunoelectrophoresis (CIE) was done as described by McKinney and Rimler (1980) using a 0.05 M sodium acetate buffer at a pH of 5.6. The CIE was run at 6 v/cm for 45 min at 10°C. Plates were observed for immunoprecipitates with a viewing chamber and then again after staining with Coomassie Brilliant Blue R-250 (LKB Application Note #249).

Results

Immunogenicity of KSCN extracted *P. multocida* X-73I antigens in chickens as determined by homologous and heterologous challenge

The data for the immunogenicity study of the purified *P. multocida* antigens are shown in Table 1. The 40s antigen fraction and the electro-focused groups 1 through 5 (which were derived from 40s antigens) were weak immunogens (25% to 41.7% survival) when compared to the crude KSCN extract (60% survival). The use of Freund's Complete Adjuvant (FCA) increased the immunogenicity of the various 40s bacterins (57.1% to 100% survival) except in the case of the group 2 bacterin which decreased from 33.3% to 0% survival. The 40p antigen fraction and the electrofocused 40p antigen groups 6 and 7 were highly immunogenic as exemplified by the 100% survival rates with or without FCA.

The data for protection of chickens against heterologous serotype challenge (Table 1) show that the controls had a high level of survival; 63% survived. The highest level of heterologous protection was found in

Table 1. Homologous and heterologous protection induced with purified potassium thiocyanate extracted antigens of Pasteurella multocida X-73I cells in New Hampshire Red Chickens

Bacterin group	pI range	Homologous challenge ^a		Heterologous challenge ^b	
		W/O adjuvant ^c	W/adjuvant ^d	W/O adjuvant ^c	W/adjuvant ^c
KSCN ext.	—	3/5 ^e	ND ^f	3/8	4/7
40s ^g	—	5/12	ND	6/8	6/7
1 ^h	8.8-9.0	3/12	5/7	5/8	5/7
2 ^h	6.3-6.6	4/12	0/7	7/8	5/7
3 ^h	5.6-6.3	4/12	4/7	4/8	5/7
4 ^h	4.9-5.3	3/12	4/7	3/8	4/7
5 ^h	4.4-4.9	3/12	7/7	4/8	1/7
40p ⁱ	—	12/12	ND	5/8	6/7
6 ^j	3.5-3.9	12/12	7/7	4/8	5/7
7 ^j	5.5-6.0	12/12	7/7	5/8	6/7
FKC ^k	—	ND	ND	4/8	4/7
Controls	—	0/12	ND	5/8	ND

^aBirds were challenged with 1,000 CFU P. multocida X-73I i.m. at day 21.

^bBirds were challenged with 300,000 CFU P. multocida P-1059I i.m. at day 21 and day 22 with 1,250,000 CFU of P. multocida P-1059I.

^cBirds were inoculated with 20 µg of antigen protein i.m. at day 1 and day 12.

^dBirds were inoculated with 15 µg of antigen protein in Freund's Complete Adjuvant s.c. at day 1.

^eNumber survived/number challenged.

^fND = not done.

^gSoluble ultracentrifuged antigens of the KSCN extract.

^hElectrofocused purified 40s antigen groups.

ⁱInsoluble ultracentrifuged antigens of the KSCN extract.

^jElectrofocused purified 40p antigen groups.

^k10⁹ formalin killed P. multocida X-73I cells per bird per inoculation.

chickens given group 2 bacterin without adjuvant. The number of deaths in the bacterin groups usually varied by only one chicken more or less from the control group making interpretation of data difficult.

Serological reactivity of chickens immunized with KSCN extracted P. multocida antigens

The results for serological reactivity of serums collected from chickens immunized with KSCN extracted and electrofocused P. multocida antigens are shown in Table 2. Chickens immunized with the crude extract were serologically reactive by g.d. (75%) and CIE (100%). The 40s antigen fraction and the electrofocused 40s antigen groups 1 through 5 were weak antigens at the dose level tested. The percentage of sera positive in g.d. ranged from 0% to 25% and in CIE the percentage positive ranged from 33% to 50%. The 40p antigen fraction and the electrofocused 40p antigen groups 6 and 7 were highly antigenic; 87.5% to 100% of the sera were positive in g.d. and 91.7% to 100% positive in CIE. Formalin-killed cells were very antigenic in chickens inducing 85.7% positive reactions in g.d. and 71.4% positive in CIE. Chickens that were inoculated with the various 40s bacterins in FCA had a higher frequency of positive response; 27%, in g.d. Only 7% receiving the aqueous 40s bacterin were positive in g.d. CIE was more sensitive for detecting positive reactors than was g.d.

Immunogenicity of KSCN extracted P. multocida antigens in mice

The data for the immunogenicity of the P. multocida antigens in protecting mice against homologous and heterologous challenge are given in Table 3. Homologous protection (90% to 100% survival) in mice was

Table 2. Serological reactivity of chickens immunized with KSCN extracted and electrofocused antigens of Pasteurella multocida X-73I as detected by gel diffusion and counterimmunoelectrophoresis

Bacterin groups	Serological tests	Gel diffusion			Counterimmunoelectrophoresis		
	Bacterin type	Aqueous	Adjuvant	Total % ^c positive	Aqueous	Adjuvant	Total % positive
	pI range	Total pos. ^a No. bled	Total pos. ^b No. bled		Total pos. No. bled	Total pos. No. bled	
Crude ext.	—	5/7	4/5	75.0	7/7	5/5	100.0
40s	—	1/9	2/3	25.0	3/9 ^d	1/3	33.3
1	8.8-9.0	0/9	0/4	0.0	n.a.	n.a.	n.a.
2	6.3-6.6	0/11	1/4	6.7	n.a.	n.a.	n.a.
3	5.6-6.3	1/10	0/3	7.7	n.a.	n.a.	n.a.
4	4.9-5.3	1/10	2/4	21.4	6/10	1/4	50.0
5	4.4-4.9	1/8	1/4	16.7	4/8	1/4	41.7
40p	—	5/6	2/2	87.5	6/6	2/2	100.0
6	3.5-3.9	5/5	4/4	100.0	5/5	4/4	100.0
7	5.5-6.0	8/8	3/4	91.7	8/8	3/4	91.7
FKE ^e	—	2/3	4/4	85.7	1/3	4/4	71.4
Controls	—	0/6	—	0.0	0/6	—	0.0

^aNumber of serologically positive birds that received an aqueous bacterin versus the total number of birds.

^bNumber of serologically positive birds that received an adjuvant bacterin versus the total number of birds.

^cPercentage of total positive reactors.

^dn.a. = Not applicable due to the pI of the antigens and the pH of the buffering system used for CIE.

^eFormalin killed in vitro grown P. multocida X-73I.

Table 3. Homologous and Heterologous protection as induced by KSCN extracted and electrofocused Pasteurella multocida X-73I antigens in mice

Bacterin group	pI range	Serotype 1 challenge ^a	Serotype 3 challenge ^b
		No. survived/ No. challenged ^c	No. survived/ No. challenged
Crude ext.	—	10/10	0/10
40s	—	0/10	0/10
1	8.8-9.0	0/10	0/10
2	6.3-6.6	0/10	0/10
3	5.6-6.3	0/10	0/10
4	4.9-5.3	0/10	0/10
5	4.4-4.9	0/10	0/10
40p	—	10/10	0/10
6	3.5-3.9	10/10	0/10
7	5.5-6.0	9/10	0/10
FKC ^d	—	9/10	0/10
In vivo C ^e	—	10/10	10/10
Controls	—	0/10	0/10

^aMice were challenged with 1,000 CFU of P. multocida X-73I on day 21.

^bMice were challenged with 1,000 CFU of P. multocida P-1059I on day 21.

^cMice were vaccinated with 20 µg of bacterial protein i.p. at day 1 and day 12.

^dMice were vaccinated with 10⁹ formalin killed P. multocida X-73I on day 1 and day 12.

^eMice were vaccinated with 10⁹ formalin killed in vivo grown P. multocida on day 1 and on day 12.

obtained with all bacterins containing 40p antigens, i.e., group 6, group 7, and 40p. The 40s antigens did not induce homologous immunity. None of the bacterins derived from in vitro culture P. multocida induced protection against heterologous challenge including formalin-killed cells cultured in vitro. In contrast, in vivo cultured P. multocida P-1059I cells killed with formalin (prepared as described in Rimler, et al., 1979) induced cross-protection. In vivo grown P. multocida X-73I can also induce cross-protection (R. B. Rimler, et al., 1979).

Toxicity of the crude extract and ultracentrifuged KSCN extracted P. multocida antigens

The toxicity of the crude KSCN extract, 40s and 40p antigens for chicken embryos is shown in Table 4. The LD₅₀ for the crude extract ranged from 0.75 µg to 14.3 µg of protein while the LD₅₀ for the 40s antigens ranged from 2.5 µg to 5.8 µg of protein. The LD₅₀ of the 40p antigens was 0.22 µg to 0.38 µg of protein.

The electrofocused 40s groups 1, 2, 3, 4, and 5 were not toxic at the levels tested. The electrofocused 40p groups 6 and 7 were toxic and had LD₅₀'s of 6.8 µg and 2.6 µg of antigen protein, respectively. Controls (not shown) of 2% ampholine-1% glycine-0.01% sodium azide and 1% glycine-0.01% sodium azide were not toxic for the embryos.

Table 4. Toxicity of KSCN extracted Pasteurella multocida X-73I antigens in chicken embryos

Antigen prep.	Trial One		Trial Two	
	Max. amnt. ^a inoculated	LD ₅₀ ^b	Max. amnt. ^c inoculated	LD ₅₀ ^b
Ext.	6.4 µg	0.75 µg (0.27, 2.29) ^c	10.0 µg	14.3 µg (1.38, 88,444)
40s ^d	5.69 µg	2.5 µg (0.82, 49.6)	10.0 µg	5.8 µg (1.86, 48.1)
Group 1 ^e	10.0 µg	N.T. ^f	—	—
Group 2 ^e	14.7 µg	N.T.	—	—
Group 3 ^e	19.2 µg	N.T.	—	—
Group 4 ^e	17.8 µg	N.T.	—	—
Group 5 ^e	20.0 µg	N.T.	—	—
40p ^g	10.0 µg	0.38 µg (0.12, 1.33)	10.0 µg	0.22 µg (0.09, 0.55)
Group 6 ^h	10.0 µg	6.8 µg (1.2, 431.1)	—	—
Group 7 ^h	10.0 µg	2.6 µg (0.5, 108.1)	—	—

^aMaximum amount of protein inoculated.

^bLD₅₀ was determined by probit analysis.

^cUpper and lower confidence limits.

^dSoluble antigens from ultracentrifuged crude extract.

^eElectrofocused antigens derived from the 40s fraction.

^fNot toxic at levels examined.

^gInsoluble antigens from ultracentrifuged crude extract.

^hElectrofocused antigens derived from the 40p fraction.

Discussion

The immunological activity of KSCN extracted 40p antigens was similar to the immunogenicity of the material associated with the pellet of ultracentrifuged extracts (i.e., free-endotoxin (FET) and lipopolysaccharide (LPS)) as has been described by Heddleston, et al., 1966; Heddleston and Rebers, 1975; and Ganfield, et al., 1976. The 40p antigens were highly immunogenic in chickens and mice against homologous challenge as evident by protection levels of 90% to 100% and high frequencies of antibody responses (87.5% to 100% of the chickens tested). The KSCN extracted 40p antigens were similar to the FET in both pI values and the presence of KDO. The serological specificity of the FET is associated with the LPS moiety (Heddleston and Rebers, 1975; Rebers and Heddleston, 1974; Heddleston, Gallagher, and Rebers, 1972) and induces serotype specific immunity in mice (Rebers, Heddleston, and Rhoades, 1967) and in our experiment mice responded only to the serotype specific antigens indicating that the 40p antigens are the serotype specific antigen.

FET isolated from noncapsulated *P. multocida* had a LD₅₀ of 5.2 µg for embryos and is toxic for mice at the 194 µg level (Ganfield, Rebers, and Heddleston, 1976). The toxicity of the KSCN extract was mainly associated with the 40p antigens. The LD₅₀ value of our electrofocused 40p group 6 and 7 was 6.8 µg and 2.6 µg of antigen protein, respectively. The electrofocused groups 6 and 7 were 10x less toxic than the initial 40p fraction. Possibly the purified components could act synergistically. The variations in the LD₅₀ might be due to varying amounts of LPS as-

sociated with the proteins of the electrofocused groups.

The supernatant (40s) of the ultracentrifuged KSCN extract contained approximately 24 antigens (McKinney and Rebers, 1980) and testing individual antigens for immunogenicity would have been prohibitive. Thus, the antigens were grouped and then tested for immunogenicity. The results showed that the 40s antigens were protective for chickens, but not for mice against homologous challenge. The 40s fraction and the electrofocused 40s antigen groups were not as immunogenic as the 40p antigens at the same dose level. The immunogenicity of the 40s fractions was increased when FCA was used. Group 2 antigens when mixed with FCA were not immunogenic. Possibly the FCA destroyed the group 2 antigens or suppressed the immunological response to these antigens. The frequency of antibody response of chickens to the 40s antigens was low as shown by 0% to 50% responding to the various 40s groups. Variations in the response of mice and chickens to P. multocida have been reported by Yaw and Kakavas (1957), Yaw, Briefman and Kakavas (1956), Heddlestone and Rebers (1969), and Heddlestone and Hall (1958). Capsulated cells or capsular polysaccharides were immunogenic for chickens and mice but noncapsulated cells (somatic antigens) were more immunogenic for chickens than for mice. Possibly the 40s fraction contained mainly somatic antigens for which the mice were nonresponsive.

The 40s fraction did induce death in chicken embryos. It was not as lethal as the 40p fraction on a protein weight bases. The 40s fraction could have been contaminated with small concentration of 40p material but this was not evident by g.d. or CIE examination. This suggests that a second toxin is present in the 40s fraction which is

capable of inducing protection in chickens but not in mice. The 40p toxin will induce protection in both chicken and mice (Ganfield, Rebers, and Heddleston, 1976; Heddleston, Rebers, and Ritchie, 1966). When the 40s antigens were separated and purified by electrofocusing, toxicity was lost. This loss indicated either that the toxin was detoxified or that the 40s antigens were only toxic when used in combination.

KSCN extracts of in vitro grown P. multocida have been reported to protect against different serotypes by Gaunt, Moffat, and Mukker (1978). Protection against different serotypes has been observed using P. multocida infected tissue or P. multocida separated from turkey blood (Heddleston and Rebers, 1972, 1974; Rebers and Heddleston, 1977; Rimler, Rebers, and Rhoades, 1979). The P. multocida cells separated from turkey blood were protective against challenge with different serotypes in chickens, turkeys, and mice. We showed that protection against a different serotype was not induced by the KSCN extracts of in vitro grown P. multocida X-73I because the control chickens were very resistant to challenge with P. multocida P-1059I. Heddleston (1962) reported that 16 week old New Hampshire chickens were more resistant to challenge with P. multocida P-1059I than were 45 week old chickens. Possibly, this phenomenon occurred in our cross-challenge experiment. Gaunt, Moffat, and Mukker (1978) used male cornish (Hubbard) chickens and that their resistance to challenges with P. multocida X-73I and P-1059I might be different than those of New Hampshire chickens. Also, the route of challenge may be of importance. Gaunt, Moffat, and Mukker (1978) used the i.p. route and we used an i.m. route. A large number of the surviving chickens developed sternal bursitis

from which viable P. multocida P-1059I was isolated indicating that the birds were only partially protected.

The virulence of P. multocida in animals varies according to species (Heddlestone and Watko, 1963; Collins, 1977). Mice are highly susceptible to both P. multocida X-73I and P-1059I. For this reason they were used to study protection against both homologous and heterologous challenge. Gaunt, Moffat, and Mukker (1978) stated that mice were protected by KSCN extracts against challenge with 3 heterologous P. multocida serotypes. Mice can be protected against either P. multocida P-1059I or X-73I when vaccines are made with in vivo cultured P. multocida. In vitro grown P. multocida P-1059I or X-73I did not protect against heterologous serotype challenges. The KSCN extracts of P. multocida X-73I did not protect against challenge with P. multocida P-1059I but in vivo grown P. multocida P-1059I did. These data are consistent with past experiments which showed that in vitro grown P. multocida induce serotype specific protection. The likelihood of being able to isolate antigens from extracts of in vitro grown cells that will protect against challenge with a heterologous serotype is much less than the likelihood of isolating these antigens from in vivo grown cells in light of our data.

Serological methods for determination of the immune status of fowls are not reliable when antigen mixtures are used. Alexander and Soltys (1973) and Heddlestone and Watko (1965) did not show correlation of antibody response with survival against challenge. Survival to challenge might depend upon a cellular response as well as humoral responses (Dua and Maheswaran, 1978; Collins, 1977). The 40p antigens induced higher

frequency of antibody responses as detected by gel diffusion or CIE. The serological reaction to purified 40s antigens or 40p antigens may be a better indicator of the state of ~~immunity~~. The 40p antigens were not the only protective antigens associated with P. multocida in chickens but were the only protective antigens for mice. A serological test to determine ~~immune~~ status should consider both the specific host response to the protective ~~immunogens~~ for that host.

If a good correlation between serological results and the ~~immune~~ status of the chickens is to be obtained, one criteria should be that it is essential to have a pure preparation(s) of the protective antigens to be used to quantitate that ~~immunity~~. A mixture of protective and non-protective antigens serves only to confuse the interpretation of the results. The precipitin test can only be used to quantitate this ~~immune~~ response if precipitating antibodies are the protective antibodies. Nonprecipitating but protective antibodies could escape detection by conventional precipitin tests. Hence, the passive protection test would be more reliable to determine the presence of nonprecipitating protective antibodies.

References

- Alexander, A. M., and M. A. Soltys. 1973. Relationship of serum agglutinins to protective immunity produced in turkeys immunized against fowl cholera. J. Comp. Pathol. 83: 191-198.
- Bain, R. V. S. 1955. Studies on haemorrhagic septicaemia of cattle. IV. A preliminary examination of the antigens of Pasteurella multocida type 1. Brit. Vet. J. 111: 492-498.
- Bhasin, J. L., and L. Lapointe-Shaw. 1980. Antigenic analysis of Pasteurella multocida serotype 1 by crossed immunoelectrophoresis: Characterization of cytoplasmic and cell envelope associated antigens. Can. J. Microbiol. 26: 676-689.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochemistry 72: 248-254.
- Collins, F. M. 1977. Mechanisms of acquired resistance to Pasteurella multocida infection: A review. Cornell Vet. 67: 103-138.
- Dua, S. K., and S. K. Maheswaran. 1978. Studies of Pasteurella multocida. VI. Nature of systemic immunity and analysis of the correlation between levels of immunity induced by various fowl cholera vaccines and protection against challenge. Avian Dis. 22: 748-764.
- Ganfield, D. J., P. A. Rebers, and K. L. Heddleston. 1976. Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from Pasteurella multocida. Infect. Immun. 14: 990-999.
- Gaunt, G., R. Moffat, and T. K. S. Mukker. 1978. Fowl cholera: Immunization of chickens with potassium thiocyanate (KSCN) extract

- of Pasteurella multocida serotype 3. Avian Dis. 21: 543-548.
- Heddleston, K. L. 1962. Studies on Pasteurella multocida: V. Two immunogenic types of Pasteurella associated with fowl cholera. Avian Dis. 6: 315-321.
- Heddleston, K. L., and W. J. Hall. 1958. Studies on pasteurellosis. II. Comparative efficiency of killed vaccines against fowl cholera in chickens. Avian Dis. 2: 322-335.
- Heddleston, K. L., and P. A. Rebers. 1969. Pasteurella multocida: Immune response in chicks and mice. Proc. 73rd Ann. Meeting U.S.A.H.A., Milwaukee, Wis., Oct. 12-17, 1969. pp. 280-284.
- Heddleston, K. L., and P. A. Rebers. 1972. Fowl cholera: Cross-immunity induced in turkeys with formalin-killed in vivo propagated Pasteurella multocida. Avian Dis. 16: 578-586.
- Heddleston, K. L., and P. A. Rebers. 1974. Fowl cholera bacterins: Host specific cross-immunity induced in turkeys with Pasteurella multocida propagated in embryonating turkey eggs. Avian Dis. 18: 213-219.
- Heddleston, K. L., and P. A. Rebers. 1975. Properties of free endotoxin from Pasteurella multocida. Am. J. Vet. Res. 36: 573-574.
- Heddleston, K. L., and L. P. Watko. 1963. Fowl cholera: Susceptibility of various animals and their potential as disseminators of the disease. 67th Ann. Prod. U.S. Livestock Sanitary Assoc., Oct. 1963. pp. 247-251.
- Heddleston, K. L., and L. P. Watko. 1965. Fowl cholera: Comparison of serologic and immunogenic responses of chickens and turkeys. Avian Dis. 9: 367-376.

- Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1972. Fowl cholera: Gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. Avian Dis. 16: 925-936.
- Heddleston, K. L., P. A. Rebers, and A. E. Ritchie. 1966. Immunizing and toxic properties of particulate antigens from two immunogenic types of Pasteurella multocida of avian origin. J. Immunol. 96: 124-133.
- Horsfall, F. L., Jr., and I. Tamm (ed.). 1965. Viral and rickettsial infections of man, 4th ed. J. B. Lippincott Co., Philadelphia, Pa.
- McKinney, K. L., and P. A. Rebers. 1980. Separation of Pasteurella multocida antigens by isoelectric focusing. Can. J. Microbiol. (prepared for submission).
- McKinney, K. L., and R. B. Rimler. 1980. Immunoelectrophoresis employing avian antisera for the detection and quantitation of Pasteurella multocida antigens. J. Immunol. Methods (prepared for submission).
- Mukker, T. K. S. 1968. Immunological and physiologic responses of calves inoculated with potassium thiocyanate extract of Pasteurella multocida type A. Am. J. Vet. Res. 39: 1269-1273.
- Mukker, T. K. S. 1979. Immunogenicity of a chaotropically extracted protective antigen(s) of Pasteurella multocida type A (bovine origin) against experimental pasteurellosis in mice. J. Gen. Microbiol. 113: 37-43.
- Mukker, T. K. S., and R. R. Nilakantan. 1972. Preliminary characterization of the crude capsular extracts of Pasteurella multocida var. bovine type 1 in phase 1. Cornell Vet. 42: 289-296.

- Prince, G. H., and J. E. Smith. 1966. Antigenic studies on Pasteurella multocida using immunodiffusion techniques. I. Identification and nomenclature of the soluble antigens of a bovine haemorrhagic septicaemia strain. J. Comp. Pathol. 76: 303-314.
- Rebers, P. A., and K. L. Heddleston. 1974. Immunologic comparison of Westphal-type lipopolysaccharides and free endotoxins from an encapsulated and a noncapsulated avian strain of Pasteurella multocida. Am. J. Vet. Res. 35: 555-560.
- Rebers, P. A., and K. L. Heddleston. 1977. Fowl cholera: Induction of cross-protection in turkeys with bacterin prepared from host-passaged Pasteurella multocida. Avian Dis. 21: 50-56.
- Rebers, P. A., K. L. Heddleston, and K. R. Rhoades. 1967. Isolation from Pasteurella multocida of a lipopolysaccharide antigen with immunizing and toxic properties. J. Bacteriol. 93: 7-14.
- Rimler, R. B., P. A. Rebers, and K. R. Rhoades. 1979. Fowl cholera: Cross-protection induced by Pasteurella multocida separated from infected turkey blood. Avian Dis. 23: 730-741.
- Yaw, K. E., and J. C. Kakavas. 1957. A comparison of the protection-inducing factors in chickens and mice of a type 1 strain of Pasteurella multocida. Am. J. Vet. Res. 18: 661-664.
- Yaw, K. E., L. Briefman, and J. C. Kakavas. 1956. A comparison of virulence for mice and chickens of different colonial variant of the three serological types of Pasteurella multocida. Am. J. Vet. Res. 17: 157-159.

CHEMOTAXIS OF FOWL MONOCYTES TO PASTEURELLA MULTOCIDA
AND ASSOCIATED ANTIGENS

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AND ASSOCIATED ANTIGENS

Abstract

The interaction of monocytes and serum from Pasteurella multocida-immunized and nonimmunized turkeys and chickens was studied for their chemotactic activity to lipopolysaccharide, free endotoxin, and P. multocida whole cells. Chemotactic factors were released from the interaction between either normal sera or antisera and the bacterial antigens. Heating of the sera affected their ability to interact and release chemotaxins. Heated normal sera were ineffective and heated antisera were less effective than unheated sera. Monocytes collected from immunized and nonimmunized fowls responded to the chemotactic factors. This occurrence of heat-labile and heat-stable chemotaxins in the sera suggests that antibody and complement are involved in the stimulation of chemotaxis.

Introduction

In the immunological study of bacterial infections, chemotaxis generally refers to the migrational response of phagocytes (Altman and Kirchner, 1974; Lachmann, Kay and Thompson, 1970; Lett-Brown, Roethoher and Leonard, 1976; Snyderman, et al., 1972; Snyderman, Shin and Hausman, 1971; Sorkin, Stecher and Borel, 1970; Ward, 1968; Ward, Lepow and Newman, 1968) to chemotaxins. The chemotaxins are mainly produced from the complement system. This occurs when bacteria (Symon, McKay and Wilkinson, 1972) or their components, such as lipopolysaccharides (Keller and Sorkin, 1967; Snyderman, et al., 1969; Symon, McKay and Wilkinson, 1972),

activate the complement system by means of the alternate or classical pathway (Frank, May and Kane, 1973; Gewurz, Shin and Mergenhagen, 1968; Lachmann, Kay and Thompson, 1970; Lett-Brown, Roethoher and Leonard, 1976; Mergenhagen, Snyderman and Phillips, 1973; Snyderman, Shin and Hausman, 1971; Snyderman, et al., 1969; Symon, McKay and Wilkinson, 1972; Ward, 1967).

This report describes the in vitro chemotaxis of chicken and turkey blood monocytes to Pasteurella multocida lipopolysaccharide (LPS), free endotoxin (FET), and P. multocida bacteria which have been suspended with turkey or chicken sera.

Materials and Methods

Animals

New Hampshire chickens and Small Beltsville White turkeys were obtained from closed flocks at the National Animal Disease Center.

Bacteria

An encapsulated strain of P. multocida X-73 serotype 1 (Heddlestone typing system) was used. It was cultivated on dextrose starch agar plates (Heddlestone, Gallagher and Rebers, 1970).

Bacterial antigens

The LPS was obtained by the Westphal procedure (Rebers and Heddlestone, 1974). The FET was isolated from the saline extract of X-73 by differential centrifugation (Ganfield, Rebers and Heddlestone, 1976; Heddlestone and Rebers, 1975).

Bacterin

Bacterins were made from X-73 formalin-killed cells and the suspension was adjusted to 10X MacFarland tube #1 by spectrophotometric means (Heddleston, Gallagher and Rebers, 1970).

Immunization

The turkeys and chickens were inoculated intravenously with 1 ml of bacterin at weekly intervals until the experiment was completed.

Serum collection

Blood was collected from the wing vein, allowed to clot at room temperature, and centrifuged at 4 C; then the serum was separated. Turkey or chicken normal serum or antiserum was then pooled and stored at - 20 C. Antiserum was obtained from blood of two turkeys or chickens. Blood was collected 5 days after the second weekly inoculation and frozen in 1-ml aliquots and used when needed. Normal serum was collected from turkeys or chickens that had no exposure to P. multocida. When needed, the various sera were heated at 56 C for 30 min to inactivate the complement components of the serum (Rose and Orlans, 1962).

Isolation of monocytes

Blood monocytes were isolated by the method of Snyderman, et al. (1969) as modified by Altman and Kirchner (1972). The Ficoll-Hypaque¹ solution had a specific gravity of 1.086, and the monocytes were resuspended to a concentration of approximately 150,000 monocytes per ml

¹Ficoll: Pharmacia, Uppsala, Sweden; Hypaque: Winthrop Laboratories, New York.

in a 10% heat inactivated normal turkey or chicken serum in Hank's balanced salt solution (HBBS). Normal monocytes were collected from turkeys and chickens that had no exposure to P. multocida. Activated monocytes were collected from turkeys and chickens used for antiserum production after they had two or more exposures to the bacterin. The activated monocytes were collected 5 days after each inoculation.

Measurement of chemotaxis

Chemotaxis was assayed according to a modification of the method of Boyden (1962) as reported by Snyderman, et al. (1969). The concentration of antigens used per chamber test well was either 2.45 μ g of LPS, 2.35 μ g of FET, or 10^8 viable P. multocida X-73 in 10% test fowl serum-HBSS (cultures in tryptose broth were standardized by spectrophotometric means at 600 nm to produce an 80% transmission that was equal to approximately 10^9 CFU/ml (Heddleston and Rebers, 1975). The concentrations of antigens used were predetermined to produce maximal chemotactic response at the minimal antigen concentration.

The upper and lower chambers of the modified Boyden chamber were separated by polycarbonate membranes with a pore size of 5 μ m (Bio-Rad Laboratories, Richmond, California). The lower chamber contained the test antigens in 10% turkey or chicken test serum. The upper chamber contained approximately 80,000 monocytes in 10% heated turkey or chicken normal serum (normal turkey serum was used for turkey monocytes and normal chicken serum for chicken monocytes). The Boyden chambers were incubated for 90 min in a closed humidified container at 37 C, after which the monocyte suspension was resuspended and aspirated, as was the

antigen solution. The membrane was stained with Wright's stain, and then with Geimsa stain. The bottom side of the membrane (side exposed to the antigen-serum solution) was examined microscopically (1000X); at least 10 fields were counted. The average number of monocytes per microscopic field was then determined. The experiments were duplicated by using two Boyden chambers and were then repeated with different batches of monocytes and sera.

Chemotactic ratios were calculated by dividing the average number of monocytes per field of the experiment using serum and P. multocida antigens by the average number of monocytes per field of the experiment using serum without P. multocida antigen (control). Different batches of monocytes had varying chemotactic activity. The variations were taken into account by running the serum-only blanks with each experiment. Average chemotactic ratios were determined by averaging the chemotactic ratios from similar repeated chemotactic tests. Chemotactic ratios greater than 1.18 indicated that chemotaxis was significant ($p < .05$ by use of the Student's t test). Chemotactic trends were perceivable by the use of this method, even though the chemotactic activity of the monocytes and serum varied.

Results

The presence of serum was needed for chemotaxis to occur. LPS, FET, and P. multocida cells in HBSS were not chemotaxins for the monocytes. Table 1 shows the chemotactic response of turkey and chicken monocytes to turkey or chicken serum mixed with LPS, FET, or P. multocida.

Table 1. Chemotactic ratios of turkey or chicken monocytes as stimulated by mixtures of Pasteurella multocida antigens and turkey or chicken serums

Antigen ^c	Serum ^d	Treatment ^e	Average chemotactic ratios of monocytes ^{a,b}			
			NTMØ ^f	ATMØ ^g	NCMØ ^h	ACMØ ⁱ
LPS	NTS	None	2.02	2.31	ND	ND
		HTD	0.84	0.80	ND	ND
	NCS	None	1.26	ND	1.18	1.29
		HTD	1.07	ND	(1.02)	(1.30)
	TAS	None	1.54	2.35	ND	ND
		HTD	1.18	0.83	ND	ND
	CAS	None	1.57	ND	1.47	1.59
		HTD	(1.12)	ND	1.16	(0.98)
FET	NTS	None	2.33	1.52	ND	ND
		HTD	0.66	1.09	ND	ND
	NCS	None	1.33	ND	0.87	1.36
		HTD	(1.02)	ND	ND	ND
	TAS	None	2.35	2.13	ND	ND
		HTD	1.74	1.19	ND	ND
	CAS	None	1.31	ND	1.60	ND
		HTD	(1.10)	ND	ND	ND
LC	NTS	None	2.23	1.19	ND	ND
		HTD	1.13	1.07	ND	ND
	NCS	None	ND	ND	1.65	0.87
		HTD	ND	ND	ND	ND

TAS	None	2.20	1.79	ND	ND
	HTD	1.02	1.14	ND	ND
CAS	None	ND	ND	ND	ND
	HTD	ND	ND	ND	ND

^aND = not done.

^bValues in parentheses represent one determination.

^cLPS = lipopolysaccharide; FET = free endotoxin; LC = live P. multocida.

^dNTS = normal turkey serum; NCS = normal chicken serum; TAS = turkey antiserum; CAS = chicken antiserum.

^eHTD = serum heated at 56 C for 30 min.

^fNTMØ = normal turkey monocytes.

^gATMØ = activated turkey monocytes.

^hNCMØ = normal chicken monocytes.

ⁱACMØ = activated chicken monocytes.

LPS, when mixed with normal turkey or chicken serum, was chemotactic for normal or activated turkey or chicken monocytes. When the normal serum was heated, the chemotactic activity was destroyed. LPS, when mixed with turkey or chicken antiserum, was also chemotactic for the monocytes; and when the antiserum was heated, the chemotactic activity was destroyed.

FET, when mixed with normal turkey or chicken serum, was chemotactic for normal and activated monocytes, except in the case when FET was mixed with normal chicken serum and normal chicken monocytes were used. Heating the normal sera from turkeys or chickens destroyed the chemotactic activities. FET and turkey or chicken antiserum was chemotactic for normal and activated turkey and chicken monocytes. When the antiserum was heated, the chemotactic activity was less than that of unheated antiserum.

Live P. multocida, when mixed with normal turkey or chicken serum, was chemotactic for normal and activated monocytes; when the serum was heated, the chemotactic activity was destroyed. Also, turkey antiserum and P. multocida were chemotactic for normal and activated turkey monocytes; heating the antiserum destroyed the chemotactic activity. The chemotactic response was not species-specific, because turkey monocytes could respond to chicken-serum chemotaxins.

Discussion

The results showed that heat-labile substances in fowl serum are responsible for the chemotaxis of fowl monocytes. Heat lability suggests

that these serum substances are derived from the complement system which can be activated by two pathways. One of the pathways is the alternate pathway (Frank, May and Kane, 1973), which is independent from the presence of antibody. In this pathway, the production of the complement chemotaxins (Lachmann, Kay and Thompson, 1970; Lett-Brown, Roethoher and Leonard, 1976; Ward, 1967) can be caused by the interaction between bacterial antigens and the properidin system, which then activates the complement system, starting with the C_3 component and consumption of C_5 through C_9 , and releasing the chemotactic components. This method of chemotaxin production could have occurred when P. multocida antigens reacted with normal turkey and chicken sera (Table 1), which stimulated chemotaxis of the monocytes. The alternate pathway is heat labile in that various components like factor B and complement (Brumfield, Benson and Pomeroy, 1961; Rose and Orlans, 1962), are heat sensitive; thus, when normal fowl serum was heated, the chemotactic activity was destroyed.

The chemotaxins could also be produced by the classical pathway of complement activation. In this pathway, complement is activated by the interaction of antibody and antigen, which then interacts with complement component C_1 , which precedes the consequential consumption of C_2 through C_9 . This process produces the same chemotaxins as the alternate pathway; it too is heat labile. Antiserum collected from immunized chickens and turkeys had chemotactic activity, and when the antiserum was heated, the activity was diminished or destroyed. The chemotaxins were probably produced by both the classical and alternate pathways. Heating the antiserum destroyed the complement system and left just antibody-antigen complexes to act as possible chemotaxins. However, the data on heated

antiserum are not conclusive enough to prove this point (Leung-Tack, Maillard and Voisin, 1977).

The production of chemotaxins in vivo could be one of multiple immune responses (Collins, 1977; Collins and Woolcock, 1973) to P. multocida infection. The in vitro data collected show a possible in vivo interaction between the chemotaxigens, such as antibody, P. multocida, and its soluble antigens, and the heat-labile, chemotaxin-producing systems, which induces chemotaxis of macrophages. The attraction of the phagocytes to the sites of infection (Rhoades, Heddleston and Rebers, 1967) could be an important step in stopping the spread of infection, especially if the phagocytes engulf and kill P. multocida (Bain, 1955; McKinney and Rebers, 1977; Woolcock and Collins, 1976; Yamaguchi and Baba, 1975).

References

- Altman, L. C., and H. Kirchner. 1972. The production of a monocyte chemotactic factor by aggamaglobulinemic chicken spleen cells. J. Immunol. 109: 1149-1151.
- Altman, L. C., and H. Kirchner. 1974. Mononuclear leucocyte chemotaxis in chicken. Definition of a phylogenetically specific lymphokine. Immunology 26: 393-405.
- Bain, R. V. S. 1955. Studies on haemorrhagic septicaemia of cattle. IV. A preliminary examination of the antigens of Pasteurella multocida type 1. Brit. Vet. J. 111: 492-498.
- Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J. Exp. Med. 115: 453-466.

- Brumfield, H. P., H. Benson, and B. J. Pomeroy. 1961. Procedure for modified complement fixation test with turkey, duck, and chicken serum antibody. *Avian Dis.* 5: 270-282.
- Collins, F. M. 1973. Growth of Pasteurella multocida in vaccinated and normal mice. *Infect. Immun.* 8: 868-875.
- Collins, F. M. 1977. Mechanisms of acquired resistance to Pasteurella multocida infection: A review. *Cornell Vet.* 67: 103-130.
- Collins, F. M., and J. B. Woolcock. 1973. Immune responses to Pasteurella multocida in the mouse. *J. Reticuloendothel. Soc.* 19: 311-321.
- Frank, M. M., J. E. May, and M. A. Kane. 1973. Contributions of the classical and alternate complement pathways to the biological effects of endotoxin. *J. Infect. Dis.* 128 (Suppl.: S176-S181).
- Ganfield, D. J., P. A. Rebers, and K. L. Heddleston. 1976. Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from Pasteurella multocida. *Infect. Immun.* 14: 990-999.
- Gewurz, M., M. S. Shin, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxic lipopolysaccharide: Consumption of each of the six terminal complement components. *J. Exp. Med.* 128: 1049-1057.
- Heddleston, K. L., and P. A. Rebers. 1975. Properties of free endotoxin from Pasteurella multocida. *Am. J. Vet. Res.* 36: 573-574.
- Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1970. Fowl cholera: Immune response in turkeys. *Avian Dis.* 14: 626-635.
- Keller, H. U., and E. Sorkin. 1967. Studies on chemotaxis. V. On the chemotactic effect of bacteria. *Int. Arch. Allergy* 31: 505-517.
- Lachmann, P. J., A. B. Kay, and R. A. Thompson. 1970. The chemotactic

- activity for neutrophil and eosinophil leukocytes of the trimolecular complex of the fifth, sixth and seventh components of human complement (C_{567}) prepared in free solution by "reactive lysis" procedure. Immunology 19: 895-899.
- Lett-Brown, M. A., D. A. Roethoher, and E. J. Leonard. 1976. Chemotactic responses of normal human basophils to lymphocytes-derived chemotactic factor and to C_{5a} . J. Immunol. 117: 246-252.
- Leung-Tack, J., J. Maillard, and G. A. Voisin. 1977. Chemotaxis for polymorphonuclear leukocytes induced by soluble antigen-antibody complexes. Immunology 33: 937-945.
- McKinney, K. L., and P. A. Rebers. 1977. Phagocytosis of Pasteurella multocida by turkey macrophages. 28th Annual North Central Poultry Disease Conference. June 7-9, 1977, Fargo, ND. Abstract p. 36.
- Mergenhausen, S. E., R. Snyderman, and J. K. Phillips. 1973. Activation of complement by endotoxin. J. Infect. Dis. 128 (Suppl.): S86-S90.
- Rebers, P. A., and K. L. Heddleston. 1974. Immunologic comparison of Westphal-type lipopolysaccharides and free endotoxins from an encapsulated and a nonencapsulated avian strain of Pasteurella multocida. Am. J. Vet. Res. 35: 555-560.
- Rhoades, K. R., K. L. Heddleston, and P. A. Rebers. 1967. Experimental hemorrhagic septicemia gross and microscopic lesions resulting from acute infections and from endotoxin administration. Can. J. Comp. Med. Vet. Sci. 31: 226-233.
- Rose, M. E., and E. Orlans. 1962. Fowl antibody: III. Its haemolytic activity with complement of various species and some properties of

- fowl complement. Immunology 5: 633-641.
- Snyderman, R., L. C. Altman, M. S. Hausman, and S. E. Mergenhagen. 1972. Human mononuclear leukocyte chemotaxis: A quantitative assay for humoral and cellular chemotactic factors. J. Immunol. 108: 857-860.
- Snyderman, R., H. S. Shin, and M. S. Hausman. 1971. A chemotactic factor for mononuclear leukocytes. Proc. Soc. Exp. Biol. Med. 138: 387-390.
- Snyderman, R., H. S. Shin, J. K. Phillips, H. Gewurz, and S. E. Mergenhagen. 1969. A neutrophil chemotactic factor derived from C_5 upon interaction of guinea-pig serum with endotoxin. J. Immunol. 103: 413-422.
- Sorkin, E., V. S. Stecher, and J. F. Borel. 1970. Chemotaxis of leukocytes and inflammation. Ser. Haematol. 3: 131-174.
- Symon, D. N. K., I. C. McKay, and P. C. Wilkinson. 1972. Plasma-dependent chemotaxis of macrophages towards Mycobacterium tuberculosis and other organisms. Immunology 22: 267-276.
- Ward, P. A. 1967. A plasmin-split fragment of C'_3 as a new chemotactic factor. J. Exp. Med. 126: 189-206.
- Ward, P. A. 1968. Chemotaxis of mononuclear cells. J. Exp. Med. 128: 1201-1221.
- Ward, P. A., I. H. Lepow, and L. J. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. Am. J. Pathol. 52: 725-736.
- Woolcock, J. B., and F. M. Collins. 1976. Immune mechanisms in Pasteurella multocida infected mice. Infect. Immunol. 13: 949-958.

Yamaguchi, Y., and T. Baba. 1975. Demonstration in tissue culture of cellular immunity to fowl cholera. Proc. First Intersect. Congress of I.A.M.S. 4: 14-18.

GENERAL DISCUSSION AND CONCLUSIONS

The results of this investigation included the successful application of a new immunoelectrophoretic procedure employing fowl serum to detect bacterial antigens. The second part of this study describes an improved procedure for the purification of P. multocida antigens by ultracentrifugation and preparative isoelectrofocusing. The third part of this study compares the immunogenicity, antigenicity and toxicity of the purified P. multocida antigens. The fourth and final part describes the interaction of P. multocida with fowl serum to induce chemotaxis of turkey and chicken blood monocytes.

The use of fowl serum for various immunoelectrophoretic techniques has been only partly successful in the past because electrophoresis cannot be performed in the presence of 1 M to 2 M NaCl. This high concentration of NaCl is needed for optimal immunoprecipitation in gels. The pH of the buffer is also important, especially when the immunoglobulins are supposed to be immobilized in the agar, i.e., rocket and two-dimensional immunoelectrophoresis. Conventional immunoelectrophoresis uses a buffer with a pH of 8.0 or 8.6 to immobilize mammalian antibodies, but this pH is not suitable for avian antiserum because the antibodies are mobile at this pH. The pI of avian immunoglobulin ranges from 5.2 to 6.6 which is lower than mammalian immunoglobulins (7 and above). Thus, using an electrophoresis buffer with a pH within the pI range of the avian immunoglobulin should immobilize the immunoglobulin during electrophoresis.

The newly developed electrophoresis buffer consisted of 0.05 M sodium acetate-acetic acid, pH 5.6. This buffer was suitable for one-

or two-dimensional, rocket- and counterimmunoelectrophoresis. The 17% salt treatment of one-dimensional immunoelectrophoresis plates improved the immunoprecipitin reaction and the resolution of the precipitation patterns. High salt treatment was not necessary for the other immunoelectrophoretic techniques. The reason for this difference is not known. Fowl serum can now be used to quantitate bacterial antigens by either rocket immunoelectrophoresis or counterimmunoelectrophoresis using the sodium acetate buffer with a pH of 5.6. Furthermore, two-dimensional immunoelectrophoresis using fowl serum can be used to qualitate and compare various extraction procedures or purity of P. multocida antigens.

In the second paper a method was described to separate and purify antigens from a crude extract of P. multocida. The antigens found in the crude extract, containing over 24 proteinaceous antigens, were separated into a high molecular weight particulate fraction and a low molecular weight soluble fraction by ultracentrifugation. These two fractions showed marked differences in serological tests. Since most of the extracted antigens were proteinaceous in nature, isoelectrofocusing was successful in separating the antigens according to their pI values. The separation and purity of the individual antigens was improved when they were refocused in a narrow pH range (3 pH units). Still, some of the antigens were found in groups of two or three and possibly refocusing for a third time in very narrow pH ranges (1 or 2 pH units) would make it be possible to resolve the antigens into single entities. Because of the large number of antigens present, this was not feasible at this time. Instead, the antigens were grouped into specific pI ranges.

The grouped antigens differed in gel diffusion, one-dimensional immuno-electrophoresis and analytical isoelectrofocusing patterns.

The third paper describes the immunogenicity and toxicity of the KSCN extracted P. multocida antigens. The high molecular weight particulate antigens, 40p, were very effective in inducing serological activity and immunity in mice and chickens. The soluble antigens, 40s, were not toxic and incapable of inducing protection in mice. However, the 40s antigens induced protection in chickens and a weak but detectable antibody response (though not as frequent as the 40p antigens).

The failure of the KSCN extract of in vitro cultured P. multocida to induce cross-protection is probably related to the fact that in vitro cultured P. multocida cells are incapable of inducing cross-protection in mice or chickens. The extraction procedure furthermore failed to uncover or release antigens which might have the capability of inducing cross-protective antigens. It is possible that extracting with KSCN might denature some antigens, in particular, the cross-protective antigen, but at this time it does not appear to be so. The extracted antigens were active in the various serological tests used and are still immunogenic and toxic. As reported in the past, cross-protective antigens are mainly associated with in vivo cultured cells and possibly the cross-protective antigen was not denatured by the extraction procedure because it was not present in the in vitro cultured P. multocida.

The immunogenicity trials using the 40s antigens clearly show the differences in the immunological responses of mice and chickens. The 40s antigens were weak immunogens for chickens but were not protective at all in mice. The results suggest that the chickens but not mice

can be immunized with the protein-polysaccharide antigens found in the 40s fractions. Since the 40s antigens are antigenically distinct from the 40p antigens, it is obvious that more than one class of protective antigens are present in a given strain of P. multocida.

The final paper describes the interaction of serum components with P. multocida and associated antigens to induce a cellular response, chemotaxis, of fowl blood monocytes. Whole P. multocida bacteria, free-endotoxin or lipopolysaccharide interacted with serum components releasing chemotaxins. Either complement, antibody or both appeared to be involved in the production of the chemotaxins by interacting with the P. multocida antigens. This was indicated by the heat-labile and heat-stable properties associated with the chemotaxins found within the fowl sera. If phagocytosis is important in preventing P. multocida infection, chemotaxis of phagocytes to the sites of infection would be a prerequisite to inhibit the multiplication of P. multocida.

It is possible to derive the following conclusions from our studies:

1. A new buffer for immunoelectrophoresis with fowl sera has been described which improves the qualitative and quantitative determination of antigens. The buffer is a 0.05 M sodium acetate buffer, pH 5.6, which reduces the migration of avian immunoglobulins and intensifies the precipitin reaction in a media of low ionic strength. This makes it possible to satisfactorily perform immunoelectrophoresis with avian antiserum.

2. A chaotropic agent, KSCN, extracted active antigenic components from washed P. multocida cells. The extract was fractionated into

definite antigenic entities by ultracentrifugation followed by isoelectrofocusing in a granulated gel. The immunogenicity of the purified antigen groups was evaluated in mice and chickens. The toxic antigens, as determined by the chicken embryo toxicity test, were immunogenic in both mice and chickens, while the nontoxic antigens were only immunogenic in chickens. The toxic antigens induced a higher frequency of detectable serological responses in chickens than did the nontoxic antigens.

3. The ability of P. multocida cells, LPS and FET to stimulate formation of chemotaxins in normal or immune chicken or turkey serum was determined. The formation of the chemotaxins are thought to be derived from the complement system by either direct interaction with the bacterial antigens, via the alternate pathway or by interaction with specific antibody. This was indicated by the presence of heat-labile and heat-stable serum components needed to stimulate chemotaxis of the blood monocytes.

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ADDITIONAL REFERENCES CITED

- Alexander, A. M., and M. A. Soltys. 1973. Relationship of serum agglutinins to protective immunity produced in turkeys immunized against fowl cholera. J. Comp. Pathol. 83: 191-198.
- Bain, R. V. S. 1955. Studies on haemorrhagic septicaemia of cattle. IV. A preliminary examination of the antigens of Pasteurella multocida type 1. Brit. Vet. J. 111: 492-498.
- Bain, R. V. S., and K. W. Knox. 1961. The antigens of Pasteurella multocida Type I. II. Lipopolysaccharides. Immunology 4: 122-129.
- Bhasin, J. L., and L. Lapointe-Shaw. 1980. Antigenic analysis of Pasteurella multocida serotype 1 by crossed immunoelectrophoresis: Characterization of cytoplasmic and cell envelope associated antigens. Can. J. Microbiol. 26: 676-689.
- Bierer, B. W., and T. H. Eleazer. 1968. Continuous use of a live vaccine in the drinking water against fowl cholera infection in turkeys. Poult. Sci. 47: 1258-1260.
- Bierer, B. W., and W. F. Scott. 1969. Comparison of attenuated live Pasteurella multocida vaccine given in drinking water every two weeks to an injected oil base bacterin administered to turkeys. Poult. Sci. 48: 520-523.
- Bond, R. E., and L. D. Olsen. 1974. Susceptibility of turkey poults to Pasteurella multocida and a comparison with older turkeys. Avian Dis. 18 (4): 619-622.
- Brogden, K. A., and P. A. Rebers. 1978. Serological examination of the Westphal-type lipopolysaccharides of Pasteurella multocida.

- Am. J. Vet. Res. 39 (10): 1680-1682.
- Brown, J., D. L. Daw , R. B. Davis, J. W. Foster, and K. K. Srivastava.
1970. Fowl cholera immunization in turkeys. I. Efficacy of various cell fractions of Pasteurella multocida as vaccines. Appl. Microbiol. 19: 837-841.
- Carter, G. R. 1952. The type specific capsular antigen of Pasteurella multocida. Can. J. Med. Sci. 30: 48-53.
- Carter, G. R. 1955. Studies on Pasteurella multocida I. A hemagglutination test for the identification of serological types. Am. J. Vet. Res. 16: 481-484.
- Carter, G. R., and E. Annau. 1953. Isolation of capsular polysaccharides from colonial variants of Pasteurella multocida. Am. J. Vet. Res. 14: 474-478.
- Coates, S. R., M. M. Jensen, and E. D. Brown. 1977. The response of turkeys to varying doses of live oral Pasteurella multocida vaccine. Poult. Sci. 56: 273-276.
- Collins, F. M. 1973. Growth of Pasteurella multocida in vaccinated and normal mice. Infect. Immun. 8 (6): 868-875.
- Collins, F. M. 1977. Mechanisms of acquired resistance to Pasteurella multocida infection: A review. Cornell Vet. 67: 103-138.
- Collins, F. M., and J. B. Woolcock. 1976. Immune responses to Pasteurella multocida in the mouse. J. Reticuloendothel. Soc. 19 (5): 311-321.
- Ganfield, D. J., P. A. Rebers, and K. L. Heddleston. 1976. Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from Pasteurella multocida. Infect. Immun. 14 (4): 990-999.

- Heddleston, K. L. 1962. Studies on Pasteurella multocida: V. Two immunogenic types of Pasteurella associated with fowl cholera. Avian Dis. 6: 315-321.
- Heddleston, K. L., and P. A. Rebers. 1969. Pasteurella multocida: Immune response in chick and mice. Proc. 73rd Annual Meeting U.S.A.H.A., Milwaukee, Wisconsin, Oct. 12-17, 1969. pp. 280-284.
- Heddleston, K. L., and P. A. Rebers. 1972. Fowl cholera: Cross-immunity induced in turkeys with formalin-killed in vivo propagated Pasteurella multocida. Avian Dis. 16 (3): 578-586.
- Heddleston, K. L., and P. A. Rebers. 1974. Fowl cholera bacterins: Host specific cross-immunity induced in turkeys with Pasteurella multocida propagated in embryonating turkey eggs. Avian Dis. 18 (2): 213-219.
- Heddleston, K. L., and K. R. Rhoades. 1978. Avian Pasteurellosis. p. 181-199 in M. S. Hofstad, B. W. Calnek, C. F. Helmboldt, W. M. Reid, and H. W. Yoder, Jr., eds. Diseases of Poultry. Iowa State University Press, Ames, Iowa.
- Heddleston, K. L., and L. P. Watko. 1965. Fowl cholera: Comparison of serologic and immunogenic responses of chickens and turkeys. Avian Dis. 9: 367-376.
- Heddleston, K. L., L. P. Watko, and P. A. Rebers. 1964. Dissociation of a fowl cholera strain of Pasteurella multocida. Avian Dis. 8: 649-657.
- Heddleston, K. L., P. A. Rebers, and E. A. Ritchie. 1966. Immunizing and toxic properties of particulate antigens from two immunogenic

- types of Pasteurella multocida of avian origin. J. Immunol. 96 (1): 124-133.
- Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1970. Fowl cholera: Immune response in turkeys. Avian Dis. 14 (4): 626-635.
- Hungerford, T. G. 1968. A clinical note on avian cholera: The effect of age on the susceptibility of fowls. Australian Vet. J. 44: 31-32.
- Knox, K. W., and R. V. S. Bain. 1960. The antigens of Pasteurella multocida type 1. I. Capsular polysaccharides. Immunology 3: 352-362.
- Maheswaran, S. K., and E. S. Thies. 1979. Influence of encapsulation on phagocytosis of Pasteurella multocida by bovine neutrophils. Infect. Immun. 26 (1): 76-81.
- Maheswaran, S. K., J. R. McDowell, and B. S. Pomeroy. 1973. Studies on Pasteurella multocida. 1. Efficacy of an avirulent mutant as a live vaccine in turkeys. Avian Dis. 17 (2): 396-414.
- Maheswaran, S. K., E. S. Thies, and S. K. Dua. 1976. Studies on Pasteurella multocida: III. In vitro assay for cell-mediated immunity. Avian Dis. 20 (2): 332-341.
- McKinney, K. L., and P. A. Rebers. 1977. Phagocytosis of Pasteurella multocida by turkey macrophages. 28th Annual North Central Poultry Disease Conference. June 7-9, 1977, Fargo, N.D. Abstract p. 36.
- Namioka, S. 1978. Pasteurella multocida - Biochemical characteristics and serotypes. p. 271-292 in T. Bergan and J. R. Norris, eds. Methods in Microbiology. Academic Press, New York.

- Nathanson, R. M., M. S. Hofstad, and E. L. Jeska. 1980. Pasteurella multocida: Antibody-mediated resistance to virulent challenge exposure in vaccinated turkeys. Am. J. Vet. Res. 41 (8): 1285-1287.
- Pasteur, L. 1880. Sur le cholera des poules; etudes des conditions de la non-recidive de la maladie et de quelques autres de ses caracteres. Compt. Rend. Acad. Sci. 90: 239-248, 1030-1033.
- Penn, C. W., and K. L. Nagey. 1976. Isolation of a protective non-toxic capsular antigen from Pasteurella multocida type B and E. Res. Vet. Sci. 20: 90-96.
- Perreau, P., and Petit, J. P. 1963. Antigene lipopolysidique de Pasteurella multocida type E. Rev. Elevage Med. Vet. Pays Trop. 16: 5-18.
- Pirosky, I. 1938. Sur l'antigene glucidolipidique des Pasteurella. Compt. Rend. Soc. Biol. 127: 98-100.
- Priestley, F. W. 1936. Some properties of the capsule of Pasteurella septica. Brit. J. Exp. Pathol. 17: 374-378.
- Prince, G. H., and J. E. Smith. 1966. Antigenic studies on Pasteurella multocida using immunodiffusion techniques. I. Identification and nomenclature of the soluble antigens of a bovine haemorrhagic septicaemia strain. J. Comp. Pathol. 76: 303-314.
- Rebers, P. A., and K. L. Heddleston. 1974. Immunologic comparison of Westphal-type lipopolysaccharides and free endotoxins from an encapsulated and a nonencapsulated avian strain of Pasteurella multocida. Am. J. Vet. Res. 35 (4): 555-560.
- Rebers, P. A., and K. L. Heddleston. 1977. Fowl cholera: Induction of cross-protection in turkeys with bacterin prepared from host-

- passaged Pasteurella multocida. Avian Dis. 21 (1): 50-56.
- Rebers, P. A., K. L. Heddlestone, and K. R. Rhoades. 1967. Isolation from Pasteurella multocida of a lipopolysaccharide antigen with immunizing and toxic properties. J. Bacteriol. 93: 7-14.
- Rebers, P. A., K. L. Heddlestone, B. Wright, and K. Gillette. 1975. Fowl cholera: Cross-protective turkey antisera and IgG antibodies induced with Pasteurella multocida-infected tissue bacterins. Carbohydrate Res. 40: 99-110.
- Rhoades, K. R., K. L. Heddlestone, and P. A. Rebers. 1967. Experimental hemorrhagic septicemia gross and microscopic lesions resulting from acute infections and from endotoxin administration. Can. J. Comp. Med. Vet. Sci. 31: 226-233.
- Rimler, R. B., P. A. Rebers, and K. R. Rhoades. 1979. Fowl cholera: Cross-protection induced by Pasteurella multocida separated from infected turkey blood. Avian Dis. 23: 730-741.
- Salmon, D. E. 1880. Investigations of fowl cholera. p. 401-426 in Report of U.S. Commissioner of Agriculture. U.S. Government Printing Office, Washington, D.C.
- Salmon, D. E. 1881. Investigation of swine plague, fowl cholera, and southern cattle fever. p. 258-306 in Report of U.S. Commissioner of Agriculture. U.S. Government Printing Office, Washington, D.C.
- Srivastava, K. K., and J. W. Foster. 1977. Characterization of an immunogenic fraction of Pasteurella multocida culture filtrates. Can. J. Microbiol. 23: 197-201.
- Srivastava, K. K., J. W. Foster, D. L. Dawe, J. Brown, and R. B. Davis. 1970. Immunization of mice with components of Pasteurella multocida.

- Appl. Microbiol. 20: 951-956.
- Woolcock, J. B., and F. Collins. 1976. Immune mechanism in Pasteurella multocida infected mice. Infect. Immun. 13 (3): 949-958.
- Yamaguchi, Y., and T. Baba. 1975. Demonstration in tissue culture of cellular immunity to fowl cholera. Proc. First Intersectional Congress of I.A.M.S. 4: 14-18.
- Yaw, K. E., and J. C. Kakavas. 1957. A comparison of the protection-inducing factors in chickens and mice of a type 1 strain of Pasteurella multocida. Am. J. Vet. Res. 18: 661-664.