Characterization and Application of Monoclonal Antibody and Bovine Neutrophil Reactivity to Pasteurella Haemolytica Antigens.

Frank William Austin
Louisiana State University and Agricultural & Mechanical College

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Characterization and application of monoclonal antibody and bovine neutrophil reactivity to *Pasteurella haemolytica* antigens

Austin, Frank William, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1988
CHARACTERIZATION AND APPLICATION OF MONOCLONAL ANTIBODY
AND BOVINE NEUTROPHIL REACTIVITY TO
PASTEURELLA HAEMOLYTICA ANTIGENS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in the

Veterinary Microbiology and Parasitology Option of

The Interdepartmental Program in

Veterinary Medical Sciences

by

Frank William Austin
B.S., Oklahoma State University, 1979
D.V.M., Oklahoma State University, 1983
August 1988
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ABSTRACT

Monoclonal antibodies (McAbs) were prepared against Pasteurella haemolytica serotype 1 (Ph-1) capsular material to identify, quantitate and purify antigens. McAbs were selected by ELISA and characterized according to their isotype, cross-reactivity with P. haemolytica and P. multocida serotypes in ELISA, antigen formalin sensitivity and ability to cause bacterial agglutination. Four groups of McAbs were established each recognizing a different epitope. Various P. haemolytica and P. multocida serotypes were found to share several epitopes demonstrating their antigenic relatedness.

A colorimetric assay, based on tetrazolium dye reduction by bovine neutrophils, was adapted for the measurement and characterization of Ph-1 cytotoxin activity. Using different cytotoxin preparations which varied in cytolytic activity, cytotoxin activity was identified as the inhibition of dye reduction. Stimulation of dye reduction by neutrophils in response to the cytotoxin preparations was also detected in the assay suggesting neutrophil activation involving the respiratory burst. McAbs and chemical methods were used to identify and quantitate cytotoxin preparation antigens.

The colorimetric assay was further applied to select and characterize a cytotoxin-neutralizing monoclonal antibody (nMcAb). The nMcAb was not reactive in ELISA, but produced substantial neutralization in the colorimetric assay. The nMcAb was identified as an IgM. McAbs selected ELISA against capsular material did not affect neutrophil dye reduction in response to cytotoxin.
Ultrastructural changes in bovine neutrophils exposed to cytotoxin were examined over different time intervals and concentrations to characterize its effects at the cellular level. Identical time and concentration dependent changes involving cell polarization with uropod formation, plasma membrane defects, and differential intracellular degranulation progressing to complete lysis were observed. Polarization with uropod formation suggested the presence of a chemotactic factor and corresponded with activation observed in the colorimetric assay.

A McAb was applied in immunoaffinity to purify the Ph-1 specific epitope from capsular material. The immunoaffinity product contained no detectable protein and at least one half the original hexosamine content. McAbs were used in ELISA to identify and quantitate the product antigens. Lipopolysaccharide and pan-pasteurella carbohydrate were selectively retained in addition to Ph-1 specific capsular polysaccharide suggesting epitope sharing among polysaccharide antigens.
SUMMARY

*Pasteurella haemolytica* serotype 1 (Ph-1) capsular material was extracted from logarithmic growth phase cells and used to immunize BALB/c mice for monoclonal antibody production. Hybridomas producing antibody to Ph-1 capsular material were selected using an ELISA procedure and repeatedly cloned by limiting dilution. Monoclonal antibodies obtained were characterized and grouped according to their isotype, cross-reactivity with 6 and 24 hour *P. haemolytica* (Ph) and *P. multocida* (Pm) serotypes in ELISA, antigen formalin sensitivity and ability to cause rapid plate agglutination. Four groups of antibodies were established which were (1) IgM Ph-1 specific agglutinating, (2) IgG3 cross-reactive with various Ph serotypes, (3) IgM cross-reactive with many Ph and Pm serotypes, and (4) IgG2A cross-reactive with all Ph and Pm serotypes. The Ph-1 specific antigen and the antigen common to all Ph and Pm serotypes were formalin sensitive. These studies demonstrate the use of differential antigen expression by bacterial serotypes to characterize monoclonal antibodies which were produced against a complex antigen source and indicates that the various Ph and Pm serotypes share several common epitopes.

INTRODUCTION

*Pasteurella haemolytica* biotype A, serotype 1 (Ph-1) is the pathogenic agent most frequently associated with severe fibrinous
pneumonia in pnemonic pasteurellosis (shipping fever) of cattle.\textsuperscript{1,2} Successful division of \textit{P. haemolytica} into discrete serotypes was accomplished using specific rabbit antisera in indirect hemagglutination and rapid plate agglutination reactions.\textsuperscript{3,4} Soluble capsular polysaccharide antigens are accepted to confer serotype specificity recognized by antisera in these reactions.\textsuperscript{4,6} Greater than 12 serotypes have been identified which can be divided into 2 biotypes, A and T, respective of their ability to ferment arabinose and trehalose.\textsuperscript{7,8} Serotypes 3,4 and 10 belong to biotype T, while the remaining serotypes do not ferment trehalose and belong to biotype A. Serological cross-reactions among the various serotypes, mostly within biotypes, have been reported using several analytical techniques.\textsuperscript{3,4,9-12} These reactions and cross reactions have defined a complex, yet distinguishable antigenic profile among the Pasteurellae.

With the development of monoclonal antibody techniques, more than a decade ago by Kohler and Milstein,\textsuperscript{13} it has become possible to study complex antigenic systems using serologic methods. Cell surface antigens of lymphocytes and bacteria represent a complex system to which monoclonal antibodies have been extensively applied. Whole cells or extracts are commonly injected into mice and monoclonal antibodies can be obtained which define specific surface antigens in a complex subpopulation of cells.\textsuperscript{14-20} Because the serotypes and biotypes of \textit{Pasteurella haemolytica} and other \textit{Pasteurella} species represent antigenically complex subpopulations of cells, it should be possible to produce monoclonal antibodies which can differentiate soluble or surface expressed antigens among these populations. Reactivity of monoclonal antibodies with cell surface antigens is commonly measured
using enzyme-linked immunosorbent assays (ELISA) for which many variations exist.\textsuperscript{14-22} Usually cells are attached to a solid phase (96 well plates) using a cross-linking agent such as glutaraldehyde, paraformaldehyde, or other fixatives.\textsuperscript{20,21,23-25} These procedures often alter antigenicity of cell surface constituents and therefore the assay results obtained.\textsuperscript{26-30} Antigen reactivity following different treatments can therefore be used to distinguish various antigens and to differentiate monoclonal antibody specificities. It is easily envisioned that this could be an important phenomena with respect to the immunogenicity and efficacy of bacterins and vaccines treated with different fixative agents.

Further antibody characterizations frequently involve determination of isotype, subisotype, the capacity to neutralize biological activities, bacteriocidal or bacteriostatic effects in the presence of complement, and insolubilization of antigens through agglutination or precipitation. Monoclonal antibody technology has not been extensively applied to \textit{P. haemolytica} or bovine pnemonic pasteurellosis. The purpose of this report is to describe the development and characterization of monoclonal antibodies against \textit{P. haemolytica} serotype 1 capsular material.

\textbf{MATERIALS AND METHODS}

\textit{P. haemolytica} antigen preparation:

\textit{Pasteurella haemolytica} biotype A, serotype 1, was maintained by lyophilization in skim milk and periodic animal passage as previously
described. Agar plates containing 10% (v/v) bovine blood, 1% (v/v) horse serum, and 1% (v/v) yeast hydrolysate were used to propagate the bacterium in candle jars at 37°C. Capsular material was extracted from 6 hour confluent cultures according to the method of Gentry. The bacteria were removed from the solubilized capsular material by centrifugation at 12,000 xg for 30 min at 4°C. The supernate containing capsular antigens was filter sterilized (0.22μm) and dialyzed (8-10 k MWCO, Fisher Sci. Co.) extensively against distilled water at 4°C over 48 hrs. The dialyzed capsular antigens were frozen at -70°C and lyophilized prior to use.

Mice and immunizations:

The BALB/c mice which were used for the production of monoclonal antibodies were bred in our facilities. One half mg capsular material in Freund's complete adjuvant (0.5 ml) was used to immunize 6-8 week old female mice by intraperitoneal injection. One month later, mice were immunized again with 0.5 mg capsular material in 0.5 ml phosphate buffered saline (PBS, pH 7.2) by the same route. Blood samples were withdrawn from the retro-orbital plexus prior to immunization and 3 days following the booster immunization for measurement of serum antibody. Successful immunization and selection of mice for monoclonal antibody production was established using ELISA (see Assay for antibody production and hybridoma cloning). Mice having the highest antibody titer to capsular material were choosen for cell fusion.
Cell fusion and hybrid selection:

Four days following the booster immunization, spleen cells from one mouse were fused with SP-2/0 murine myeloma cells at a 4:1 ratio in the presence of 50% (v/v) 1450 MW polyethylene glycol. After the fusion, cells were gently washed with and diluted in RPMI-1640 containing 15% (v/v) fetal calf serum, 0.01mM hypoxanthine, 1.6x10^{-2} mM thymidine, 10 mM sodium bicarbonate, 1% (v/v) non-essential amino acids, and 10mM L-glutamine (HT-medium) to a concentration of 7.5x10^6 splenocytes/ml. Aliquots (0.1ml) were delivered to the wells of four 96 well plates which contained 5x10^6 BALB/c thymocytes/well as feeder cells. The mixed cell population was cultured for 24 hr at 37C in the presence of 5% CO_2. Hybrid cells were selected by the daily feeding of 0.1 ml volumes of HT-medium containing 4x10^{-4} mM aminopterin (HAT-medium) for four consecutive days. Following hybrid selection the cells were maintained and expanded in HT-medium. Plates were visually assessed for clonal growth on a daily basis during hybrid cell selection using an inverted microscope.

Assay for antibody production and hybridoma cloning:

Supernates from hybridoma cell cultures or mouse sera were evaluated for specific antibody content by ELISA using capsular material as the antigen. Briefly, 50 µl volumes containing 5µg capsular material in TEN buffer 0.05M Tris, 0.001M NaEDTA, 0.15M NaCl, pH 7.4 were air dried in 96 well polystyrene plates overnight. The dessicated antigen was fixed with 50 µl paraformaldehyde per well for 5 min and washed 3 times with TEN buffer. Wells were blocked with
200 μl aliquots of 2% (w/v) BSA in phosphate buffered saline (PBS, 0.15M, pH 7.2) for 2 hr and washed. Hybridoma cultural supernates or mouse sera (50 μl) were added and allowed to bind for 45 min at room temperature. The wells were washed 3 times and 50 μl volumes of goat anti-mouse IgG, IgM horse-radish peroxidase conjugated secondary antibody (1:1500 dilution in TEN buffer) was added to the wells and incubated for 45 min at room temperature. Unbound conjugated antibody was washed from the wells 3 times and hybridomas secreting antibody specific for capsular material were identified using orthophenylenediamine as a substrate. Plates were visually inspected for positive reactions and 10 wells were selected for cloning.

Following selection of those hybridomas secreting antibody specific for capsular antigens, cells were cloned by limiting dilution in 96 well plates. BALB/c thymocytes (5x10^6/well) were used as feeder cells. Cloned hybridomas were expanded in 25 cm^2 flasks in HT-medium and cryopreserved in the same medium containing 20% (v/v) FCS and 10% (v/v) DMSO.

Peritoneal ascitic fluid, for each of the cloned hybridomas, was produced in adult BALB/c mice conditioned one week in advance with 0.5 ml pristane. Preconditioned mice were inoculated with 10^6 viable hybrid cells. Peritoneal ascitic fluid was harvested by periodic abdominoacentesis beginning 7 days after inoculation, clarified by centrifugation, and stored at -70C.

**Determination of monoclonal antibody isotype:**

Isotypes of the 10 selected monoclonal antibodies were determined, using hybridoma cultural supernates, with two different commercially
available isotyping kits based on immunodiffusion and antibody capture.

**Assay of monoclonal antibody specificity:**

Monoclonal antibodies were assayed for their specificity to other *P. haemolytica* and *P. multocida* serotypes using the ELISA procedure with slight modifications. *P. haemolytica* serotypes and biotypes 1-12\(^4,7\) and *P. multocida* serotypes 1-16\(^33\) were defined and propagated as described for Ph-1. Both 6 and 24 hr cultures of the bacteria were prepared and suspended in PBS. Bacterial suspensions were adjusted to \(1 \times 10^9\) CFU/ml using a spectrophotometer and 50 µl aliquots of a 1:26 dilution, determined optimum by checkerboard titration, were used as antigens in the ELISA.

**Determination of antigen formalin sensitivity:**

Ph-1 whole cells or homologous capsular material were prepared as described and dessicated into 96 well plates. The dessicated antigen were either untreated or treated with 50 µl 10% (v/v) buffered formalin and/or paraformaldehyde for 5 min. The wells were washed three times and the ELISA completed as described.

**Determination of rapid slide agglutination of Ph-1 by ascitic fluids:**

Peritoneal ascitic fluids were clarified by centrifugation at 2500 xg for 5 min, residual pristane removed with a pipette and the ascitic fluid passed through a 0.22 µm filter. Fifty µl aliquots of the clarified ascitic fluids from each cloned hybridoma were mixed with 50 µl volumes of \(1 \times 10^9\) CFU/ml of the *P. haemolytica* serotypes on a cleaned
slide. The slides were slowly rocked at room temperature and observed for rapid agglutination.

RESULTS

Following hybrid cell selection and expansion in 96 well plates, nearly every well contained rapidly growing hybridomas 10 days after the fusion. Specific antibody production against \textit{P. haemolytica} serotype 1 capsular antigens was identified in 250 cultures (74\% of total). These positive wells were variable in their degree of hybrid cell growth and antibody reactivity based on ELISA substrate absorbance. Ten wells that varied in absorbance, all which contained vigorously growing hybridomas, were chosen for cloning by limiting dilution and further study. These hybridomas were numbered 1 through 10 and cloned greater than 5 times and selected by ELISA over several months. During cloning 2 hybridomas proved to be unstable and failed to produce antibody as detected by ELISA. A third hybridoma became contaminated and was discarded.

Isotypes of the 10 originally selected hybridomas were determined using cultural supernate in two different methods (Table 1.1). The results of immunodiffusion and antibody capture were in exact agreement, however, immunodiffusion was apparently not as sensitive as antibody capture and failed to identify many antibodies. Antibodies were identified in either the IgM or IgG class and many hybridomas were found to produce the same isotype and sub-isotype of antibody.
### Table 1.1 - Determination of Monoclonal Antibody Isotype

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Immunodiffusion</th>
<th>Antibody Capture</th>
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<tbody>
<tr>
<td>1</td>
<td>IgG3</td>
<td>IgG3</td>
</tr>
<tr>
<td>2</td>
<td>ND*</td>
<td>IgG2A</td>
</tr>
<tr>
<td>3</td>
<td>IgM</td>
<td>IgM</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>IgG2A</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>IgG3</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>IgG3</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>IgG1</td>
<td>IgG1</td>
</tr>
<tr>
<td>9</td>
<td>IgM</td>
<td>IgM</td>
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<tr>
<td>10</td>
<td>IgM</td>
<td>IgM</td>
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* ND = Not determined

Reactivity of the seven remaining monoclonal antibodies with *P. haemolytica* serotypes (Table 1.2) and *P. multocida* serotypes (Table 1.3) was determined using whole bacterial cells as the antigens in ELISA. Monoclonal antibodies were grouped according to their reactivity among these serotypes. Four patterns of reactivity were identified: (1) *P. haemolytica* serotype 1 specific, (2) cross reactive with *P. haemolytica* serotypes 1, 5-8, and 12 not cross reactive with any *P. multocida* serotypes, (3) cross reactive with *P. haemolytica* serotypes 1, 2, 5-8, and 12 with 6 and 24 hr culture variability also
cross reactive with *P. multocida* serotypes 1-7, 9, 12, 15, 16, and (4) cross reactive with all *P. haemolytica* and *P. multocida* serotypes. Monoclonal antibodies grouped on the basis of serotype reactivity were of the same isotype and sub-isotype.

Table I.2 - Reactivity of Monoclonal Antibodies with *P. haemolytica*

<table>
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<tr>
<th>Whole Cell Serotypes</th>
<th>P. haemolytica serotype</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
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<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Denotes differences in reactivity between 6 and 24 hr cultures with both antibodies.*
Table 1.3 - Reactivity of Monoclonal Antibodies with *P. multocida* Whole Cell Serotypes

<table>
<thead>
<tr>
<th><em>P. multocida</em> serotype$^+$</th>
<th>Monoclonal Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
</tr>
</tbody>
</table>

$^+$No difference in reactivity between 6 and 24 hr cultures. $^*$Number 6 monoclonal antibody positive for serotype 16 only.

Formalin treatment of *P. haemolytica* serotype 1 whole cells or capsular material was found to completely alter the antigenicity, reducing the reactivity of some monoclonal antibodies in ELISA (Table I.4). The *P. haemolytica* serotype 1 specific antigen, recognized by
monoclonal antibody number 3, and the antigen common to all *P. haemolytica* and *P. multocida* serotypes, recognized by monoclonal antibody number 2, was apparently destroyed by formalin treatment. Paraformaldehyde treatment did not adversely affect antigenicity in this system. Neither formalin nor paraformaldehyde was necessary for capsular material antigenicity in ELISA.

Table 1.4 - Reactivity of Monoclonal Antibodies with Formalin or Paraformaldehyde Treated *P. haemolytica* serotype 1 Whole Cells or Capsular Material in ELISA

<table>
<thead>
<tr>
<th>Antigen Treatment*</th>
<th>Monoclonal Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3  1,5,6  9,10  2</td>
</tr>
<tr>
<td>Formalin/paraformaldehyde</td>
<td>-  +  +  -</td>
</tr>
<tr>
<td>Formalin/no paraformaldehyde</td>
<td>-  +  +  -</td>
</tr>
<tr>
<td>No formalin/paraformaldehyde</td>
<td>+  +  +  +</td>
</tr>
<tr>
<td>No formalin/no paraformaldehyde</td>
<td>+  +  +  +</td>
</tr>
</tbody>
</table>

*Whole cells or capsular material were treated following adsorption to 96 well plates.

Rapid slide agglutination of *P. haemolytica* serotype 1 by clarified peritoneal ascitic fluids was produced only by monoclonal antibody number 3. Other monoclonal antibodies were incapable of agglutinating *P. haemolytica* serotype 1 or the remaining serotypes.
The ability of monoclonal antibody number 3 to agglutinate serotype 1 corresponded with its ELISA reactivity (Tables 1.2 and 1.3).

A summary characterizing the monoclonal antibodies prepared against *P. haemolytica* serotype 1 capsular material is presented in Table I.5. Monoclonal antibodies were placed in four groups based on their characteristic isotypes, serotype reactivity, antigen formalin sensitivity, and agglutination. The results of these determinations indicated several of the antibodies recognized the same epitope.
Table I.5 - Characterization of Monoclonal Antibodies Prepared Against Pasteurella haemolytica serotype 1

<table>
<thead>
<tr>
<th>Group Number(s)</th>
<th>Group Antibody Number(s)</th>
<th>Isotype</th>
<th>Serotype Reactivity</th>
<th>P. haemolytica</th>
<th>P. multocida</th>
<th>Formalin</th>
<th>Agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>IgM</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1,5,6</td>
<td>IgG3</td>
<td>1,5-8,12</td>
<td>None*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>9,10</td>
<td>IgM</td>
<td>1,3,5-8,12</td>
<td>1-7,9,12,15,16</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>IgG2A</td>
<td>all</td>
<td>all</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Monoclonal antibody number 6 reactive with *P. multocida serotype 16 only.*
DISCUSSION

Monoclonal antibodies were developed against *P. haemolytica* serotype 1 capsular material. The hybridomas producing these monoclonal antibodies were selected from a total of 250 antibody producing wells generated by fusing the immune spleen cells with SP-2/0 myeloma cells. Ten different wells containing vigorously growing hybridomas were chosen on the basis of antibody binding with serotype 1 capsular material. Monoclonal antibody number 3 had the highest ELISA reactivity, while antibodies 1, 5, 6, and 2 produced moderate ELISA absorbances, and antibodies 9 and 10 displayed low ELISA reactivity (data not shown). These antibodies were characterized according to isotype, bacterial serotype specificity, antigen sensitivity to formalin and agglutination properties. The serotype distribution of antigens recognized by the monoclonal antibodies were determined using primarily ELISA with defined bacterial cells. These determinations allowed establishment of four different groups of antibodies, each recognizing an apparently different epitope. The first epitope, recognized by monoclonal antibody number 3, is *P. haemolytica* serotype 1 specific and involved with bacterial agglutination. The second epitope, defined by antibodies 1, 5, and 6, is present on many *P. haemolytica* serotypes, but not *P. multocida* serotypes. The third epitope, bound by antibodies 9 and 10, is present on many *P. haemolytica* and *P. multocida* serotypes. Finally, the fourth epitope, present on all *P. haemolytica* and *P. multocida* serotypes, is recognized by monoclonal antibody number 2. In this study, only *P. haemolytica* and *P. multocida* serotypes were examined, however, this system may be extended to and possibly include other species of *Pasteurella*, or other
genera within the Brucellaceae family, or even other Gram-negative bacteria. This system exemplifies the use of antigen distribution among bacterial serotypes to distinguish different monoclonal antibodies.

The P. haemolytica serotype 1 specific antigen is defined routinely by indirect hemagglutination and rapid plate agglutination. Serotype specificity is believed to reside in capsular polysaccharide antigens of P. haemolytica. Serotype 1 specific agglutinating antibody has been suggested as necessary for protection from experimental disease. Immunization of cattle with crude capsular extracts has afforded some degree of resistance to experimental challenge, however, formalized bacterins have been shown to have a neutral or deleterious effect. The results of this study indicates monoclonal antibody number 3 recognizes the serotype 1 specific capsular polysaccharide of P. haemolytica and causes bacterial agglutination. Furthermore, it is suggested that the serotype 1 specific antigen is formalin sensitive, which may account, at least in part, for the ineffectiveness of formalinized bacterins to impart disease resistance.

Serologic cross-reactivity among the established serotypes, within each biotype, of P. haemolytica has been discovered by indirect hemagglutination, rapid plate agglutination, ELISA, and crossed immunoelectrophoresis. Crossed immunoelectrophoresis further demonstrated cross-reactions between biotypes of P. haemolytica. However, the antigens responsible for this cross-reactivity have remained uncharacterized. Monoclonal antibodies 1, 5, and 6 characterized in this study were considerably cross-reactive with many
P. haemolytica serotypes, but not between the biotypes. In another study, these monoclonal antibodies were shown to be specific for lipopolysaccharides by immunoblotting and the antigen was localized within the bacterial capsule using protein A-gold labeled conjugate in electron microscopy. The lipopolysaccharide epitope recognized by these antibodies was not sensitive to formalin. These results supply evidence that a lipopolysaccharide epitope is common among many serotypes, but not between biotypes of P. haemolytica. Thus, some degree of cross-reactivity among serotypes but not between biotypes, of P. haemolytica may be attributable to a common lipopolysaccharide epitope.

The similarity of antibodies 1, 5, and 6 and antibodies 9 and 10 reacting with P. haemolytica serotypes was further distinguished using P. multocida serotypes recognized only by the latter group. Serologic cross-reactivity among P. haemolytica and P. multocida serotypes has not been reported in the literature. Monoclonal antibodies 9 and 10 were cross-reactive among many serotypes of these species, but not between biotypes of P. haemolytica. Immunoblot analysis of the antigen recognized by these antibodies revealed a 29KD protein, most likely of outer membrane origin, however, its exact locale could not be determined using protein A-gold probes due to the antibody isotype. Antigenicity of the 29KD protein was not affected by formalin treatment. The 29KD protein represents a potential source of cross-reactivity among P. haemolytica and P. multocida serotypes.

Common antigens of Gram-negative bacteria have been reported in P. haemolytica and other bacteria. Monoclonal antibody number 2
was cross-reactive with all *P. haemolytica* and *P. multocida* serotypes examined. It is unknown whether antibody number 2 recognizes other species of *Pasteurella*, or other members of the family *Brucellaceae*, or other Gram-negative bacteria; however, this antibody did not react with *Escherichia coli*, used as bacterial antigen control in the ELISA (data not shown). The antigen could not be detected by immunoblotting and its nature remains undetermined. It was formalin sensitive and this property may also account, at least in part, for the failure of formalinized bacterins to impart protection from disease. Nevertheless, this antigen represents a source of considerable cross-reactivity among *P. haemolytica* serotypes, biotypes, and *P. multocida*.

In summary, development and characterization of the reported monoclonal antibodies has lead to the acquisition of powerful tools to study the antigens of *P. haemolytica*. Four groups of antibodies were established based on their characteristics each corresponding to a different antigen. This study exemplified the use of differential antigen expression among *Pasteurella* serotypes to distinguish monoclonal antibodies prepared and selected from a complex antigen source. Further immunologic, biochemical, and biological experiments are needed to more fully characterize the antibody groups, the specific antigens recognized, and their relationship to disease.


42. Schnorr KL, Todd WJ, Corstvet, RE, Austin FW, Nobles D. Identification and localization of <i>Pasteurella haemolytica</i> antigens by monoclonal antibodies. The First International Veterinary Immunology Symposium, pg 133.


A colorimetric assay, based on mitochondrial reduction of a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) was adapted for the measurement of Pasteurella haemolytica serotype 1 cytotoxin using bovine neutrophils. Cytotoxin was produced in a defined medium and concentrated by ultrafiltration. Five cytotoxin preparations, a preconcentrate, a 15:1 concentrate, an ultrafiltrate and 15:1 concentrates inactivated at either 56°C or by boiling for 1 hr followed by clarification, were used to measure cytotoxin activity in the assay. Chemical and immunologic methods were used to identify and quantitate antigens in the cytotoxin preparations. Neutrophil cytotoxicity was identified as inhibition of tetrazolium dye reduction which indicated that neutrophils were killed. At different concentrations each of the cytotoxin preparations, except the ultrafiltrate, caused neutrophil stimulation of tetrazolium dye reduction relative to unexposed cells whether cytotoxicity was present or not. Results of this study indicate the MTT colorimetric assay can measure bovine neutrophil cytotoxicity or stimulation. Neutrophil stimulation of tetrazolium dye reduction may reflect activation via the respiratory burst in response to cytotoxin preparation antigens.
INTRODUCTION

The pathogenesis of bovine pneumonic pasteurellosis (shipping fever) is currently recognized to involve the interaction of many host, pathogen and environmental factors.\textsuperscript{1,2} The severe fibrinous pneumonia characterizing this disease is most commonly associated with lower respiratory tract infection by Pasteurella haemolytica serotype 1.\textsuperscript{1-3} In the acute stages of pneumonic pasteurellosis, a significant step in the pathogenesis is the rapid accumulation of primarily neutrophils in alveolar spaces.\textsuperscript{4-7}

During logarithmic-phase growth, \textit{P. haemolytica} has been shown to liberate a soluble cytotoxin (leukotoxin) which is specifically cytolytic for ruminant pulmonary macrophages, peripheral blood mononuclear cells, and neutrophils.\textsuperscript{8-12} Cytotoxin is believed to contribute to the pathogenesis of the pulmonary lesion by directly impairing phagocyte function and hence bacterial clearance from the lung. Cytotoxin may further contribute to the pathogenesis by causing the release and/or generation of inflammatory mediators from neutrophils. \textit{P. haemolytica} cytotoxin has been characterized as a heat labile, trypsin sensitive protein with an approximate molecular weight of 105,000. The cytolytic activity can be neutralized by antibody from a variety of sources.\textsuperscript{13-16} Resistance to experimentally induced and naturally occurring pneumonic pasteurellosis has been correlated with high titers of cytotoxin neutralizing serum antibodies.\textsuperscript{17-19} However, cytotoxin preparations contain several soluble antigens of \textit{P. haemolytica} including capsular polysaccharides, lipopolysaccharides and possibly other unidentified components.\textsuperscript{20-22} The diversity of serum antibodies and the impurities of cytotoxin
preparations antigens have hampered identification of many, possibly important interactions between neutrophils and these components.

Most assays designed to quantitate cytotoxin have relied on plasma membrane damage as an indicator of activity. These assays include measurement of trypan blue dye exclusion, chromium release, microtiter neutral red dye-elution, and monolayer integrity. Additionally, a luminol dependent chemiluminescent (LDCL)-inhibition assay has been described, using bovine neutrophils, for detecting cytotoxin activity. These assays have also been used to measure cytotoxin neutralization by antibody in serum and nasal or bronchoalveolar washings. The LDCL-inhibition assay was found to be the most sensitive in detecting cytotoxin activity when compared to assays dependent on cell lysis and membrane integrity as indicators of cytotoxicity. It was hypothesized that the LDCL-inhibition assay was more sensitive because it measured metabolic impairment of the neutrophil.

Most recently a microtiter colorimetric assay based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye reduction by bovine neutrophils was reported to quantitate cytotoxin and its neutralization. The MTT colorimetric assay was adapted from the methods originally described by Mossmann for the quantitation of cellular proliferation and cytotoxicity. The MTT colorimetric assay has since been reported to measure cellular activation independent of proliferation. The principle of this assay is the reduction of yellow MTT dye to a dark blue insoluble formazan by metabolically active mitochondria and its subsequent photometric quantitation.

MTT colorimetric assays offer a unique means of measuring neutrophil
response to toxins and antigens because neutrophils undergo a respiratory burst involving increased mitochondrial activity.\textsuperscript{30-32}

Assays of neutrophil metabolism in response to \textit{P. haemolytica} antigens in cytotoxin preparations are needed to gain a better understanding of the pathogenesis in pneumonic pasteurellosis. The purposes of this study were to adapt, optimize, and validate the MTT colorimetric assay using bovine neutrophils for the measurement of cytotoxin activity and to identify component antigens in cytotoxin preparations using monoclonal antibodies and chemical methods. The MTT colorimetric assay was also adapted for the selection and assessment of a cytotoxin neutralizing monoclonal antibody and to determine the effects of other monoclonal antibodies against antigens in cytotoxin preparations on neutrophil responses.

**MATERIALS AND METHODS**

**Bacterial cultures and cytotoxin production:**

\textit{Pasteurella haemolytica} biotype A, serotype 1, originally isolated from the trachea of a feedlot calf, was maintained by lyophilization in skim milk and periodic animal passage as previously described.\textsuperscript{33} Lyophilized cultures were reconstituted and streaked for isolation on agar plates containing 10\% (v/v) citrated bovine blood, 1\% (v/v) horse serum, and 1\% (v/v) yeast hydrolysate. Isolated colonies were selected and suspended in Dulbecco's phosphate buffered saline (PBS, pH7.4) for confluent inoculation of fresh media. Agar plates were incubated in candle jars at 37\textdegree C for 18 to 24 hr. Confluent cultures were harvested, suspended in Dulbecco's PBS, and adjusted to an optical density of 0.25 at 650 nm using a 1:10
dilution. This suspension contained $1 \times 10^9$ CFU/ml as determined by plate count.

*P. haemolytica* cytotoxin was produced in a defined medium using modifications of previously described methods. A 10.0 ml sample of the bacterial suspension was used to inoculate 500 ml RPMI-1640, containing 10mM L-glutamine and 10 mM sodium bicarbonate, giving a final inoculation density of $2 \times 10^7$ CFU/ml. Following incubation for 5.5 hr at 37°C in the presence of 5% CO$_2$, the bacteria were pelleted by centrifugation at 12,000 xg for 30 min at 4°C. The supernate was collected, passed through a 0.22 μm filter, and concentrated 15:1 using a PM-30 membrane fitted in an ultrafiltration cell at 4°C. Samples of preconcentrated cytotoxin and cytotoxin ultrafiltrate were retained for subsequent analysis. Aliquots of concentrated cytotoxin were heat inactivated at either 56°C for 1 hr or by boiling for 1 hr followed by a high speed centrifugation step and 0.22 μm filtration. The bacterial pellets and cytotoxin preparations were cultured on the appropriate media to ensure purity and sterility respectively. Cytotoxin preparations were stored at -70°C.

**Analysis of the cytotoxin preparations:**

Protein concentrations of the cytotoxin preparations were determined using the method of Bradford. Quantitation of hexosamines was performed according to previously published methods. Antigens present in the cytotoxin preparations were quantitated in ELISA using previously defined monoclonal antibodies (Chapter 1). Fifty μl dilutions of the cytotoxin preparations in Dulbecco's PBS were dessicated in the wells of 96 well plates overnight. The
adsorbed antigens were not treated with a fixative and monoclonal antibodies in ascitic fluid (1:20 dilution) were used to quantitate the antigens. The remaining ELISA steps were completed as described (Chapter 1). The antigen titer was expressed as the inverse of the highest dilution producing an absorbance value 1.5 times greater than normal mouse serum used as a control. Cytotoxic activity present in the 5 cytotoxin preparations was determined using bovine neutrophils and trypan blue dye exclusion. Equivalent proportions of neutrophils and cytotoxin preparations were used in the trypan blue dye exclusion and MTT colorimetric assays for cytotoxin activity.

**Preparation of bovine neutrophils:**

Bovine neutrophils were prepared using modifications of the method described by Boyum. \(^{36}\) Briefly, heparinized blood (10 U/ml) was centrifuged at 500xg for 30 min at 22\(^\circ\)C and the buffy coats and upper third of the packed cell volume were collected. The contaminating erythrocytes were lysed with an equal volume of ice cold 0.87% \(\text{NH}_4\text{Cl}\) (pH 7.2) for 15 min. The resulting leukocytes were pelleted at 200 xg for 5 min and washed twice in calcium-magnesium free Hank's balanced salt solution (pH7.4). The leukocyte suspension was carefully layered on discontinuous density gradients containing Ficoll-sodium diatrizoate 1.077 g/ml on top of 1.119 g/ml. Neutrophils were isolated at the 1.077/1.119 g/ml interphase by centrifugation at 400xg for 15 min at 22\(^\circ\)C. The mononuclear cell layer at the upper gradient interphase was discarded. Neutrophils were washed three times prior to suspension in Dulbecco's PBS for enumeration and use. This procedure routinely produced cell suspensions consisting of at least
90% neutrophils with an overall viability of greater than 95% when evaluated by differential stains and trypan blue dye exclusion. The neutrophil suspension was adjusted to a final working concentration of $5 \times 10^6$ cells/ml.

**Evaluation of MTT formazan solvents:**

One hundred µl aliquots of the neutrophil suspension ($5 \times 10^6$ cells/ml) were delivered to the wells of a 96 well flat bottom tissue culture plate using a multichannel pipettor. The plate was centrifuged for 5 min at 500xg to facilitate neutrophil adherence. Aliquots (25 µl) of MTT dye at 5 mg/ml Dulbecco's PBS were added to the wells and the neutrophils were allowed to reduce the dye for 4 hr at 37°C. Different organic solvents, detergents, and combinations (100µl) were added to the wells and the plate was agitated on a micro-ELISA shaker. Solvents were visually evaluated for their ability to rapidly and completely solubilize MTT formazan formed by neutrophil monolayers in 96 well plates.

**Validation and optimization of the MTT colorimetric assay:**

Two fold test tube dilutions of the neutrophil suspension were performed between $5 \times 10^6$ cells/ml and $7.81 \times 10^4$ cells/ml in Dulbecco's PBS. Aliquots (100 µl) of these suspensions were dispensed in replicates of 5 into 96 well plates which were then centrifuged to encourage cell adherence. Twenty five µl aliquots of MTT dye (5 mg/ml) were added to each well. Plates were incubated at 1 hour intervals between 1.5 and 4.5 hr at 37°C. Following MTT incubation with neutrophils, MTT formazan was solubilized with hexamethyl
phosphoric triamide (HMPT) (100 μl) by vortexing for 7 min. Solubilized formazan was quantitated spectrophotometrically with an ELISA plate reader set at a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.00. Control blanks, consisting of replicate wells in which MTT was added to the maximum cell #/well immediately prior to solubilization, were subtracted from absorbance values and the net determined. The mean and standard deviation of the replicate tests were determined and plotted.

In a separate similar experiment, methanol or formalin fixed and washed neutrophils were mixed with viable neutrophils at various proportions while keeping the total number constant at either 5 X 10^6 cells/ml or 2.5 X 10^6 cells/ml. Absorbance was measured at 1 hour intervals between 2.5 and 4.5 hr at 37C. The mean and standard deviation of 4 replicate wells was determined and plotted.

MTT colorimetric assay for determination of cytotoxin activity:

Serial two fold (50 μl) dilutions of the cytotoxin preparations in Dulbecco's PBS were performed in separate 96 well plates. Fifty μl SP-2/o murine myeloma cell conditioned medium (RPMI-1640 with 15% (v/v) FCS, 0.01 mM hypoxanthine, 0.01 mM thymidine, 10 mM sodium bicarbonate, 1% (v/v) non-essential amino acids and L-glutamine) was added to each well. Aliquots (100 μl) of the neutrophil suspension (5 X 10^6 cells/ml) were added to the wells and the plates were incubated for 20 min at 37C. Following incubation, the plates were centrifuged and gently washed with three 150 μl volumes of Dulbecco's PBS and aspirated to remove intracellular products released from the
neutrophils. Neutrophil cytolysis was also examined in wells using an inverted light microscope. Twenty-five µl aliquots of MTT solution were added to the wells and the neutrophils were allowed to reduce the dye for 4 hr at 37°C. The resulting formazan was solubilized and quantitated as described. Controls consisted of replicate wells treated identically but using Dulbecco's PBS in place of the cytotoxin preparations and wells in which MTT was added immediately prior to the solubilization step. The mean and standard deviation of gross data from 4 replicates for each cytotoxin preparation dilution was determined and plotted.

RESULTS

Initial experiments demonstrated that bovine neutrophils reduced MTT to a deep blue insoluble formazan as has been published for lymphocytes (Figure II.1). However, acid isopropanol only partially dissolved MTT formazan using the described experimental conditions. Different organic solvents and detergents were therefore evaluated for their ability to dissolve MTT formazan produced by adherent neutrophils. Rapid and complete solubilization of MTT formazan was achieved using HMPT (50% final concentration) and shaking for 7 min. However, prolonged incubation (>1 hour) of HMPT with MTT solution resulted in spontaneous reduction (data not shown). Other solvents were found to be unsatisfactory for a variety of reasons (Table II.1).
Table II.1 - Evaluation of MTT Formazan Solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>none</td>
</tr>
<tr>
<td>ethanol</td>
<td>none</td>
</tr>
<tr>
<td>propanol</td>
<td>incomplete</td>
</tr>
<tr>
<td>isopropanol (100%, 70%, 50%)</td>
<td>incomplete</td>
</tr>
<tr>
<td>0.04 N HCl in isopropanol</td>
<td>incomplete</td>
</tr>
<tr>
<td>isopropanol with 1 to 10% TritonX-100</td>
<td>incomplete</td>
</tr>
<tr>
<td>isopropanol with 1 to 10% SDS⁺</td>
<td>gelation of wells</td>
</tr>
<tr>
<td>isopropanol in DMSO (75:25, 50:50)</td>
<td>incomplete</td>
</tr>
<tr>
<td>DMSO</td>
<td>incomplete</td>
</tr>
<tr>
<td>DMF</td>
<td>none</td>
</tr>
<tr>
<td>Acetone</td>
<td>none</td>
</tr>
<tr>
<td>Pyridine</td>
<td>of plate</td>
</tr>
<tr>
<td>HMPT</td>
<td>rapid and complete</td>
</tr>
</tbody>
</table>

* used at 100% unless indicated

⁺Abbreviations: SDS = sodium dodecyl sulfate; DMSO = dimethyl sulfoxide; DMF = dimethyl formamide; HMPT = hexamethyl phosphoric triamide.
Figure II.1 - Intracellular aggregates and extracellular crystals of MTT formazan produced by bovine neutrophils; light microscopy, X 1,000.
A plot of neutrophil number per well against absorbance at 570 nm revealed a non-linear relationship at each concentration over time (Figure II.2).

Figure II.2: Nonlinearity of absorbance verses neutrophil number at various time intervals in the MTT colorimetric assay.
Rearrangement of this data showed there was a linear relationship between absorbance and time at a constant neutrophil number (Figure II.3).

Figure II.3 - Linear relationship between absorbance and MTT incubation time at different neutrophil numbers per well.
When either formalin (data not shown) or methanol fixed and washed cells were incorporated into this system absorbance results were not adversely affected (Figure II.4).

Figure II.4 - Effect of methanol fixed neutrophils mixed with normal neutrophils in the MTT colorimetric assay. Formalin fixed neutrophils produced identical results (not shown).

The optimum conditions for the MTT colorimetric assay were chosen to be $5 \times 10^5$ neutrophils per well with a MTT incubation time of 4 hr and using HMPT as a formazan solvent.
Different dose response curves were produced using each of the 5 cytotoxin preparations. Preconcentrated cytotoxin resulted in an immediate linear concentration dependent increase in absorbance followed by increased dye reduction relative to unexposed neutrophils (Figure II.5).

Figure II.5. Dose response of bovine neutrophils exposed to the preconcentrated cytotoxin preparation in the MTT colorimetric assay. *indicates absorbance test value obtained using unexposed neutrophils.
Neutrophils responded with more variance to the higher dilutions of preconcentrated cytotoxin. Cell lysis was not evident in plate wells using an inverted microscope, even at low dilutions of preconcentrated cytotoxin. Trypan blue dye exclusion assays revealed minimal cytotoxicity and morphological change upon exposure to the preconcentrated preparation (Table II.2).

Table II.2. Cytotoxicity of the Cytotoxin Preparations Determined Using Trypan Blue Dye Exclusion.

<table>
<thead>
<tr>
<th>Cytotoxin Preparation*†</th>
<th>% Cytotoxicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconcentrate</td>
<td>20</td>
</tr>
<tr>
<td>15x Concentrate</td>
<td>90-95</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>5</td>
</tr>
<tr>
<td>56C/1hour - 15x Concentrate</td>
<td>10</td>
</tr>
<tr>
<td>Boiled/1hour - 15x Concentrate</td>
<td>5</td>
</tr>
</tbody>
</table>

*Trypan blue dye exclusion assay performed using same proportion of neutrophils and cytotoxin preparations as in colorimetric assay.
†Used at 100%, no dilution.

A sigmoidal relationship between absorbance and concentrated cytotoxin dilution was established using the MTT colorimetric assay. Increasing the cytotoxin concentration lowered the initial starting absorbance of the preconcentrated preparation and developed a baseline up to a 1:8 dilution (Figure II.6).
Figure II.6 - Sigmoidal dose response of bovine neutrophils exposed to the 15:1 concentrated cytotoxin preparation in the MTT colorimetric assay. *indicates absorbance produced by normal neutrophils.

This baseline was followed by a linear concentration dependent increase in absorbance with increasing cytotoxin dilution similar to the preconcentrated preparation. The concentrated cytotoxin preparation also caused enhanced dye reduction at high dilutions relative to unexposed cells. The baseline of this curve consisted of
totally lysed cells and MTT reduction to formazan was not evident when viewed with an inverted microscope. The concentrated cytotoxin preparation produced 90-95% cytotoxicity as assessed by trypan blue dye exclusion (Table II.2).

The ultrafiltrate from cytotoxin concentration, containing antigens less than 30 KD, produced a linear, concentration independent relationship when absorbance was plotted against dilution (Figure II.7).

Figure II.7. Dose response of bovine neutrophils to cytotoxin ultrafiltrate in the MTT colorimetric assay. *absorbance value of neutrophils not exposed to cytotoxin.
Dye reduction by neutrophils appeared to be independent of ultrafiltrate dilution and extreme variation was obtained at each assay point. No difference in neutrophil morphology was observed among the wells using an inverted microscope. Neutrophils exposed to cytotoxin ultrafiltrate were not undergoing cytolysis as evidenced by trypan blue dye exclusion (Table II.2).

Inactivation of the concentrated cytotoxin preparation, by heating at 56°C for 1 hr produced a bell shaped curve in the colorimetric assay (Figure II.8).
Figure II.8. Dose response of bovine neutrophils exposed to 15:1 concentrated cytotoxin which had been inactivated with mild heat (56°C for 1 hr). *denotes absorbance value produced by unexposed cells.

The baseline and lowered initial starting absorbance of the fully active concentrated preparation was absent. However, MTT reduction by neutrophils increased with respect to dilution, then decreased to an absorbance value characteristic of unexposed cells. Neutrophil lysis was not apparent in the plate wells and cytotoxicity was not evident visually using trypan blue dye.
Boiling the concentrated cytotoxin preparation for 1 hr, followed by clarification, resulted in a different dose response by the neutrophils (Figure II.9).

![Graph showing dose response of neutrophils to boiled and clarified concentrated (15:1) cytotoxin. *indicates normal neutrophils.](image)

Figure II.9. Dose response of neutrophils to boiled and clarified concentrated (15:1) cytotoxin. *indicates normal neutrophils.

MTT reduction was greatest when the boiled preparation was used at the highest test concentration and then decreased with dilution of the cytotoxin preparation. The cytotoxin ultrafiltrate and heat inactivated preparations caused neutrophils to respond with more variance as indicated by the large standard deviations characterizing
each dilution. Cell lysis was not evident in plate wells and cytotoxicity was not evident using trypan blue dye with these preparations.

Chemical and immunologic analysis of the cytotoxin preparations demonstrated differences in the protein and antigen content while hexosamine concentration remained relatively constant (Table II.3). The ultrafiltrate, from cytotoxin concentration, contained a nearly equivalent hexosamine concentration relative to the concentrated preparations, but only the pan-pasteurella carbohydrate antigen was detected at significant levels as detected by monoclonal antibodies in ELISA. Heating the concentrated preparation at 56°C for 1 hr did not alter the ELISA reactivity of the antigens. Boiling the concentrated preparation, followed by clarification, significantly reduced the amount of 29 kilodalton protein and it was undetectable using ELISA. The remaining antigens were not affected by boiling.
Table II.3 - Chemical and Immunologic Characterization of the Cytotoxin Preparations.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cytotoxin Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Con-centrate</td>
</tr>
<tr>
<td>Protein (μg/ml)</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>Hexosamine (μM/ml)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

McAb ELISA:

<table>
<thead>
<tr>
<th>Assay</th>
<th>Pre-Con-centrate</th>
<th>15:1 Concentrate</th>
<th>Ultra-filtrate</th>
<th>56°C/1hr</th>
<th>Boiled /1hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide</td>
<td>64</td>
<td>&gt;256</td>
<td>0</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Pan-pasteurella CH₃O</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Serotype 1 specific Ag</td>
<td>32</td>
<td>&gt;256</td>
<td>0</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>29KD protein</td>
<td>64</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;256</td>
<td>0</td>
</tr>
</tbody>
</table>

*Monoclonal antibodies were used at constant concentrations (1:20 ascitic fluid dilution) in ELISA. Antigen titer was expressed as the inverse of the highest dilution producing an absorbance value 1.5 times greater than the absorbance obtained with normal mouse serum.
DISCUSSION

In the present study, bovine neutrophils were observed to reduce MTT dye to a deep blue formazan under defined conditions in Dulbecco's PBS. The MTT formazan occurred as intracellular aggregates and extracellular crystals. Extracellular crystal formation indicated partial solubility of MTT formazan in Dulbecco's PBS. Therefore, a solvent was sought to dissolve MTT formazan without removing the well contents to ensure measurement of all reduced MTT. Acid isopropanol, used successfully by other investigators,\textsuperscript{28,29} was not effective under our experimental conditions because it would not completely solubilize MTT formazan when diluted by the well contents. HMPT was found to be a rapid and complete formazan solvent that was effective in the presence of the well contents. HMPT did not interfere with absorbance at a test wavelength of 570 nm and a reference wavelength of 630 nm and effectively solubilized neutrophils and the formazan they produced.

A non-linear relationship between absorbance and neutrophil number per well was established using the described procedures. This is in direct contradiction to other reports which describe a linear relationship between these parameters.\textsuperscript{28,29} Although many conditions in this study were varied from those originally described, the reason for this non-linearity remains largely undetermined. However, a cooperative effect by the neutrophils in MTT reduction is a plausible explanation. Our assay procedure produces a linear relationship between absorbance and MTT incubation time at constant cell numbers. The slope of these linear relationships at different cell numbers increased with respect to increasing cell concentration which supports a cooperative effect of the neutrophils in dye reduction. When
absorbance was plotted against MTT incubation time by other investigators, non-linear relationships were obtained.\textsuperscript{28,29} It was hypothesized that progressive cell death during MTT incubation was responsible for the non-linearity. Later this was proven and corrected values revealed a linear relationship over time.\textsuperscript{29} The linear relationship between absorbance and time in this study indicated a constant rate of MTT reduction. The rate of MTT reduction was not affected by mixing either methanol or formalin fixed and washed neutrophils with normal cells in the assay.

Optimization and validation studies of the MTT colorimetric assay using bovine neutrophils resulted in the decision to use HMPT as a MTT formazan solvent, 5X10\textsuperscript{5} neutrophils per well, and a MTT incubation time of 4 hr for the measurement of cytotoxin activity. The latter two parameters were chosen because they produced a neatly confluent monolayer and measurable absorbance values respectively.

Cytotoxin activity in the MTT colorimetric assay was measured using different cytotoxin preparations which varied in their ability to cause cytotoxicity as assessed by trypan blue dye exclusion assays. The preconcentrated cytotoxin preparation inhibited MTT reduction at low dilutions relative to unexposed neutrophils. Increasing the cytotoxin concentration caused further inhibition of MTT reduction and totally lysed cells. Inactivated cytotoxin preparations did not cause inhibition of MTT reduction. The inhibition of MTT reduction was interpreted as cytotoxin activity in the MTT colorimetric assay.

Enhanced MTT reduction by the neutrophils occurred in response to all cytotoxin preparations except the ultrafiltrate. Neutrophils were stimulated to reduce more MTT at different concentrations among the
cytotoxic activity was present or not. These findings indicate the MTT colorimetric assay is capable of measuring more than neutrophil cytolysis. Enhanced MTT dye reduction most likely reflects neutrophil activation via the respiratory burst. Neutrophil activation may account for their rapid and intense influx into pulmonary tissue following experimental challenge.4-7

From the results obtained, it is unclear whether cytotoxin at low concentrations, inactivated cytotoxin, other component antigens, or combinations of them are responsible for neutrophil stimulation of MTT reduction. Since most protein was removed from the boiled preparation, which caused neutrophil stimulation of MTT reduction, it is unlikely that protein in itself was responsible for neutrophil activation. Similarly, because the only detectable antigen in the ultrafiltrate was pan-pasteurella carbohydrate, which did not cause neutrophil activation, it is unlikely this antigen was solely responsible for enhanced MTT reduction. Lipopolysaccharide and capsular polysaccharide were the only two antigens, detectable using the described methods, which were common to all preparations resulting in neutrophil stimulation. Clearly, more extensive fractionation or purification of antigens in the cytotoxin preparations are needed to identify components responsible for neutrophil activation.

Because cytotoxin activity was characterized in the presence of SP-2/o murine myeloma cell conditioned medium, the MTT colorimetric assay should be readily adaptable for the selection of a cytotoxin neutralizing monoclonal antibody. Measurement of cytotoxin activity in
the presence of other body fluids, such as immune and non-immune serums, nasal or bronchoalveolar washings, may extend the use of this assay for studying other interactions. Furthermore, the incorporation of monoclonal antibodies to component antigens of cytotoxin preparations may aid identification of neutrophil metabolic responses to particular antigens or their immune complexes. The MTT colorimetric assay has potential application for investigating the metabolic response of neutrophils from other species to a wide variety of antigens, toxins and antibodies.
BIBLIOGRAPHY


CHAPTER 3

CHARACTERIZATION OF A NEUTRALIZING MONOCLONAL ANTIBODY PREPARED AGAINST PASTEURELLA HAEMOLYTICA SEROTYPE 1 CYTOTOXIN

SUMMARY

Pasteurella haemolytica serotype 1 (Ph-1) cytotoxin was produced in a defined medium, concentrated by ultrafiltration and used to immunize BALB/c mice for monoclonal antibody production. A hybridoma producing antibody capable of cytotoxin neutralization was selected using a tetrazolium dye (MTT) colorimetric assay with isolated bovine peripheral blood neutrophils as target cells. Neutrophil response to other defined monoclonal antibodies, selected by ELISA, against component antigens in the cytotoxin preparation was also evaluated in the MTT colorimetric assay. Reactivity of the cytotoxin neutralizing antibody to 6 and 24 hr cultures of known P. haemolytica (Ph) serotypes as well as Ph-1 whole cells, capsular material, and cytotoxin preparations treated in various ways was determined using an ELISA procedure. Ascitic fluid containing the neutralizing antibody was evaluated for the extent of cytotoxin neutralization using the MTT colorimetric assay. Using standard serologic methods the isotype of the neutralizing antibody was identified as IgM. The neutralizing antibody was not reactive in ELISA with Ph whole cells, capsular material, or cytotoxin preparations regardless of serotype, culture age, or treatment. Neutralizing activity was detectable at a 1:200,000 dilution of ascitic fluid in the MTT colorimetric assay. Monoclonal antibodies selected by ELISA did not affect neutrophil response to the cytotoxin preparation in the MTT colorimetric assay.
INTRODUCTION

Bovine pneumonic pasteurellosis, commonly known as shipping fever, is a severe fibrinous bronchopneumonia causing major economic losses to the North American feedlot cattle industry.\textsuperscript{1,2} Pasteurella haemolytica serotype 1 (Ph-1) is the pathogenic agent most frequently isolated in association with this disease.\textsuperscript{1-3} \textit{P. haemolytica} has been shown to secrete, into cultural supernate, a heat labile, immunogenic protein cytotoxin (leukotoxin) which is specifically cytolytic for ruminant alveolar macrophages and peripheral blood leukocytes.\textsuperscript{4-6} Cytotoxin is currently viewed as a potential virulence factor of \textit{P. haemolytica}, believed to contribute to the pathogenesis of pneumonic pasteurellosis through its lytic effect on pulmonary defense cells. The interaction of cytotoxin with neutrophils is thought to be important because of the neutrophil's early and predominant influx into lung tissue following experimental challenge,\textsuperscript{7,8} ability to clear bacteria through phagocytosis, and potential to cause tissue damage.\textsuperscript{9,10} The association of high cytotoxin neutralizing serum antibody titers with disease resistance may indicate the importance of cytotoxin in the pathogenesis of pneumonic pasteurellosis.\textsuperscript{11-13} Likewise, the lack of pathophysiologic changes in neutropenic cattle experimentally challenged with \textit{P. haemolytica} are thought to reflect the importance of neutrophils in the disease.\textsuperscript{9}

However, Ph-1 liberates several soluble antigens into cultural supernate in addition to cytotoxin which are known to affect the neutrophil. Other component antigens of cytotoxin preparations include lipopolysaccharides, capsular polysaccharides, other carbohydrates and proteins (Chapter 2). Attempts to purify cytotoxin
from other soluble antigens of P. haemolytica have resulted in a substantial loss of cytotoxic activity. Consequently, direct assays for cytotoxin, independent of its cellular effects, have not been developed.

Because cytotoxin is immunogenic and its effects are neutralizable by antibody we decided to use these properties to develop a monoclonal antibody against cytotoxin. Specific monoclonal antibody against cytotoxin could aid in its identification, quantitation, and large scale purification using contemporary immunologic techniques. Furthermore, specific neutralization of cytotoxin may allow identification of novel interactions of neutrophils with other component antigens in cytotoxin preparations. Additionally, the effect of specific antibodies against component antigens of cytotoxin preparations is unknown and could also identify more subtle interactions with neutrophils. Assays and reagents concerning the interaction of specific antibodies and antigens with neutrophils are needed to gain a better understanding of the pathogenesis in pneumonic pasteurellosis.

The response of neutrophils to active and heat inactivated cytotoxin preparations in the MTT colorimetric assay has been characterized (Chapter 2). At different concentrations, both active and inactive preparations were found to stimulate neutrophil reduction of tetrazolium dye. This assay was carried out in the presence of SP-2/o murine myeloma cell conditioned medium for equivalence with neutralization by monoclonal antibody. The primary purpose of this study was to select and characterize a neutralizing monoclonal antibody against Ph-1 cytotoxin using the MTT colorimetric assay.
Additional objectives of this investigation were to determine the effect of other component antigens in cytotoxin preparations (Chapter 2) on bovine neutrophil responses using defined monoclonal antibodies in the MTT colorimetric assay.

MATERIALS AND METHODS

P. haemolytica serotype 1 cytotoxin:

Ph-1 cytotoxin was produced in RPMI-1640 containing 10mM sodium bicarbonate and 10mM L-glutamine, concentrated 15:1 by ultrafiltration, and stored at -70C as previously described (Chapter 2).

MTT colorimetric assay of cytotoxin activity:

Cytotoxic activity in the concentrated cytotoxin preparation was measured and standardized using the MTT colorimetric assay described in Chapter 2. The assay was performed in the presence of SP-2/o murine myeloma cell conditioned medium for equivalence with cytotoxin neutralization assays.

MTT colorimetric assay for cytotoxin neutralization:

A 1:8 dilution of the concentrated cytotoxin preparation was used in the MTT colorimetric assay for cytotoxin neutralization. Cytotoxin neutralization was determined by the introduction and comparison of either neutralizing or non-neutralizing serums, hybridoma cultural supernates, or peritonal ascitic fluids (50μl) in place of the SP-2/o murine myeloma cell conditioned medium in the assay of cytotoxin.
activity. Neutralization was allowed to occur for 15 min prior to adding the neutrophils.

**The extent of cytotoxin neutralization:**

Neutralization by peritoneal ascitic fluids was determined using a 10 fold dilution series in the colorimetric assay. Defined monoclonal antibodies generated against Ph-1 capsular material (Chapter 1) were also evaluated in the assay. Checkerboard combinations of these antibodies, in equal proportions of hybridoma cultural supernate, were further examined for cytotoxin neutralization. Wells in which neutrophils were exposed to RPMI-1640 in place of cytotoxin were used as positive neutralization controls to represent live cells. Negative controls consisted of neutrophils exposed to a 1:8 dilution of concentrated cytotoxin. Results were expressed as the mean of quadruplicates plus or minus one standard deviation.

**Production of monoclonal antibody:**

Six to eight week old female BALB/c mice were immunized by intraperitoneal injection with 0.5 ml concentrated cytotoxin. Four weeks later, mice were immunized a second time using the same route and inoculum. Two weeks following the second immunization, mice were immunized again by intravenous inoculation with 0.1 ml concentrated cytotoxin. Three days after the last immunization, sera was collected from the retro-orbital plexus and evaluated for cytotoxin neutralization in the MTT colorimetric assay. A single mouse, producing the highest absorbance value in the colorimetric assay for cytotoxin neutralization, was selected for hybridoma production.
Splenocytes, from the mouse neutralizing the most cytotoxin, were fused with SP-2/o murine myeloma cells on the fourth day following the last immunization and hybridomas were selected with HAT medium as previously described (Chapter 1). Hybridoma culture supernates were evaluated for cytotoxin neutralization using the MTT colorimetric assay. Hybridomas secreting antibody capable of cytotoxin neutralization were cloned by limiting dilution. Ascitic fluids were produced by injecting cloned hybridomas into the peritoneal cavity of BALB/c mice preconditioned one week in advance with 0.5 ml pristane.

**ELISA reactivity of the cytotoxin neutralizing monoclonal antibody:**

Reactivity of the cytotoxin neutralizing monoclonal antibody with Ph-1 whole cells, capsular material, and concentrated cytotoxin preparations which had been either untreated, fixed with formalin, paraformaldehyde, or both were determined in an ELISA procedure (Chapters 1 and 2). In a separate ELISA experiment, *P. haemolytica* serotypes 1 through 12 whole cells from 6 and 24 hr cultures were used as antigens. Monoclonal antibodies developed against serotype 1 capsular material were used as positive controls. Reactivity in ELISA was determined using hybridoma cultural supernates as the primary antibody solution.

**Isotype determination:**

The isotype of the cytotoxin neutralizing monoclonal antibody was determined using commercially available kits based on antibody capture ELISA and immunodiffusion. Hybridoma cultural supernate was used in isotype determinations.
RESULTS

Figure III.1 illustrates the MTT colorimetric assay results of cytotoxic activity present in the 15:1 concentrated cytotoxin preparation.

Figure III.1 - Cytotoxic activity present in the 15:1 concentrated cytotoxin preparation using the MTT colorimetric assay. A 1:8 dilution of the concentrated cytotoxin preparation was used in the assay for cytotoxin neutralization. *Indicates absorbance value produced by normal neutrophils.
Cytotoxic activity was in excess of neutrophil number up to a 1:8 cytotoxin dilution, producing a baseline of totally lysed cells. At a 1:8 and higher cytotoxin dilutions, absorbance increased with respect to dilution. Therefore, a 1:8 dilution of the concentrated cytotoxin preparation was used in the MTT colorimetric assay for cytotoxin neutralization.

Normal mouse sera was incapable of cytotoxin neutralization in the MTT colorimetric assay (Table III.1).
Table III.1 - Antibody Solutions Capable of Cytotoxin Neutralization in the MTT Colorimetric Assay.

<table>
<thead>
<tr>
<th>Antibody Solution</th>
<th>Cytotoxin Neutralization</th>
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<tbody>
<tr>
<td>Normal mouse serum</td>
<td>-</td>
</tr>
<tr>
<td>Capsular material immune mouse serum</td>
<td>+</td>
</tr>
<tr>
<td>Cytotoxin immune mouse serum</td>
<td>+</td>
</tr>
<tr>
<td>Capsular material monoclonal antibodies: anti-*+lipopolysaccharide</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
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</table>

*Monoclonal antibodies present in either hybridoma cultural supernate or peritoneal ascitic fluid.

†Checkerboard combinations of monoclonal antibodies against capsular material antigens also evaluated.
Sera from mice immunized with either capsular material (Chapter 1) or concentrated cytotoxin effectively neutralized cytotoxin activity. Therefore, defined monoclonal antibodies generated against *P. haemolytica* serotype 1 capsular material antigens (Chapter 1) were evaluated for cytotoxin neutralization. These antibodies did not neutralize cytotoxic activity. Checkerboard combinations of these antibodies were also evaluated for neutralization without success (data not shown).

Following the fusion of splenocytes, from cytotoxin immune mice, with SP-2/o murine myeloma cells, selection of approximately 1000 hybrid cells was accomplished using HAT medium. One line from these hybrids, termed neutralizing monoclonal antibody (nMcAb), was selected using culture supernates in the MTT colorimetric assay for cytotoxin neutralization (Figure III.2). The hybridoma producing the nMcAb was cloned by limiting dilution and selected using the MTT colorimetric assay for cytotoxin neutralization.
Figure III.2 - Cytotoxin neutralization by monoclonal antibodies in the MTT colorimetric assay. nMcAb = neutralizing monoclonal antibody; #1 = anti-lipopolysaccharide; #2 = anti-pan-pasteurella carbohydrate; #3 anti-serotype 1 capsular polysaccharide; #10 = anti-29 KD protein. Hybridoma cultural supernates were not diluted. Control A represents live cells not exposed to cytotoxin. Control B represents dead cells exposed to a 1:8 dilution of concentrated cytotoxin.

The extent of cytotoxin neutralization by the nMcAb in peritoneal ascitic fluid is displayed in Figure III.3.
Cytotoxin neutralization was not detectable using ascitic fluids containing monoclonal antibodies against serotype 1 capsular material antigens (Chapter 1). The non-neutralizing ascitic fluids, containing antibodies against either lipopolysaccharide, pan-pasteurella carbohydrate, serotype 1 capsular polysaccharide or the 29KD protein of P. haemolytica, produced identical results in the MTT colorimetric assay for neutralization. Cytotoxin neutralization by the nMcAb was detectable out to a 1:200,000 ascitic fluid dilution where one standard deviation of the neutralizing and non-neutralizing ascitic fluids converged.
When the nMcAb in hybridoma cultural supernate was evaluated for reactivity with *P. haemolytica* antigens in ELISA consistently negative results were obtained. The nMcAb was not reactive with *P. haemolytica* serotype 1 whole cells, capsular material, or concentrated cytotoxin preparations. The nMcAb was not reactive with *P. haemolytica* serotypes 1 through 12 whole cells. Neither antigen formalization nor fixation with paraformaldehyde imparted ELISA reactivity. Monoclonal antibodies generated against Ph-1 capsular material (Chapter 1) were used as positive controls, substantiating the negative ELISA reactivity of the nMcAb.

Both antibody capture ELISA and immunodiffusion confirmed the presence of murine antibody in hybridoma cultural supernates with neutralizing activity and identified the isotype as an IgM.

**DISCUSSION**

The experimental results demonstrated that *P. haemolytica* cytotoxin activity can be neutralized by monoclonal antibody. Cytotoxin neutralizing activity was present in cultural supernate and peritoneal ascitic fluid derived only from the hybridoma selected using the MTT colorimetric assay. The antibody nature of cytotoxin neutralizing activity is supported by the presence of murine IgM in hybridoma cultural supernate which neutralized cytotoxic activity while other monoclonal antibodies and SP-2/0 murine myeloma cell conditioned medium could not. High cytotoxin neutralizing titers were obtained with peritoneal ascitic fluid from hybridomas producing the nMcAb compared to other non-neutralizing monoclonal antibodies using the MTT colorimetric assay.
Although nMcAb binding to *P. haemolytica* serotype 1 antigen preparations in ELISA could not be demonstrated, regardless of the conditions evaluated, a cooperative research team has shown nMcAb binding to a 105 kilodalton protein separated by electrophoresis and blotted to nitrocellulose.\(^a\) This 105 kilodalton protein has recently been shown to constitute a major component of cytotoxin.\(^6\) It is unknown why nMcAb binding could not be demonstrated in ELISA, yet detectable using immunoblotting techniques. Polyclonal rabbit antibodies prepared against the 105 KD protein, which have neutralizing activity, have been shown to bind to logarithmic growth phase *P. haemolytica* cells using indirect immunofluorescence.\(^6\) These antibodies also bound to a 95 KD primary proteolytic breakdown product identified by immunoblotting and it was unclear which of these proteins was labeled in immunofluorescence. Other lower molecular weight forms have also been detected.\(^14,15\) Proteolytic degradation or denaturation of the cytotoxin epitope responsible for neutralization could be responsible for the lack of nMcAb reactivity in ELISA.

Cytotoxin activity was also neutralized by sera from mice immunized with either *P. haemolytica* serotype 1 capsular material or cytotoxin (Table 1). Therefore, monoclonal antibodies developed against capsular material, specific for lipopolysaccharide, pan-pasteurella carbohydrate, capsular polysaccharide, and a 29 KD protein (Chapter 1) were evaluated in the MTT colorimetric assay.

\(^{a}\)Maheswaran SK, University of Minnesota, St. Paul: Personal communication 1987.
These antibodies were incapable of cytotoxin neutralization and did not affect neutrophil reduction of MTT dye in response to antigens in the cytotoxin preparation (Figure 2). Apparently, the component antigens identified or their antigen-antibody complexes have little effect on the response of neutrophils in the assay.

The main advantages of the MTT colorimetric assay were speed and sensitivity, allowing a large number of samples to be evaluated for cytotoxin neutralization. These features allowed efficient screening of hybridoma cultured supernates necessary to detect monoclonal antibody capable of neutralizing *P. haemolytica* cytotoxin. The use of the assay was further extended to determine the extent of cytotoxin neutralization by peritoneal ascitic fluids and the effect of different antibodies and combinations on neutrophil response. The results of the assay were microscopically and grossly apparent, aiding in rapid qualitative assessment. The MTT colorimetric assay is a rapid, sensitive, and versatile means of studying bovine neutrophil interaction with bacterial toxins, antigens, and specific antibodies.
BIBLIOGRAPHY


CHAPTER 4
STRUCTURAL CHANGES IN BOVINE NEUTROPHILS EXPOSED TO
PASTEURELLA HAEMOLYTICA SEROTYPE 1 CYTOTOXIN

SUMMARY

Pasteurella haemolytica serotype 1 (Ph-1) cytotoxin was produced in a defined medium, concentrated by ultrafiltration and used to characterize structural changes in isolated bovine peripheral blood neutrophils. Neutrophils were exposed to Ph-1 cytotoxin over various time intervals and concentrations for examination using light and transmission electron microscopy. Neutrophil cytotoxicity was observed to involve cell polarization with uropod formation, plasma membrane defects, and differential intracellular degranulation progressing to complete degeneration and lysis within 20 min. Identical cytotoxic changes were noted throughout both parameters suggesting cytotoxicity is both time and concentration dependent.

INTRODUCTION

Bovine pneumonic pasteurellosis or shipping fever is a severe fibrinous pneumonia causing major economic loss to the feedlot cattle industry.1,2 Although numerous predisposing factors are thought to influence the pathogenesis of this disease, infection of the lower respiratory tract with Pasteurella haemolytica serotype 1 (Ph-1) is the major cause of pneumonia and death associated with bovine respiratory disease.1-4 P. haemolytica has been shown to produce a protein cytotoxin (leukotoxin) which is toxic for bovine alveolar macrophages, peripheral blood mononuclear cells, and neutrophils.5-7
This cytotoxin may contribute to the pathogenesis of pneumonic pasteurellosis by altering cellular lung defense mechanisms and causing neutrophil lysis that releases inflammatory mediators.

The cytotoxin of \textit{P. haemolytica} was initially characterized as a high molecular weight, heat sensitive protein that was liberated during logarithmic phase growth.\textsuperscript{8,9} Cytotoxin was later shown to cause cytocidal effects in ruminant leukocytes, but did not adversely affect nonruminant cells.\textsuperscript{10,11} All serotypes of \textit{P. haemolytica} produce cytotoxin which is immunogenic and neutralizable by antibody from a variety of sources.\textsuperscript{12-15} High serum antibody cytotoxin neutralizing titers have been correlated with resistance to experimentally induced pneumonic pasteurellosis.\textsuperscript{15-17} Recently, the cytotoxin has been cloned and expressed in \textit{Escherichia coli} allowing its identification, genetic, and biochemical properties to be more extensively characterized.\textsuperscript{19-21} However, little is known about the effect of cytotoxin at the cellular level. Several methods have been developed to quantitate cytotoxin activity, using ruminant cells, which includes vital dyes,\textsuperscript{51} chromium release, luminol-dependent chemiluminescence-inhibition, and microscopy.\textsuperscript{6-17} These studies have indicated that the degree of structural, functional and metabolic effects are related to cytotoxin dose and exposure time.

The severe fibrinous bronchopneumonia produced by \textit{P. haemolytica} is characterized by the accumulation of predominately neutrophils in alveolar spaces.\textsuperscript{22,23} Neutrophils are considered important in the pathogenesis not only because of their intense pulmonary accumulation, but also because of their ability to clear bacteria and release inflammatory mediators. Cytotoxin is recognized to destroy the
phagocytic capabilities of neutrophils and lead to cell lysis.\textsuperscript{7,8} Upon uncontrolled degranulation or lysis, neutrophils release potent oxygen dependent and independent microbiocidal factors which can further damage pulmonary tissue and exacerbate disease.\textsuperscript{24-27} Likewise, stimulated neutrophils mobilize and metabolize arachadonic acid to form many important inflammatory mediators.\textsuperscript{28} The interaction of \textit{P. haemolytica} cytotoxin with bovine neutrophils is clearly an important occurrence in the pathogenesis of pneumonic pasteurellosis and the topic of many investigations. However, the mechanism of cytotoxin's cytocidal effects on neutrophils has remained obscure.

To gain a better understanding of cytotoxin's mechanism of neutrophil cytotoxicity and hence its role in the pathogenesis of pneumonic pasteurellosis a further characterization of the toxins interaction with neutrophils is needed. The purposes of this study were to microscopically characterize the structural changes in bovine neutrophils exposed to \textit{P. haemolytica} cytotoxin over different time intervals and cytotoxin concentrations.

\textbf{MATERIALS AND METHODS}

\textit{P. haemolytica} cytotoxin:
Cytotoxin from \textit{Pasteurella haemolytica} biotype A, serotype 1 was produced in a defined medium, concentrated by ultrafiltration (15:1), and quantitated using bovine neutrophils as target cells in a colorimetric assay as previously described (Chapter 2). Cytotoxin dilutions were performed in Dulbecco's PBS (pH 7.4).
Bovine neutrophils and exposure to cytotoxin:

Bovine neutrophils were isolated from peripheral blood by double discontinuous density gradient centrifugation (Chapter 2). Isolated neutrophils were suspended in Dulbecco's PBS to a final concentration of 5×10⁶ cells/ml. Approximately 5-10% of this cell population was mononuclear cells which co-purified with the neutrophils. Aliquots of the neutrophil suspension (2.0 mls) were exposed to 1.0 ml volumes of a 1:8 dilution of concentrated cytotoxin for 5 min intervals to 20 min in the temporal study. In the dose study, 2.0 ml aliquots of the neutrophil suspension were exposed to 1.0 ml volumes of a 2-fold cytotoxin dilution series for 20 min. To halt cytotoxicity, cells were fixed overnight with 3.0 mls 0.2M sodium cacodylate buffer (pH7.2) containing 3% glutaraldehyde. Control neutrophils were exposed to Dulbecco's PBS or RPMI-1640 containing 10 mM sodium bicarbonate and L-glutamine under identical conditions. Duplicate samples were prepared for light and transmission electron microscopy. The temporal and dose studies were performed as separate experiments.

Microscopy of Neutrophils:

Light microscopy was performed on wet mounted slides stained with either new methylene blue or trypan blue. Slides were examined at 400x and 1000x magnification. Preparation of the neutrophils for transmission electron microscopy was adapted from the encapsulation method of Kellenberger, et al.²⁹ Briefly, the cells were gently washed with fixative, stained with 1% OsO₄, and rewashed. The osmium fixed pellet was encapsulated with 3% agarose containing uranyl acetate and glutaraldehyde. Following encapsulation, samples were alcohol
dehydrated and infiltrated with epon/araldite and allowed to polymerize overnight at 60°C. Samples were sectioned at 60-80 nm and picked up on 200-300 mesh copper grids. The grids were then stained with uranyl acetate and lead nitrate solutions. \(^{30,31}\) Grids were examined using a Zeiss EM 10-A transmission electron microscope.

RESULTS

Light Microscopy:

Light microscopy of wet mounted control neutrophils, not exposed to cytotoxin, demonstrated basically round and highly light refractile cells. The multilobed nuclei of these cells were centrally located and the cytoplasmic granules appeared evenly distributed. Cellular aggregation was not evident grossly or microscopically. Control neutrophils readily excluded trypan blue dye.

Following a five min exposure to cytotoxin, the majority of cells lost their refractility and round morphology becoming oval to elongated. Prominant cytoplasmic projections developed and cytoplasmic granules were observed to be polarized to the opposite side from these projections. Plasma membrane damage was confirmed by the uptake of trypan blue dye. Cellular aggregation was apparent, but not pronounced.

After ten min exposure to cytotoxin, cell morphology became more oval to elongated. Large cytoplasmic projections extended from cell surfaces. Many cells were recognized to have plasma membrane defects large enough for granules and nuclei to exit the cytoplasm through these projections. Cellular aggregation was more pronounced and most cells readily took up trypan blue dye.
By 15 to 20 min neutrophil cytotoxicity was judged as complete, only aggregated ghost cells and cellular debris remained. All neutrophils were severely affected, highly irregular cell shapes containing few intracytoplasmic granules were observable. No differences were noted between the temporal and dose studies, similar morphological changes were observed in short exposures to high cytotoxin concentrations. Exposure of cells to high cytotoxin concentrations (<1:8 dilution) for prolonged times (>20 min) caused complete neutrophil lysis. Light microscopic examination of the neutrophil debris revealed low numbers of intact mononuclear cells which were apparently resistant to cytotoxin at the described times and concentrations.

**Transmission electron microscopy:**

Transmission electron microscopy of normal neutrophil morphology revealed cells with a round shape containing a centrally located multilobed nucleus, confirming light microscopy observations. The cell surfaces were uniformly involuted and cytoplasmic granules were abundant, evenly distributed, and displayed ellipsoidal to round profiles, indicating rod to spherical shapes. Differences in size and electron density of the granules were also evident. Mitochondria were easily differentiated from the granules based on these characteristics (Figure IV.1).
Figure IV.1. Transmission electron micrograph of a normal bovine neutrophil. Note round shape, centrally located nucleus, and well defined, evenly distributed granules of various sizes, shapes, and densities. X10,000.

Following a 5 min exposure to cytotoxin (1:8 dilution) considerable structural changes occurred. Prominant uropods developed from cell surfaces (Figure IV.2).
Figure IV.2. Electron micrograph of a bovine neutrophil exposed to cytotoxin (1:8 dilution) for 5 min. Note elongated shape, eccentric nucleus, polarized granules, and prominent cytoplasmic projection (uropod). Granules appear larger, less distinct and more electron dense. X10,000.

Membranes limiting these uropods had a moth eaten appearance, indicating damage which was confirmed by the inability to exclude trypan blue dye in light microscopy. The cells became polarized, in that nuclei were shifted to an eccentric site and granules were
mobilized to the opposite side from the uropods limited by damaged membrane (Figure IV.2). All granules appeared larger, more electron dense, and their sharpness became less distinct. Higher magnification of these enlarged fuzzy granules revealed alteration of their integrity characterized by changes reminiscent of small vacuolar or tubular formations (Figure IV.3).

Figure IV.3. Alteration of bovine neutrophil granule integrity occurring at 5 min cytotoxin (1:8 dilution) exposure suggestive of small vacuolar or tubular formations. X60,000.
After 10 min exposure to cytotoxin larger and broader uropods limited by incomplete membranes were commonly observed (Figure IV.4).

Figure IV.4. Bovine neutrophil with 2 large, broad cytoplasmic projections (uropods) typical of cells following a 10 min cytotoxin (1:8 dilution) exposure. The plasma membrane limiting the uropods has a moth eaten appearance and large dense persistent granules containing folded cord like structures dominate in the cytoplasm. X10,000.
A dramatic decrease in granule numbers was observed and the remaining granules were much larger and more electron dense. The remaining large granules contained electron dense folded cord-like structures which exhibited a striking periodicity at higher magnifications (Figure IV.5A and B).

Figure IV.5. Transmission electron micrographs showing granules in neutrophils exposed to cytotoxin (1:8 dilution) for 10 min. (A) folded cord-like intragranular structures of increased electron density. X31,500. (B) Higher magnification of Fig. IV.5A demonstrating periodicity. X63,000.
At 15 min, the large broad uropods commonly present in the 10 min exposures, were rarely observed. Round to oval cell shapes with a highly irregular surface and incomplete membranes predominated (Figure IV.6).

Figure IV.6. Neutrophil following a 15 min cytotoxin (1:8 dilution) exposure. Note lack of a prominent uropod and plasma membrane integrity. There is a decrease in the number of persistent granules which are forming concentrically laminated structures (arrow). X10,000.
The number of large, dense, persistent granules decreased further and many were in the process of forming myelin figures. These concentrically laminated structures with a central electron density were observed to replace the large dense granules persisting at 10 min cytotoxin exposure (Figure IV.7).

Figure IV.7. Concentrically laminated myelin figure formed from the large persistent granules. Note lack of integrity and moth eaten appearance of plasma membrane.
At 20 min, cell shape remained round to oval with an irregular surface, resembling the 15 min exposure. However, many cytoplasmic vacuoles were present that contained a flocculant material and few to no granules remained (Figure IV.8).

Figure IV.8. Typical neutrophil at 20 min post cytotoxin (1:8 dilution) exposure. There is a lack of membrane integrity and cytoplasmic granules. Numerous vacuoles containing a flocculant material are present in the cytoplasm. X10,000.
The nuclei of the remaining cells lost their tightly wound form and became swollen.

At a low cytotoxin concentration (1:512 dilution) for 20 min, most neutrophils formed uropods and became polarized. Nuclei shifted to an eccentric location and granules were always present at a site opposite from the cytoplasmic extensions. Granule morphology appeared normal in that size shape, and density remained unchanged (Figure IV.9).

Figure IV.9. Transmission electron micrograph of a bovine neutrophil exposed to a 1:512 cytotoxin dilution for 20 min. The cells displays a polarized morphology and 2 uropods at the posterior aspect. Plasma membrane integrity and granule morphology remain normal.
Plasma membranes were not observed to be damaged using electron microscopy and very few neutrophils took up trypan blue at this cytotoxin concentration in light microscopy.

Doubling the dose of cytotoxin (1:256 dilution) produced noticeable changes in the plasma membrane and granules. Prominant uropods developed, identical to those occurring in the temporal study, which were limited by a membrane with a moth eaten appearance. Granules appeared enlarged, less clearly demarcated, and more electron dense. These structural changes were indistinguishable from those occurring at 5 min in the temporal study (Figure IV.10).

Figure IV.10. Neutrophil exposed to a 1:256 cytotoxin dilution for 20 min. Note identity with Figure 2. X10,000.
Higher concentrations of cytotoxin (1:64 dilution) produced large broad uropods incompletely limited by a plasma membrane. Granule number decreased and the persistent ones became swollen, more electron dense, and contained the folded cord-like structures (Figure IV.11).

Figure IV.11. Neutrophil exposed to a 1:64 cytotoxin dilution for 20 min. Note identity with Figure 4. X10,000.
These structural changes were identical to those occurring at 10 min exposure to a 1:8 cytotoxin dilution.

The large broad uropods occurring during exposure to a 1:64 cytotoxin dilution were not evident in neutrophils exposed to 1:32 dilution. Increasing cytotoxin concentration produced round to oval shaped cells with an irregular margin, paralleling those changes typifying a 15 min exposure to a 1:8 cytotoxin dilution (Figure IV.12).

Figure IV.12. Neutrophil exposed to a 1:32 cytotoxin dilution for 20 min. Note similarities with Figure 6. X10,000.
No differences were observed between a 1:16 and 1:8 cytotoxin dilution in the dose study which were typical of cells in Figure 8. Structural changes were remarkably similar throughout the time and dose studies.

DISCUSSION

Following stimulation with chemotactic factors neutrophils commonly undergo structural changes characterized by an elongated polarized form. Polarized neutrophils develop lamellapodium or pseudopods towards the leading edge of stimulation. Posterior cytoplasmic projections or uropods form the tail of polarized cells just behind the nucleus. During polarization cytoplasmic granules are mobilized anteriorly, towards the pseudopods, for degranulation within phagosomes. Cellular polarization of membrane receptors and contractile filaments has also been observed to occur following chemotactic factor stimulation. Membrane receptors are diffusely distributed over the surface of unstimulated cells, but are found predominately in the uropod of polarized cells. After cytotoxin exposure, neutrophils in this study were observed to elongate and assume a polarized orientation, suggesting stimulatory chemotactic factors are present in the cytotoxin preparation. The presence of chemotactic factors in cytotoxin preparations could be related to the early and intense pulmonary neutrophil accumulation following experimental challenge. The cytoplasmic projections observed herein developed posteriorly with respect to the polarized orientation, tentatively identifying these structures as uropods. These structures bear remarkable similarity to uropods induced by chemotactic factors in other studies. These uropods increased in
size and lost membrane integrity as cytotoxin dose and exposure time increased. The disruption of the uropod plasma membrane integrity suggests a receptor mediated mechanism of cytotoxicity since receptors are known to be concentrated in this area following stimulation. The contention of a receptor mediated mechanism of cytotoxicity may explain the ruminant cell specific action of cytotoxin.

Investigations into the degranulative process of human bovine neutrophils has revealed a complex event in which more than one signal is involved. Calcium ionophores (A23187), phorbol myristate acetate, and immune complexes cause a time and dose dependent release of enzymatic activity associated with the specific granules while azurophil granules remain cell associated. Following phagocytosis or exposure to soluble stimuli, bovine neutrophils discharge by true secretion both their large and specific granules. Azurophil granules are fully retained. These findings have supported a selective independent mobilization of cytoplasmic granules upon exposure to particulate as well as soluble stimuli. This differential degranulation was also noted in neutrophils exposed to cytotoxin by the persistence of the large dense granules containing the cord-like structures exhibiting periodicity. The differential intracellular degranulation of bovine neutrophils exposed to cytotoxin could be related to the mechanism of cytotoxicity.

Human neutrophil granules have been reported to contain a central crystal of well defined periodicity. Additionally, bovine neutrophil large (third) granules are accepted to contain a central core of matrix material. The persistent large granules described in
this study also contained subgranular structures exhibiting periodicity. These granules were later observed to form myelin figures. However, the significance and function of these structures remain undetermined. The number and morphology of the persistent granules are consistent with descriptions of the large (third) granules in bovine neutrophils.\textsuperscript{43,47} This large granule is reported to contain primarily lactoferrin and other less prominent cationic proteins.\textsuperscript{43} It is interesting to note that lactoferrin and other iron binding compounds may supply a usable source of iron for toxinogenesis by \textit{P. haemolytica}.\textsuperscript{48} Lactoferrin release by neutrophils exposed to cytotoxin may increase cytotoxin production in vivo and exacerbate disease.

All ruminant leukocytes are reported to be sensitive to \textit{Pasteurella} cytotoxin.\textsuperscript{49-52} The differential susceptibility of bovine leukocytes to cytotoxin has been previously reported and neutrophils were found to be the most sensitive.\textsuperscript{53} In this study, a neutrophil population containing 5-10\% mononuclear cells was exposed to an excess of cytotoxin activity (15:1 concentrate) for prolonged times (>20 min) and resistant cells were identified as mononuclear using light microscopy (not shown). Our results suggest that neutrophils are more sensitive to cytotoxin than mononuclear cells and supports the contention of differential leukocyte susceptibility.
BIBLIOGRAPHY


CHAPTER 5

PURIFICATION OF THE SEROTYPE 1 SPECIFIC EPITOPE FROM
PASTEURELLA HAEMOLYTICA USING MONOCLONAL ANTIBODY IMMUNOAFFINITY

SUMMARY

A murine monoclonal IgM agglutinating antibody was used in immunoaffinity to purify the serotype 1 specific epitope from Pasteurella haemolytica (Ph-1). The Ph-1 specific antibody was precipitated from peritoneal ascitic fluid, dialyzed, evaluated for activity, and covalently attached to CNBr activated Sepharose 4B at alkaline pH. Retention of purified antibody activity and coupling efficiency to the support material were greater than 99% when evaluated by ELISA, agglutination, and protein determinations. KSCN was selected as an eluant based upon reversible dissociation of bacterial agglutination and titrated for the lowest effective concentration. Immunobead activity was observed microscopically by immobilization of encapsulated Ph-1 and reversible dissociation after elution with 0.4M KSCN. Specificity of immobilization was confirmed using P. haemolytica serotypes 2 and 5 which were not bound and by blocking Ph-1 binding with homologous capsular material. Saline extractable capsular material from Ph-1 was used as an antigen source and reacted with an equal volume of immunobeads. The immobilized antigen was separated and washed by centrifugation. Following elution of the Ph-1 specific epitope, the product was dialyzed and analyzed using chemical and immunologic methods. The purified Ph-1 specific epitope contained no detectable protein and at least one-half the original hexosamine
content. Using defined monoclonal antibodies in ELISA, titration of the immunoaffinity product and original capsular material revealed specific retention of lipopolysaccharide, pan-pasteurella carbohydrate, and serotype 1 capsular polysaccharide, suggesting epitope sharing among hexosamine antigens of Ph-1.

INTRODUCTION

*Pasteurella haemolytica* serotype 1 (Ph-1) is recognized as the major cause of severe fibrinous pneumonia in cattle termed pneumonic pasteurellosis or shipping fever. Although greater than 12 serotypes of *P. haemolytica* have been defined, it is predominately serotype 1 and less frequently serotype 2 or *P. multocida* which are associated with this disease.

The serotypes of *P. haemolytica* have been defined by either indirect haemagglutination or rapid plate agglutination using diffusible surface antigens or encapsulated bacteria respectively. An age-dependent capsular material has been demonstrated on early logarithmic growth-phase bacteria. This capsular material is readily diffusible and easily removed without adversely affecting bacterial viability. Polysaccharides present in capsular material are thought to confer serotype specificity among the Pasteurellae.

Because bacterial capsular polysaccharides are recognized as pathological determinants in numerous diseases, the role of capsular material in pneumonic pasteurellosis has been the subject of many investigations. Immunization with live encapsulated Ph-1 or capsular extracts has resulted in enhanced resistance to experimental
Large amounts of capsular material have been associated with Ph-1 isolated from cattle with pneumonic pasteurellosis; little to no capsule exists on the organism isolated from nondiseased animals. Furthermore, high antibody titers to polysaccharide antigens, derived from saline extractable capsular material of Ph-1, has been correlated with disease resistance. These findings signify the importance of capsular material and especially the Ph-1 specific antigen in the pathogenesis of pneumonic pasteurellosis.

However, capsular material extracted from Ph-1 is known to contain lipopolysaccharides and outer membrane proteins in addition to capsular polysaccharides. The relative contribution of these component antigens to the importance attributed to capsular material remains undetermined. Versatile procedures are needed to purify capsular antigens in order to determine their importance. Several procedures have been adopted or developed to separate capsular antigens. The purposes of this study were to establish an optimized monoclonal antibody immunoaffinity procedure to purify the serotype 1 specific epitope from \textit{P. haemolytica} and identify the product antigen(s). We believe this is the first immunological purification of bacterial polysaccharide antigens.

\textsuperscript{a}Corstvet, RE, unpublished observation.
MATERIALS AND METHODS

Monoclonal antibodies:

The development and characterization of murine monoclonal antibodies has been described (Chapter 1). A Ph-1 specific IgM agglutinating antibody was used as the ligand in immunoaffinity. Other defined monoclonal antibodies to antigens of P. haemolytica were used to evaluate the immunoaffinity product. Hybridomas were injected into the peritoneal cavity of pristane primed BALB/c mice for ascitic fluid production. Monoclonal antibodies were fractionated from ascitic fluid by precipitation in 50% saturated ammonium sulfate at 4C. The precipitated antibodies were collected by centrifugation and resuspended in PBS (pH 7.2) to one half the original ascitic fluid volume. Residual ammonium sulfate was removed by dialysis against PBS (0.1M, pH 7.2) at 4C for 48 hr. Purified monoclonal antibodies were filter sterilized (0.22 μm) and stored at -70C prior to use. The retention of purified antibody activity relative to peritoneal ascitic fluid was evaluated using antibody ELISA and agglutination titers (Chapter 1) and Bradford protein determinations. All ELISA titers were expressed as the inverse of the highest dilution producing an absorbance value 1.5 times greater than normal mouse serum (1:100 dilution) used as a control.

Antigen source:

Capsular material from Ph-1 serotype 1 was extracted from 6 hour cultures at 41C for 1 hour in PBS using the method of Gentry. Extracted capsular antigens were extensively dialyzed against 4 changes
of PBS over 48 hr at 4C and filter sterilized (0.22 μm). Capsular material was characterized prior to immunoaffinity using protein and hexosamine determinations. Antigens present in capsular material were quantitated using defined monoclonal antibodies in ELISA (Chapter 1).

**Eluant selection:**

Eluant selection was based on dissociation of bacterial agglutination and reversibility upon dilution with distilled water. The selected eluant was then titrated for the lowest concentration causing dissociation prior to use.

**Support material and coupling conditions:**

CNBr activated Sepharose 4B was used as the support material and the monoclonal antibody was covalently attached at 10 mg/ml preswollen beads at pH8.3 in NaHCO₃ (0.1M) buffer containing 0.5M NaCl for 2 hr at room temperature. The post-coupling supernate was retained for subsequent analysis. Remaining binding groups on the support material were blocked with 0.1M Tris buffer pH 8.5 for 2 additional hr. Unbound protein was desorbed from the immunobeads by repeated alternate washes of acetate buffer (0.1M, pH 4.0) and the coupling buffer, each containing 0.5 M NaCl. The immunobeads were stored at 4C and repeatedly washed with PBS prior to use. Antibody coupling efficiency was determined by comparing the purified antibody to the post-coupling supernate using ELISA and agglutination titers and protein determinations. Immunobead activity was determined microscopically by observing for immunobilization of encapsulated Ph-1. The efficacy of
the selected eluant was confirmed by observing bacterial elution from the immunobead surface. Specificity of bacterial immobilization was evaluated using either encapsulated \textit{P. haemolytica} serotype 2 or 5 and blocking Ph-1 binding with homologus capsular material.

\textbf{Antigen separation and analysis:}

Capsular material was reacted with an equal volume of immunobeads, in a polypropylene test tube, for 30 min at 37°C while gently rocking. The serotype 1 specific epitope was separated and washed 3 times with PBS-Tween (pH 7.2, 0.05% Tween 20) by centrifugation at 50 Xg for 2 min at 22°C. All supernates from these washes were saved for further analysis. The serotype 1 specific epitope was eluted from the immunobeads with an equal volume of 0.4 M KSCN for 15 min at 22°C while gently rocking. The eluant was removed from the serotype 1 specific epitope by dialysis against PBS at 4°C. Separation efficiency during immunoaffinity was determined by comparing protein\textsuperscript{25} and hexosamine\textsuperscript{26} determinations of capsular material to the PBS-Tween washes. The dialyzed immunoaffinity product was analyzed using protein and hexosamine determinations and defined monoclonal antibodies in ELISA (Chapter 1).

\textbf{RESULTS}

Following precipitation, dialysis, and filter sterilization of the Ph-1 specific monoclonal antibody, ELISA and agglutination titers were observed to increase relative to peritoneal ascitic fluid (Table V.1).
Table V.1 - Retention of Antibody Activity and Protein Concentration of Purified Monoclonal Antibody.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ascitic Fluid</th>
<th>Purified Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA titer*</td>
<td>$10^7$</td>
<td>$&gt;10^{11}$</td>
</tr>
<tr>
<td>Agglutination titer*</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Protein concentration</td>
<td>NT</td>
<td>11.43 mg/ml</td>
</tr>
</tbody>
</table>

*Titers expressed as inverse of highest dilution imparting a positive result.

NT = not tested

ELISA antibody titer increased more than the agglutination titer.
Protein concentration of the purified antibody was determined for optimal coupling to CNBr activated Sepharose 4B and subsequent evaluations.

Saline extractable capsular material from Ph-1 used as an antigen source in immunoaffinity, was determined to contain 60 µg/ml protein and 0.20 µM/ml hexosamine. Titration of capsular material in ELISA using defined monoclonal antibodies, at constant concentration, allowed quantification of specific antigens for subsequent comparison with the immunoaffinity product (Table V.2).
**Table V.2 - Characterization of Saline Extractable Capsular Material of *P. haemolytica* serotype 1.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (μg/ml)</td>
<td>60</td>
</tr>
<tr>
<td>Hexosamine (μM/ml)</td>
<td>0.20</td>
</tr>
<tr>
<td>McAbs in ELISA*</td>
<td></td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>32</td>
</tr>
<tr>
<td>Pan-Pasteurella CH₂O</td>
<td>&gt;512</td>
</tr>
<tr>
<td>Ph-1 specific Ag</td>
<td>16</td>
</tr>
<tr>
<td>29 KD Protein</td>
<td>8</td>
</tr>
</tbody>
</table>

*Capsular antigens were titrated in ELISA and defined monoclonal antibodies (McAbs) were used to identify the titer expressed as the inverse of highest capsular antigen dilution giving a positive result. CH₂O = carbohydrate, Ag = antigen.

KSCN was selected as an immunoaffinity eluant based on reversible dissociation of encapsulated Ph-1 agglutination by the Ph-1 specific monoclonal antibody. Dilution of the KSCN dissociated agglutination reaction with distilled water confirmed reversibility of the effect by causing agglutination to reoccur. However, the degree of bacterial agglutination was visually reduced upon dilution. Titration of KSCN for the lowest effective concentration causing dissociation of agglutination revealed 0.4M to be the optimal concentration for antigen elution. Reversibility of agglutination was easily achieved.
with minimum dilution of the 0.4M KSCN dissociated agglutination. 
\( \text{Na}_2\text{S}_2\text{O}_3 \) was found to cause dissociation of agglutination, but this 
effect was not as complete as with KSCN and could not be reversed upon 
dilution. Glycine-HCL (0.2M,pH 2.5) did not cause dissociation of 
agglutination (Table V.3).

Table V.3 - Selection of an Optimal Eluant for Immunoaffinity Based on 
Dissociation of Bacterial Agglutination.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>Dissociation</th>
<th>Reversibility</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSCN</td>
<td>+4</td>
<td>+2</td>
<td>0.4M</td>
</tr>
<tr>
<td>( \text{Na}_2\text{S}_2\text{O}_3 )</td>
<td>+4</td>
<td>Neg</td>
<td>NT</td>
</tr>
<tr>
<td>Glycine-HCL</td>
<td>Neg</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>(pH 2.5 0.2M)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NT = not tested

Antibody coupling efficiency to CNBr activated Sepharose 4B was 
determined by comparing the purified antibody to the post-coupling 
supernate (Table V.4). The lack of demonstrable ELISA and 
agglutination titers and negligible amount of protein in the 
post-coupling supernate indicated greater than 99% of the Ph-1 
specific antibody was covalently attached to the support material.
Table V.4 - Monoclonal Antibody Coupling Efficiency to CNBr Activated Sepharose 4B.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Purified Antibody</th>
<th>Post-coupling supernate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA titer</td>
<td>$&gt;10^{11}$</td>
<td>0</td>
</tr>
<tr>
<td>Agglutination titer</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Protein</td>
<td>11.43 mg/ml</td>
<td>&lt;5.0 µg/ml</td>
</tr>
</tbody>
</table>
Figure V.1 - Phase contrast micrograph of encapsulated *P. haemolytica* serotype 1 immobilized on immunobead surface. X400.
Phase contrast microscopy demonstrated immunobead activity by immobilization of encapsulated Ph-1 on the surface (Figure V.1). The number of bacteria bound was found to be related to the amount of capsular material in solution since this immobilization could be completely blocked by preincubation of the immunobeads with capsular material. Specificity of bacterial immobilization was confirmed using either *P. haemolytica* serotypes 2 or 5 which were not bound (Figure V.2).
Figure V.2 - Phase contrast micrograph of encapsulated *P. haemolytica* serotype 2 and immunobeads demonstrating specificity by a lack of immobilization. *P. haemolytica* serotype 5 was not immobilized and micrograph is typical following elution with 0.4M KSCN. X400.
Elution of encapsulated Ph-1 from the immunobead surface with 0.4M KSCN was complete, typical of Figure V.2, and reversible upon dilution with distilled water, however, only a small number of bacteria would reassociate (not shown). Immunobeads regenerated by antigen elution with 0.4M KSCN and extensive washing with PBS consistently bound numerous encapsulated serotype 1 bacteria representative of Figure 1.

Separation of Ph-1 capsular antigens during immunoaffinity was evaluated using chemical determinations of the washes relative to the original capsular extract (Table V.5).

Table V.5 - Chemical Determination of Immunoaffinity Antigen Separation.

<table>
<thead>
<tr>
<th>Assay</th>
<th>original</th>
<th>start</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (μg/ml)</td>
<td>60</td>
<td>36</td>
<td>&lt;18</td>
<td>&lt;10</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Hexosamine (μM/ml)</td>
<td>0.20</td>
<td>0.10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not determined because below test limits.

Protein and hexosamine concentrations decreased consistently throughout washing indicating high fidelity in separation. Protein concentration approached zero with repeated washing while one half of the original capsular hexosamines were retained by the immunobeads.
Chemical analysis of the immunoaffinity product revealed a lack of detectable protein and approximately one half the hexosamine content as the original capsular extract (Table V.6).

Table V.6 - Analysis of the Immunoaffinity Product.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (µg/ml)</td>
<td>zero</td>
</tr>
<tr>
<td>Hexosamine (µM/ml)</td>
<td>0.12</td>
</tr>
<tr>
<td>McAbs in ELISA*</td>
<td></td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>16</td>
</tr>
<tr>
<td>Pan-pasteurella CH₂O</td>
<td>&gt;512</td>
</tr>
<tr>
<td>Ph-1 specific Ag</td>
<td>16</td>
</tr>
<tr>
<td>29 KD protein</td>
<td>zero</td>
</tr>
</tbody>
</table>

*Immunoadfinity product was titrated in ELISA and defined monoclonal antibodies (McAbs) were used to determine the titer expressed as the inverse of of the highest dilution producing an absorbance value 1.5 times greater than normal mouse serum used as a control. CH₂O = carbohydrate, Ag = antigen.

Titration of the immunoaffinity product in ELISA, using defined monoclonal antibodies at constant concentrations, revealed 3 molecules were selectively purified by the procedure (Table V.6). Lipopolysaccharide and pan-pasteurella carbohydrate co-purified with serotype 1 specific antigen. One half of the original capsular
extract lipopolysaccharide was recovered using these methods. Although the titer of the pan-pasteurella carbohydrate was greater than assayed, in both the original capsular extract and immunoaffinity product, the overall absorbance values were greater in the immunoaffinity product indicating selective retention of this molecule (not shown). The titer of the Ph-1 specific antigen was the same in the original capsular extract and the immunoaffinity product. The 29 KD protein was absent, as expected since all protein was removed by washing, from the product.

DISCUSSION

Reversible dissociation of bacterial agglutination was used in this study to select an optimal eluant for immunoaffinity. Glycine - HCL (0.1M, pH 2.5) and Na₂S₂O₃ were found to be unsuitable as eluants using this criteria. Glycine - HCL, of low pH has been used successfully as an IgG eluant in a variety of previous studies. Apparently, a shift in pH had no effect on the IgM - Ph-1 complex as it does with IgG antigen complexes and agglutination was not dissociated. Na₂S₂O₃ caused dissociation of agglutination, however, reversibility could not be achieved upon dilution with distilled water. The reason for irreversibility encountered with Na₂S₂O₃ remains unknown, but permanent antigen or antibody denaturation are possibilities. Chaotropic thiocyanates have been previously used as successful immunoaffinity eluants in a variety of applications. KSCN was found to satisfy our criteria in the selection of an eluant and was titrated for the lowest concentration causing dissociation. The lowest dissociative concentration was considered optimal because it produced
mild elution conditions which were easily reversible. These results indicate properties of immunoglobulins, particularly agglutination and possibly precipitation, can be used in the selection and optimization of immunoaffinity eluants.

Immunobead activity was assessed by microscopic observation of encapsulated bacterial immobilization. The agglutinating activity of the Ph-1 specific IgM monoclonal antibody was undoubtedly responsible for this phenomena. Following establishment of immunobead activity, the specificity of bacterial immobilization was evaluated using \textit{P. haemolytica} serotypes 2 and 5 and by blocking Ph-1 binding with homologous capsular material. Encapsulated Ph-1 could be reversibly dissociated from the bead surface using the optimized eluant. These findings supported previous rational and conclusions and provided a fast, easy, and practical means of assessing immunobead activity prior to use. Other immunoaffinity procedures have only to rely on product evaluation, sometimes days to weeks later, as an assessment of activity. Thus, agglutination by immunoglobulins can be applied to test the feasibility and specificity of using different antibody coupling procedures, support materials, and eluants in immunoaffinity.

Separation and washing of the immunobead antigen complex was performed by low speed centrifugation. This proved to be a fast, easy means of separating and washing the bound complex. Another advantage of this method was that volumes of wash and eluant solutions were easily controllable and held constant for subsequent evaluations. Common problems encountered with affinity columns are dilution of the product upon elution, pH neutralization, and identification of the product in numerous fractions. In this study, volumes of wash and
eluants equivalent with the antigen volume were used allowing easy tracking and evaluation of the components. This method also deleted the need for special, expensive, and sometimes cumbersome mechanical equipment.

The procedures used in this study were evaluated by chemical and immunological methods. Antibody activity was assessed using ELISA and agglutination titers which were observed to increase following purification and concentration of monoclonal antibody from peritoneal ascitic fluid. This may have been due to removal of inhibitory substances, such as pristane, lipids, or proteolytic enzymes during purification. The agglutination titer did not increase as much as the ELISA titer and was attributed to solubilization of capsular antigens, since Ph-1 binding could be blocked with capsular material. These titers with protein determinations were also used to evaluate covalent antibody attachment to the support material and indicated the described conditions removed all antibody activity from solution.

Capsular material from Ph-1 has been shown to contain both protein and hexosamine antigens quantifiable by chemical and immunological methods. Chemical determinations of the capsular material, wash solutions, and immunoaffinity product allowed evaluation of the separation procedures. Protein concentrations decreased consistently and one-half the hexosamines were removed during washing, indicating the Ph-1 specific antigen was hexosamine in nature. This conclusion supports the work of other investigators who have identified the Ph-1 specific antigen as hexosamine.

Following antigen elution and dialysis, chemical analysis of the immunoaffinity product revealed a lack of detectable protein and
one-half the original hexosamine content. ELISA identification and titration of the product antigens demonstrated selective retention of lipopolysaccharide, pan-pasteurella carbohydrate and the Ph-1 specific capsular polysaccharide; all hexosamine containing antigens. The LPS ELISA titer, in the product, was decreased by one half and the pan-pasteurella carbohydrate titer remained greater than assayed. However, the absorbance values (not shown) of the pan-pasteurella carbohydrate were higher in the product than the original capsular material. The Ph-1 specific capsular polysaccharide titer remained the same following immunoaffinity. The reason for the selective retention of the 2 unexpected antigens remains undetermined, however several possibilities exist.

Non-specific retention of antigens through immunoaffinity procedures is a common problem. Support materials may non-specifically absorb antigens by hydrophobic, hydrophilic, or ionic interactions. These interactions have been exploited in many affinity procedures to purify various molecules. Likewise, it is not inconcievable that, blocking reagents can modify support surfaces leading to non-specific absorption. The relatively mild washing conditions used herein may not have been stringent enough to remove lipopolysaccharide and pan-pasteurella carbohydrate non-specifically absorbed to the support material. Another possibility includes the presence of other immunoglobulins, in peritoneal ascitic fluid, which bind lipopolysaccharide and pan-pasteurella carbohydrate. However, one would expect non-specific absorption to occur only in trace amounts, leading to low, but detectable levels of contaminants. The probability of non-specific retention seems low because of the large and nearly
equivalent amount of antigens in the product relative to the original capsular material.

In support of the specific retention of the two unexpected antigens, the Ph-1 specific epitope could be present on several molecules. Using monoclonal antibodies, *Escherichia coli* K5 capsular polysaccharide has been shown to share an antigenic determinant with the enterobacterial common antigen. Similarly, lipid has been identified on the capsular polysaccharides of many Gram-negative bacteria and common lipid carriers have been implicated in the synthesis of lipopolysaccharides, peptidoglycan, and capsular polysaccharides. Likewise, keto-deoxy-octonoic moieties, known to comprise the core of lipopolysaccharides, have been located on capsular polysaccharides. These studies indicate that polysaccharide epitopes can be shared by a variety of different carbohydrate containing molecules. Recently, it was suggested that serotype specific antigens of *P. haemolytica* are a part of the lipopolysaccharide complex, specifically the O-antigen side chain.

Other investigators have reported serotype specificity resides in capsular polysaccharides. Our results suggest that the Ph-1 specific epitope is present on capsular polysaccharide, lipopolysaccharide, and the pan-pasteurella carbohydrate. Epitope sharing among hexosamine antigens could have important implications in polysaccharide immunogenicity.


CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Basic research considered necessary for a better understanding of the pathogenesis in pneumonic pasteurellosis was conducted. The rational construction of a component vaccine is dependent on an adequate comprehension of the pathogenesis. Therefore, a characterization of monoclonal antibody and bovine neutrophil reactivity to Pasteurella haemolytica serotype 1 (Ph-1) antigens was performed to aid in achieving these goals.

The monoclonal antibodies (McAbs) prepared and characterized against Ph-1 capsular material provides some basic materials to identify, quantitate, and purify antigens. Information concerning the pathogenesis may be obtained by identifying the amount of Ph-1 antigens present in inoculums and bronchoalveolar lavage fluids. The identification and distribution of Ph-1 antigens in pneumonic lung tissue using the McAbs and immunohistochemical techniques may also aid in understanding the pathogenesis. Identification and large scale purification of specific Ph-1 antigens important in the pathogenesis will be required to construct an effective component vaccine. The McAbs developed and characterized in this research can aid in understanding the pathogenesis and in developing a vaccine.

An understanding of neutrophil interaction with Ph-1 antigens is important in the comprehension of the pathogenesis in pneumonic pasteurellosis. The logical development of a component vaccine should address the interaction of neutrophils with Ph-1 antigens. The MTT colorimetric assay adapted herein provides a means of measuring
neutrophil cytotoxicity and stimulation. The activation of neutrophils by Ph-1 antigens may explain their rapid and intense accumulation in pulmonary tissue following experimental challenge. An effective component vaccine should result in neutralization of cytotoxic activity and block activation of neutrophils. The Ph-1 antigen(s) responsible for neutrophil activation needs to be identified and effectively blocked.

The cytotoxin-neutralizing monoclonal antibody (mMcAb) prepared and partially characterized herein merits further study. Alternatives to demonstrate mMcAb binding to cytotoxin should be sought to identify and quantitate cytotoxin in inoculums and clinical samples. Because mMcAb binding to cytotoxin was demonstrable by immunoblotting it is probable that the antibody can be used to purify cytotoxin using immunoaffinity. The absolute contribution of cytotoxin to the pathogenesis could then be determined and pure cytotoxin could be used in a component vaccine.

Structural changes in neutrophils exposed to cytotoxin are important to comprehend the pathogenesis in pneumonic pasteurellosis. Neutrophil polarization with uropod formation is suggestive of activation by a chemotactic factor. Visualization of structural changes in neutrophils offers an alternative to the MTT colorimetric assay as a means of identifying the Ph-1 component(s) causing activation. Activation of neutrophils should be inhibited by an effective vaccine.

The large scale purification of Ph-1 antigens is necessary for determining their contribution to the pathogenesis and for the construction of a vaccine. Immunoaffinity procedures developed in this
research provides a means of epitope purification. Purified epitopes can then be studied to determine their contribution to neutrophil activation and the pathogenesis in vivo. Purified epitopes identified as important in the pathogenesis can then be included in a component vaccine.

This research supplies some materials, methods and results for a greater understanding of the pathogenesis in pneumonic pasteurellosis and construction of a component vaccine. Further research is necessary using monoclonal antibodies and neutrophils to more fully understand the pathogenesis in pneumonic pasteurellosis and construct an effective component vaccine.
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