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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN DERIVED FROM DIFFERENT ANIMAL SPECIES

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ABSTRACT

The enterotoxins (ETs) of *Clostridium perfringens* isolated from enterotoxemic or diarrheic alpacas, pigs, calves, dogs, and horses were obtained from sporulated cell extracts. The ETs from alpaca, pig, and calf isolates were chromatographed on Sephadex G-100. Monoclonal antibodies (MAbs) against ETs derived from alpaca, pig, and calf *C. perfringens* isolates were produced. The MAbs were used in neutralization of cytotoxicity and immunoblot assays to study cross-reactivity among all five ETs. Using neutralization of cytotoxicity technique, each MAb exhibited neutralization against the cytopathic effect of all five ETs on Vero cells. The neutralization ratio in this study was 1 ng ET: 50 ng MAb. On immunoblots, the anti-alpaca ET and anti-pig ET cross-reacted with partially purified ETs from pig and alpaca respectively. In addition, anti-alpaca ET and anti-pig ET cross-reacted with partially purified ETs from calf and horse, but not ET from dog isolates. The anti-calf ET cross-reacted with alpaca, calf, dog, and pig but not with ET from the horse isolate. The immunoblots also indicated that the MAbs recognized aggregated ET subunits. Our data indicated that *C. perfringens* ETs derived from different animal species are very similar.

Keywords: *C. perfringens*; Enterotoxin; Monoclonal Antibody
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INTRODUCTION

Clostridium perfringens enterotoxin (CPE) is a single polypeptide chain with a MW of about 35 kDa.[14] The native CPE contains all 20 amino acids and consists of 320 amino acid residues.[27] Subjection of enterotoxin (ET) to detergent gel electrophoresis results in anomalous aggregation of ET into high MW polymers.[2,19,22] The proteolytic activity of CPE during cell extraction and purification results in minor or major cleavage products.[18]

C. perfringens enterotoxin is an important virulence factor involved in human and veterinary gastrointestinal illnesses.[14] *C. perfringens* ET is responsible for symptoms associated with *C. perfringens* food poisoning,[1,6] and is a causative agent of diarrhea in dogs[10] and pigs,[9] neonatal enteritis in calves,[20] acute enteric disease of horses,[17] and enterotoxemia in alpaca and llamas.[19] Recent studies[12-14] have indicated that CPE has cytotoxic action by specific binding to a proteinaceous receptor(s) on plasma membranes of CPE-sensitive mammalian cells. The specific binding

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and insertion of ET cause permeability alterations of the plasma membrane, disruption of the cellular-osmotic equilibrium, inhibition of macromolecular synthesis and eventually cell death. *C. perfringens* ET specifically binds to mammalian cells[16] such as Vero (African green monkey kidney) cells. Vero cells are very sensitive to CPE, and enterotoxin binding to Vero cell receptors and ET insertion into the plasma membrane is rapid and irreversible.[5,14] The enterotoxin causes morphology, viability and macromolecular synthesis alteration of these cells.[13,14]

The enterotoxigenic properties of CPE isolated from alpaca enterotoxemia were explained by studying the physicochemical, biological and immunological characteristics of the ET.[19] The production and characterization of MAbs to CPE isolated from human and alpaca strains have been studied.[8,19,27]

In the present study because of the enterotoxigenic role of ET in veterinary medicine, we were pursuing two objectives. The first objective was to produce neutralizing MAbs to partially purified CPE of alpaca, calf, and pig. The second objective was to assess cross-reactivity among CPEs using these MAbs. The alpaca *C. perfringens* strain 1012, implicated in alpaca enterotoxemia, was included as a reference strain for comparison purposes.

MATERIALS AND METHODS

Production and purification

Stock cultures of *C. perfringens* strains were maintained in cooked meat medium (CMM) (Difco Lab). The CMM cultures were transferred into fluid thioglycolate (FT) medium (BBL), heated at 75°C for 20 min and then incubated for up to 3 hours at 37°C. The FT medium was then transferred into Duncan and Strong (DS) sporulation medium.[19] The DS cultures were incubated at 37°C for 18-21 hours for maximum sporulation. The cells were harvested from DS cultures at 10,000 g, 4°C for 20 min. The cells were washed once in sterile cold distilled water as above. The pellets were resuspended in cold 20 mM phosphate buffer, pH 6.8. The cells were than sonicated in a sonic-bath until the spores were free of sporangia, as determined microscopically. The cell sonicates were centrifuged as before to collect the ET in the supernatant. The ETs from the alpaca H-1012, calf 14745, and pig 18503 isolates were purified by the method described by Granum and Whitaker.[4] The purification method was composed of two final precipitations (40% and 15% of ammonium sulfate, pH 7.2) and gel filtration chromatography, on Sephadex G-100.

Enterotoxin detection

For ET detection, dot blot assays and Vero cell assays were performed.

Dot blot assays

To assure that ET was present in the samples, an antibody capture on nitrocellulose-dot blot was performed on each isolated ET before and after column chromatography, based on Harlow and Lane's[7] protocol. The alpaca strain H-1012 ET-specific monoclonal antibody (MAb) employed as the primary antibody, was prepared earlier by Ramirez.[19] ET free toxoids of *C. perfringens* type C and D were used as negative controls.

Vero cell culture assays

Vero cells were cultured in 25 cm^2 tissue culture flasks (Corning, Newark, CA) with minimal essential medium (MEM) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 5% fetal bovine serum (Sigma). Confluent monolayers of cells were removed by trypsinization with 0.25% trypsin in buffer solution (Sigma) and harvested at 800 g for 5 min. The cells were resuspended in supplemented MEM, inoculated into 24 well culture plates (Nunclon, Naperville, IL) and incubated at 37°C in a CO_2 incubator for 24 hours. The cells were then washed three times with phosphate-buffered saline (PBS) pH 7.0, and ETs derived from *C. perfringens* isolated from alpaca, pig, calf, dog, and horse, in supplemented MEM, were added. Enterotoxin doses (0, 0.1, 10 and 100 ng per well) of each isolate were added to the monolayers in duplicate. Inoculated Vero cells were incubated at 37°C in a CO_2 incubator for 30 min. The supernatant of a sporulating culture was employed as a negative control.

Monoclonal antibody production

Female BALB/c mice were injected intraperitoneally (i.p.) with 7 µg of purified enterotoxins (from alpaca, calf, and pig) individually emulsified in 0.1 mL of Freund's complete adjuvant and 0.1 mL of sterile phosphate-buffered saline (PBS), pH 7.0. After 3 weeks, the mice were injected i.p. with 10 µg of purified ET emulsified with 0.1 mL of incomplete Freund's adjuvant and 0.1 mL of sterile PBS, pH 7.0. Three days before fusion, the mouse to be used for fusion was injected intravenously (i.v.) with 15 µg of the same ET in 0.2 mL of PBS, pH 7.0. On the day of fusion, the mouse was sacrificed and the spleen was removed. Spleen cells and a non-secreting myeloma cell line, Sp 2/0, were washed and mixed together at a ratio of 1:4 (myeloma: splenocytes). One mL of 50% polyethylene glycol (PEG) (4000 MW, Merck) was added to every 1.6×10^8 spleen cells. The fused cells were then centrifuged at 800 g for 5 min, and RPMI 1640 containing penicillin, streptomycin, L-glutamine, sodium pyruvate and 15% fetal bovine serum (CRPMI) was added. The cells contained in 100 µL were placed in each well of a 96 well plate (Nunclon) and the plates were incubated at 37°C in an atmosphere containing 5% CO_2. The cells of each well were fed with 15% CRPMI-

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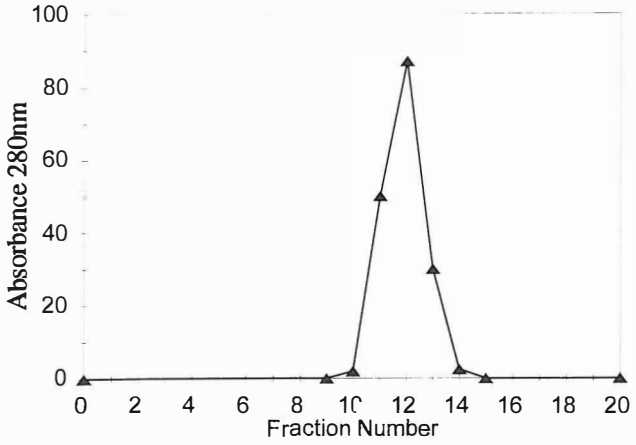

Fig. 1. Single elution profile from Sephadex G-100 gel chromatography of *C. perfringens* enterotoxins.

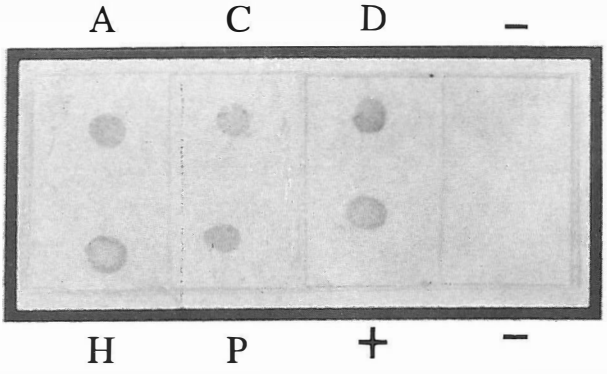

Fig. 2. Dot blot assay for *C. perfringens* enterotoxin. A=alpaca H-1012, C= calf 14745, D=dog 6624, H=horse 14027, P= pig 18503, +/- = positive/negative controls.

HAT (hypoxanthine-aminopterine-thymidine) medium over the next 4 consecutive days. Subsequently HT (hypoxanthine-thymidine) in CRPMI medium was added to each well every 2-3 days. Cell growth was monitored under an inverted microscope. Hybridoma cells were cloned twice by cloning dilution.

Mouse ascites fluid production

Female BALB/c mice were injected i.p. with 0.5 mL Pristane (Sigma) per mouse two weeks prior to injection of 10^6 hybridoma cells. The growing hybridoma cells were injected i.p. and the ascites fluids containing MAbs were collected 10 days later.

Immunoblotting

All five ETs as mentioned above along with prestained standard markers (Sigma) were subjected to electrophoresis as described by Laemmli.[11] A 38.5:1 acrylamide-bisacrylamide stock solution (Eastman Kodak, CO) was prepared and filtered for casting of the gels. Sodium dodecyl sulfate (SDS)-polyacrylamide gels consisted of a 3% stacking gel and a 10% resolving gel. The samples were run at 60 V constant voltage for stacking gel and then at 150 V constant voltage for the separating gels until the tracking dye was 0.5 cm from the bottom of the gel. The proteins were transferred from the 10% acrylamide gel to nitrocellulose membrane by electroblotting as described by Towbin et al.[24] using Tris-EDTA-NaCl (TEN) buffer pH 7.3 at 45 V for 3 hours. The non-specific binding sites on the blot were blocked in 5% non-fat dried milk in TEN buffer pH 7.3 for one hour. The diluted purified MAb (1:100) of anti-alpaca H-1012, anti-pig 18503 and anti-calf 14745 were individually reacted against the ETs, overnight at room temperature (RT), on a rotary shaker. The blots were washed three times with TEN buffer containing 0.05% Tween 20, pH 7.4 to wash off unbound antibodies. Diluted affinity purified goat anti-mouse horse radish peroxidase conjugated antibody was added and incubated at RT for one hour on a shaker. The blots were then washed as before. Reactive MAbs were detected by addition of 6 mg of diaminobenzidine (DAB) in 10 mL of 50 mM Tris-HCl, pH 7.6 and the presence of 30% H_2O_2 for 15 min. The reaction was stopped by washing the blot with PBS, pH 7.0.

Neutralization of ETs by MAbs

Confluent monolayers of Vero cells were prepared in 96 well culture plates. Ten ng of purified enterotoxin isolated from alpaca, calf, dog, horse and pig *C. perfringens* isolates were reacted with 10, 25, 50, 100, 250 and 500 ng purified MAbs in supplemented MEM by incubating the mixture of ET-MAb at 37°C for 15 minutes in a humidified incubator containing 5% CO_2. The mixtures of ET-MAb were then added to Vero cells, and the plates were incubated at 37°C in a CO_2 incubator for 30 min. The neutralization activity of the MAbs against ETs on Vero cells was determined by microscopic examination of the inoculated wells. Normal mouse serum and hybridoma supernatant containing MAb against bunyavirus were employed as negative controls.

RESULTS

ET purification

Gel filtration chromatography on Sephadex G-100 of enterotoxin from the alpaca H-1012, calf 14745 and pig 18503 isolates yielded one characteristic elution peak similar to typical enterotoxin profiles (Fig. 1).

Dot blot assays

The antibody capture on nitrocellulose membraned dot blot for each ET sample yielded positive reactions among all tested isolates with MAb specific to ET from alpaca strain

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H-1012 *C. perfringens* isolate in contrast to the negative controls (Fig. 2).

Vero cell assays

The results showed that CPE from all five different strains produced dose-dependent morphological damage to confluent monolayers of Vero cells. The morphological changes which were visible with the light microscope included changes of the shape characteristic of normal Vero cells (Fig. 3) to a spherical form when the cells were incubated with 0.1 ng of ETs. The morphological changes progressed to decreased cell viability and cell detachment from the surface of the culture wells by incubation of Vero cells with 10 ng of ETs. Complete cell lysis and death (Fig.4) occurred when the monolayers were incubated with 100 ng of ET.

Neutralization assays

The ability of each MAb to neutralize the cytopathic effect of enterotoxins was observed when enterotoxins were incubated with MAbs prior to inoculation onto confluent monolayers of Vero cells. The inhibition of the cytopathic effect of enterotoxins by MAbs was dose dependent. Ten to 100 ng of MAbs neutralized up to 30% of enterotoxin activity, 250 ng of MAbs neutralized up to 60% and 500 ng of MAbs neutralized up to 95% of enterotoxin cytopathic effects. Fig. 8 illustrates the same results of the percent inhibition of cytopathic effects of all five enterotoxins by different quantities of different MAbs. The neutralization ability of the MAbs in this study was 1:50. There was no observed alteration of the ET cytopathic effect on Vero cells when normal mouse serum or hybridoma supernatant containing MAb against bunyavirus, as negative controls. were incubated with enterotoxins under the same conditions.

Immunoblotting

Figs. 5 and 6 show the blots which were allowed to react with anti-alpaca H-1012 and anti-pig 18503 MAbs respectively. The blots demonstrated reactions of MAbs with epitopes shared by four purified enterotoxins from alpaca, calf, horse, and pig but not with epitope(s) of ET from the dog isolate. The antialpaca H-1012 MAb cross-reacted with two protein bands of >102 kDa present in ET from calf, horse and pig. It also cross-reacted with a double protein band of approximate MW of 75 kDa in calf ET and a protein band of 102 kDa in pig ET (Fig. 5). The anti-pig 18503 MAb produced an identical immunoblot to the one produced by the anti-alpaca H-1012 MAb (Fig. 6).

Fig. 7 illustrates the blot which was allowed to react with anti-calf 14745 MAb. The MAb recognized epitopes shared among alpaca, calf, dog, and pig but not with epitope(s) of the ET from the horse isolate. The anti-calf 14745 MAb cross-reacted with two protein bands of >102 kDa in pig ET and one protein band of 102 kDa in dog ET.

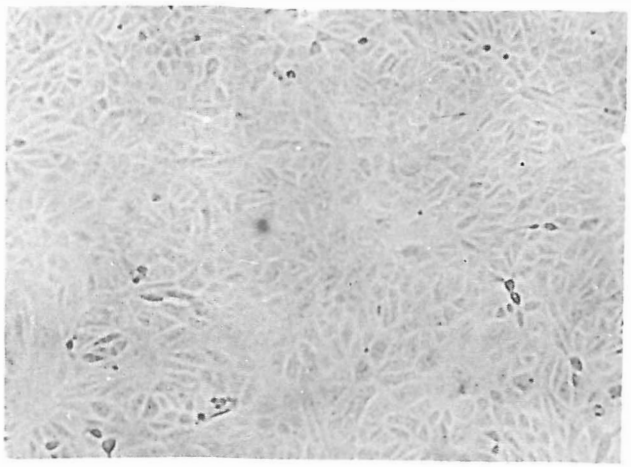

Fig. 3. Normal confluent monolayer of Vero cells.

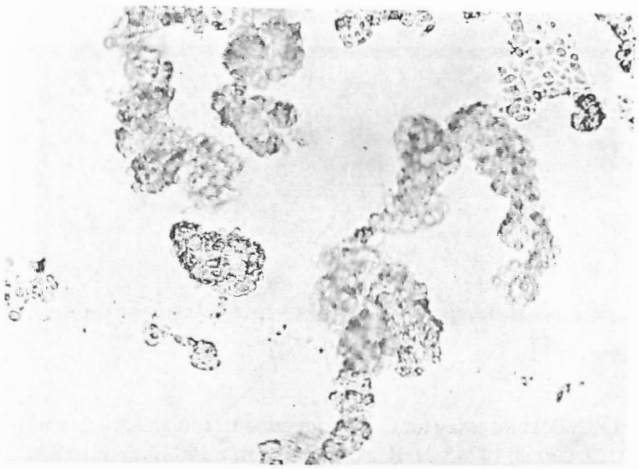

Fig. 4. Biological activity of *C. perfringens* ET on the confluent monolayer of Vero cells.

DISCUSSION

It has been reported that ammonium sulfate precipitation results in concentration of the material and removal of a large amount of contaminating protein.[19] At 40% saturation of ammonium sulfate, the ET was coprecipitated with macromolecular protein (s).[4,21] By dissolving the precipitated protein in a small volume of 0.02 M phosphate buffer, pH 6.8, 15% saturation of ammonium sulfate was sufficient to reduce the concentration of the ET in the supernatant, whereas contaminating proteins did not precipitate at this concentration of ammonium sulfate.[4,19]

The ammonium sulfate precipitated ET from human *C. perfringens* isolate on Sephadex gel filtration chromatography yielded two elution profiles of minor and major peaks.[1,4,19,21] Treatment of the crude cell extract with ribonuclease or deoxyribonuclease prior to Sephadex filtration chromatography did not eliminate the minor peak

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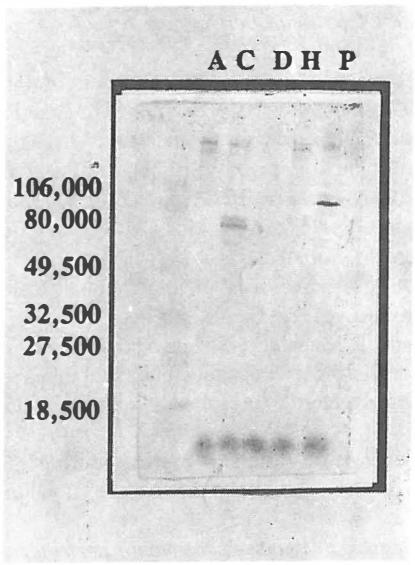

Fig. 5. Immunoblot of *C. perfringens* enterotoxins isolated from A=alpaca H-1012, C= calf 14745, D= dog 6624, H = horse 14027, and P=pig 18503 detected by anti-CP ET from alpaca H-1012 MAb (1: 100).

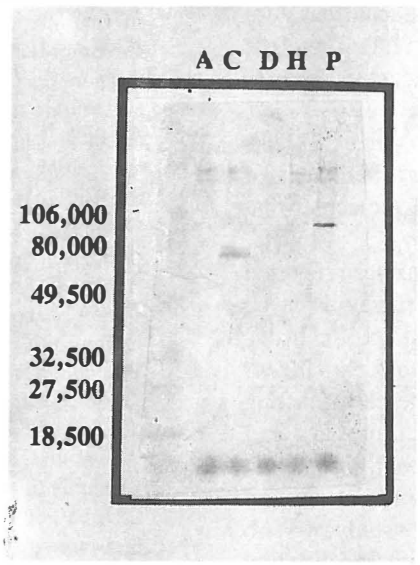

Fig. 6. Immunoblot of *C. perfringens* enterotoxins isolated from A=alpaca H-1012, C=calf 14745, D=dog 6624, H=horse 14027, and P=pig 18503 detected by anti-CP ET from pig 18503 MAb (1: 100).

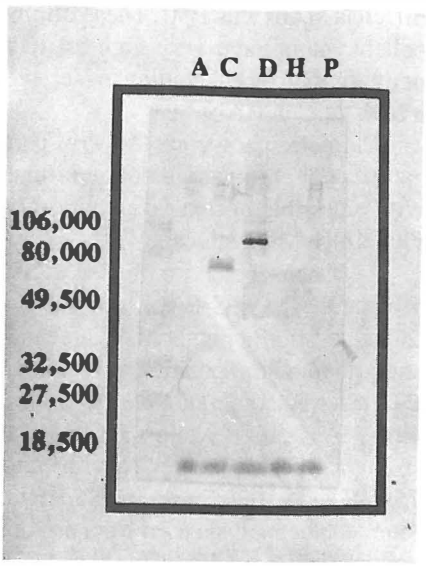

Fig. 7. Immunoblot of *C. perfringens* enterotoxins isolated from A=alpaca H-1012, C=calf 14745, D=dog 6624, H=horse 14027, and P=pig 18503 detected by anti-CP ET from calf 14745 MAb (1: 100).

on DEAE Sephadex.[23] Protein from the minor peak cross-reacted serologically with the ET from the major peak.[23]

Ramirez[19] reported that precipitated ETs from sporulated cells from alpaca *C. perfringens* isolate had a distinctive elution profile from the elution pattern obtained from the ETs from human *C. perfringens* isolate. The three different alpaca *C. perfringens* enteropathogenic strains employed for ET purification produced a single pattern of elution.

In the present study by gel filtration chromatography, we obtained a single peak pattern from ammonium sulfate precipitated ETs from all three samples of alpaca, pig, and calf *C. perfringens* isolates.

Studies on the mode of *C. perfringens* ET action have indicated that the ET acts via plasma membrane permeability alteration in sensitive mammalian cells.[12,13] Cultured Vero cells have proved to be a useful model system for studying the mode of action of the toxin.[13,19] In the Vero cell assay system, the ET causes rapid inhibition of macromolecular synthesis, loss of viability and morphological alterations.[12,13] It has been reported that specific binding of *C. perfringens* ET to Vero cell receptor is necessary to obtain biological action.[24] Previous studies showed that MAbs block specific binding of *C. perfringens* ET. In this study a Vero cell system was included in order to assay the gross morphological damage in the monolayer exposed to partially purified ETs. The biological activity of ETs in Vero cells was neutralized by different amounts of MAbs. The inhibition of the cytopathic effect of each ET was observed when the ET-MAb mixture was incubated at 37°C for 15 min, before being inoculated onto a confluent monolayer of Vero cells.

As the amount of MAbs was increased the degree of neutralization increased as well. Partial neutralization of the enterotoxins by the MAbs was observed when 10-100 ng and 250 ng of MAbs were reacted with 10 ng of each ET individually. However, 500 ng of the MABs almost completely (95%) neutralized the biological activity of 10 ng of partially purified ET. The cytotoxic effect of *C. perfringens* ET was demonstrated to be dose-dependent. There was no observed inhibition of the cytopathic effect on Vero cells when MAbs against bunyavirus or normal mouse serum were incubated with 10 ng of ET under the same conditions. In this study the ratio of neutralization of ET by

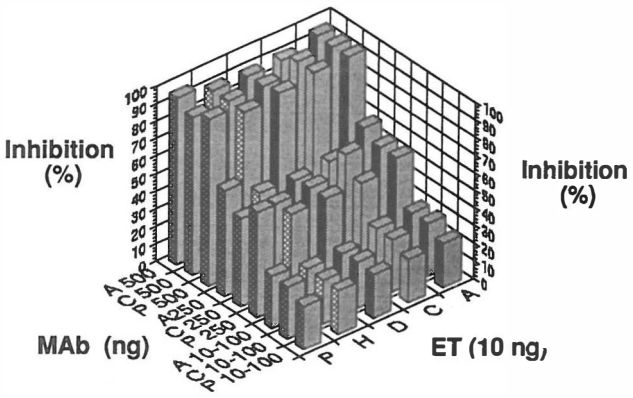

Fig. 8. Three dimensional plot of the inhibition of the cytopathic effect of ETs by anti-ET MAbs. A=alpaca H-1012, C=calf 14745, D=dog 6624, H=horse 14027, P=pig 18503.

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different MAbs was 1:50. These findings indicate that Vero cell receptors have very high affinity for ET. Therefore neutralization of ET binding to Vero cell receptors requires a high ratio of MAbs.

The molecular weight (MW) of partially purified ETs as estimated by chromatographic elution seems to be associated with a possible normal aggregation of ET molecules that yields high MW values.

Anomalous behavior of CPE in SDS has been reported previously. The SDS-PAGE and western blot assay displayed ET aggregation at higher MW value than 34 kDa.[8,19,25] In this study, the binding specificities of the MAbs to *C. perfringens* ETs detected some antigenic epitopes. The blots reacting with MAbs raised against ET from alpaca, pig, and calf strains had positive reactions with epitopes of MW of about 75, 102 and >102 kDa due to ET aggregation. The western immunoblot analysis used for a preliminary study of MAb epitopes indicated diversity among ETs from five different strains. The MAbs raised against partially purified ET from alpaca, pig, and calf strains recognized multiple subunit epitopes of different ETs. These results indicated the possible presence of conserved amino acid regions or sequences among ETs, leading to cross-reactivity and shared epitopes among those ET subunits. From our studies it was observed that epitopes exist which are not shared by some ETs. It is possible that certain epitopes of ET from pig and of ET from calf at the site of ~ 75 kDa and ~ 102 kDa respectively were denatured and thus could not be recognized by the MAbs. Further studies are needed to determine the presence of conserved amino acid sequences among these ETs.

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