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Purification of Anthrax Toxin Protective Antigen Component and Characterization of its Binding Interaction with Bovine Kidney Cells

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PURIFICATION OF ANTHRAX TOXIN PROTECTIVE ANTIGEN COMPONENT AND CHARACTERIZATION OF ITS BINDING INTERACTION WITH BOVINE KIDNEY CELLS

by

Daniel Dalton Martin

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ABSTRACT

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by

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Utah State University, 1986

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Department: Biology (Microbiology)

Protective antigen component of B. anthracis toxin was produced and purified to the >99% level. Toxin was purified from culture supernatant utilizing concentration and liquid chromatography techniques. Purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The purified protective antigen retained biological and antigenic activity as evidenced respectively by lethality in Fischer 344 rats when injected in combination with lethal factor, and by positive results on the Ouchterlony double diffussion assay.

Radioiodinated protective antigen was used both in the in vivo and the in vitro experiments.

In vivo distribution of labelled protective antigen was determined in Fischer 344 rats. Assay of organ tissues for labelled protective antigen aided in the decision to use Maden-Darby bovine kidney cells for the cell cultures in the protective antigen binding studies.

Protective antigen binding studies, all performed at 37°C, evaluated criteria for receptor existence. Labelled protective antigen was found to bind specifically and reversibly to Maden-Darby bovine kidney cells. Receptors proved to be saturable. Scatchard analysis showed a relatively high dissociation constant ($K_D = 17 \times 10^{-9}$ M) compared to other toxins in similar studies. This indicated moderately low affinity for protective antigen.

The receptor was also partially characterized. It was shown that cholera toxin subunit B blocked the binding of labelled protective antigen to Maden-Darby bovine kidney cells and that the protective antigen receptor was insensitive to trypsin treatment. Both of these observations suggest a ganglioside as the receptor for protective antigen.

(90 pages)

LITERATURE REVIEW AND INTRODUCTION

The pathological effects of most bacterial diseases are related to toxic substances released from the bacterial cell, rather than to the effects of the organism itself (Smith and Stoner, 1967). Diseases such as diphtheria, cholera, tetanus, and botulism illustrate this statement.

Bacterial Toxins

Definition. Toxins, or poisonous substances of biological origin, are a very diverse group. Consequently it is difficult to arrive at an all-encompassing definition. Bonventre (1970) attempted to define microbial toxins more specifically, classifying toxins as high molecular weight proteins which are antigenic. This definition, however, excludes endotoxins which are lipopolysaccharides and many fungal toxins such as T-2 which are of low molecular weight and presumably not antigenic. In general terms then, bacterial toxins can be defined as any poisonous substance produced by a bacterium whether it be excreted from the cell or released upon cell lysis.

History. The discovery of bacterial toxins was an outgrowth of early work by Pasteur and Koch in isolating causative organisms of disease in the late 19th century. In the years following, toxins were also demonstrated as

the principle cause of symptoms for many diseases. Prophylactic immunization with toxoids (modified toxins) proved effective in some cases for disease prevention. By the early 20th century, scientific research on bacterial toxins was well underway (Stephen and Pietrowski, 1983). The pathological effects of several bacterial diseases were shown to be a direct result of toxin production (i.e. diphtheria, botulism, anthrax, tetanus, cholera, shigella, and plague). The study of bacterial toxins continued to be a focus of research. Understanding of pathogenesis, cellular function, and ligand-receptor interaction as well as vaccine development are some of the benefits derived from toxin research.

Classification. There exists a variety of toxin classification schemes based on location of the toxin with regard to the bacterial cell, chemical structure, biological effect of toxin, and number and interaction of components (Bonventre, 1970).

The terms endotoxin and exotoxin (i.e. inside the cell or in the culture medium), which refer to the location of the toxin with respect to the organism which produces it, have found wide usage in 'toxinology'. Bonventre (1970) suggests, however, that these terms are outdated since exotoxins, once thought to be metabolites, can be found within the organism in young cultures (Bonventre and Kempe, 1960). Endotoxin of a gram-negative organism can also be found extracellularly in amounts

greater than could be accounted for by autolysis (Crutchley et al., 1968).

The chemical composition of toxins is varied. Toxins may be lipopolysaccharides as in Yersinia pestis, the organism responsible for plague (Hartley et al., 1974). Many toxins are proteins, peptides or lipoproteins as are those associated with Clostridium botulinum, Corynebacterium diphtheriae, Vibrio cholerae, Escherichia coli, Bacillus anthracis, and Pseudomonas aeruginosa (Van Heyningen, 1970; Gill, 1978).

The effect(s), and more particularly the site and mode of action as a system of classification, provides valuable information as well as a precise means of identifying a given toxin. Unfortunately, the effect(s) and/or site of action for many toxins is not known, which precludes this method of classification for general use. For the purpose of this review, the "components" classification is useful. There are three classes in this scheme: simple, mixed, and complex (Bonventre, 1970). Simple toxins are those which exist as monomers, dimers, or polymers of a monomeric subunit. Their effects are additive. The molecule can be active or may require modification by cleavage or chemical/physical modification before becoming active (Bonventre, 1970). Mixed and complex toxins, according to Bonventre (1970) are not readily distinguished from each other but both require more than one component of different molecular make up in order to

become an active toxin. Mixed toxins are composed of more than one component which are chemically distinct and do not form chemical bonds. The components of a mixed toxin are inactive alone. Bacillus anthracis produces a mixed toxin (Lincoln and Fish, 1970). Complex toxins are also composed of more than one component, but the components are chemically bound together. Some complex toxins are defined as subunit toxins that can be separated into functionally distinct subunits. V. cholerae toxin (DeWolf et al., 1981) and Clostridium tetani toxin (Morris et al., 1980) for example have been demonstrated to have at least two subunits. Other examples of subunit toxins are C. diphtheriae, P. aeruginosa, E. coli, and C. botulinum toxins (Stephen and Pietrowski, 1983).

Biological effects of toxins. The modes of actions of bacterial toxins have been the subject of much research since the turn of the century. The advent of cell culture allowed research into the action of toxins at a cellular level. Toxins require interaction with the cell membrane to produce an effect. Middlebrook and Dorland (1984) suggested that toxins be classified as either membrane damaging or intracellular acting. Toxins may damage membranes by modifying phospholipids, increasing membrane permeability, sequestering cholesterol, or acting in a detergent-like manner. Other unknown mechanisms may exist to damage cell membranes (Stephen and Pietrowski, 1983; Alouf, 1977; Craig et al., 1981). Membrane damaging

toxins include phospholipases (e.g. C. perfringens, toxins), hemolysins such as those produced by gram-negative bacteria, and other lysins such as some of the toxins of staphylococcal and streptococcal origins (Freer and Arbuthnott, 1976). Clostridium perfringens alpha toxin was the first toxin for which an enzymatic mode of action was shown. In 1941 MacFarlane and Knight, as reported by Stephen and Pietrowski (1983), demonstrated that this alpha toxin was a phospholipase which cleaves and releases phosphorylcholine from phosphatidyl choline. In some cases, cell membrane damage was associated with receptor binding of the toxin to the membrane. Research with staphylococcal leukocidin by Woodin (1970) and Noda et al. (1982) has shown that this toxin component binds with the GM1 ganglioside. Apparently, without penetrating into the cell it exerts its effect of increasing potassium permeability and increasing prostaglandin production and release. Bhakdi et al.(1984) demonstrated a correlation between toxin binding (perhaps associated with channel formation) and the hemolytic membrane damage of staphylococcal alpha toxin. Intracellular-acting toxins seem to have a common mechanism of action involving: 1)toxin binding to cell membranes; 2)internalization or translocation across that membrane; and 3) interaction with an intracellular target (Middlebrook and Dorland 1984).

Toxin binding to cell membranes suggests the presence of a receptor. In some cases a specific toxin receptor

has been identified. Gangliosides are a group of cell surface complex glycosphingolipids which serve as receptors for a number of toxins and endogenous mediators (Osborne et al., 1982). VanHeyningen (1983) stated that the normal physiological function of gangliosides has not yet been clearly defined. A number of studies (Van Heyningen, 1983; Osborn et al., 1982; Cuatrecasas, 1973; Ganser et al., 1983) have shown that ganglioside GM1 is highly specific for cholera toxin. Other studies with cholera toxin and GM1 interaction have shown that the number of binding sites varies from one cell type to another: for example, sites vary from a few thousand in erythrocyte cells (Gill and King, 1975) to more than two million in some mucosal cells (Holmgren et al., 1975). The degree of binding of the toxin per receptor nevertheless seemed to remain constant (VanHeyningen, 1983). It appears that an initial toxin-GM1 complex is necessary for a second component of the cholera toxin to enter the cell and exert its effect of activating adenylate cyclase (Osborn et al., 1982; DeWolf et al., 1981).

Mechanisms for internalization of toxin across the cell membrane has also been a subject of investigation. Research with diphtheria toxin has demonstrated receptor mediated endocytosis. Donovan et al. (1982) found that diphtheria toxin binds to the cell lipid bilayer and forms transmembrane channels. Another study with diphtheria toxin also suggested that it could destabilize the

integrity of the endocytosed vesicle membrane resulting in release of toxin into the cytoplasm (Hudson and Neville, 1985). Phosphoinositide was found to act as the cell membrane binding site for a component of diphtheria toxin (Donovan et al., 1982). Insertion of one diphtheria toxin fragment into the cell membrane facilitates the entry of an enzymatic fragment of the toxin into the cytoplasm (Collier, 1975 and Pappenheimer, 1977). The enzymatic fragment alters and renders inactive the elongation factor-2 (EF-2) involved in protein synthesis (Middlebrook and Dorland, 1981; Moynihan and Pappenheimer, 1981). Several peptides have been found to cross the cell membrane in order to elicit an effect, among which are: the toxins of C. diphtheriae, P. aeruginosa, V. cholerae, and E. coli; colicins; and the plant toxin abrin (Gill, 1978).

As already indicated, the intracellular action of toxins seem to center around interference with protein synthesis and/or activation of adenylate cyclase. Diphtheria toxin has been one of the major toxins studied with regard to interference of protein synthesis. In 1975 Collier suggested that diphtheria toxicity was due to inhibition of protein synthesis and proposed a sequence of events including receptor-mediated endocytosis, release into the cytosol, and inactivation of EF-2 by fragment A of the diphtheria toxin, which would in turn interrupt protein synthesis. In 1977 Collier (1977) attributed inhibition of protein synthesis to the P. aeruginosa toxin as well.

Research since 1975 has supported and elaborated upon Collier's model of action for diphtheria toxin (Gill, 1978; Moynihan and Pappenheimer, 1981; Clemens, 1984).

Activation of adenylate cyclase has been attributed most specifically to cholera toxin (Kabir et al., 1978; Holmgren, 1978). Gill and King (1975) reported that NAD was required as a cofactor for this action. Recent research has demonstrated that cholera toxin acts by ADP-ribosylating a membrane protein that is a guanine nucleotide-binding regulatory component of adenylate cyclase (Kaslow et al., 1981; Middlebrook and Dorland, 1984).

Contributing to the pathogenesis of toxins is the fact that cells of the immune system are also affected, thereby decreasing host resistance. Pertussis toxin was shown to inhibit chemotaxis in human polymorphonuclear leukocytes (Lad et al., 1985; Goldman et al., 1985). Anthrax toxin has been shown to decrease phagocytic activity (O'Brien et al., 1985). Friedman and Kohn (1976) demonstrated that cholera toxin inhibited interferon action.

Practical applications of toxin research. The study of toxins has proven very valuable for development of immunization against and treatment of diseases caused by toxin-producing organisms. Today, with the study of toxin-cell interaction, new and valuable possibilities exist. It has been pointed out by Middlebrook and Kohn (1981) that the study of binding properties of toxins has and

will find important relevance in medical research in the production of hybrid hormones and toxins. Utilizing the binding properties of a toxin and/or the physiological effects of a toxin component opens up new avenues of medical therapy for treatment of tumor cells and reversal of certain pathologies. The use of hybrid toxins for "target-killing" of tumor cells has been the subject of much research in recent years (Gilliland et al., 1980; Krolick et al., 1980; Blythman et al., 1981; Olsnes, 1981; Moolten et al., 1982; Colombatti, et al, 1983).

Bacillus anthracis: History and Pathogenesis of Disease

Bacillus anthracis is a rod shaped, Gram-positive, spore-forming, facultatively anaerobic bacterium (Buchanan and Gibbons, 1974). Although it is pathogenic for man, *B. anthracis* is zoonotic causing disease primarily in sheep, cattle, and other lower animals. The organism was first associated with the anthrax disease in animals as early as 1850, but was conclusively proven to be the source of the lethal infection by Koch in 1877. From his work with *B. anthracis*, Koch formulated the postulates which confirm the causal relationship between a microorganism and the corresponding disease (Freeman, 1979).

It is extremely difficult to erradicate anthrax because the causative organism is a spore-former infecting lower animals. The spore is highly resistant to extreme environmental conditions. Spores have been isolated from

the soil up to sixty years after a natural deposition (Wilson and Russell, 1964). This makes contaminated soil a source of recurring infection in grazing cattle. Contaminated bone meal incorporated into cattle feed has also been demonstrated to be a potential source of infection for cattle (Davies and Harvey, 1972).

As noted, B. anthracis is pathogenic for man and can be transmitted to man from infected animals. Three routes of entry have been observed: 1) percutaneous, 2) pulmonary, and 3) gastric. The disease is not normally transmitted from human to human. Man is most commonly infected by handling contaminated animal materials and contracting a cutaneous infection. Handling hides and brushes, for example, from contaminated animals may be a means of infecting humans via the skin (Kendall, 1959). The infection results in a localized boil or abcess which, if treated medically at this point, will not result in septicemia. Although anthrax septicemia is a critical condition, it does not always result in death if treated promptly (Tahernia and Hashemi, 1972; Nalin et al., 1977). Infection via the respiratory route is the most serious of the three natural routes of infection in man. Its frequency is relatively low compared to infection arising from the cutaneous source. Respiratory infection has been demonstrated to have greater occurrence among persons who sort contaminated sheep's wool, and thus, has come to be known as "wool sorters' disease" (Dalldorf et al., 1971).

This infection can rapidly proceed to a fatal toxemia. Intestinal infection of humans is uncommon but can be rapidly fatal (Nalin et al., 1977). Spore-contaminated meats are the likely source (Sirisanthana et al., 1984).

Depending on the strain, B. anthracis may or may not be encapsulated. The capsular material of most bacteria is composed of polysaccharide whereas that of B. anthracis is made of the polypeptide D-glutamic acid (Record and Wallis, 1956; Zwartouw and Smith, 1956). The capsule seems to enhance the virulence of the organism by acting as an "aggressin" (Zwartouw and Smith, 1956), which enables the organism to resist phagocytosis by the host's immune response.

Anthrax Toxin

As in many bacterial diseases, it appears that the anthrax organism itself is not physically responsible for the lethal effect of advanced infection. A combination of toxic substances released from the bacterial cells under specific growth conditions (Smith and Stoner, 1967) is responsible. It is also believed that the anthrax toxin adds to the virulence of the organism (Smith, 1958; Keppie et al.,1963; and Klein et al., 1963).

In vitro production of anthrax toxin in prepared media (Puziss and Wright, 1954; Wright et al., 1954; Harris-Smith et al., 1958; and Ristroph and Ivins, 1983) has allowed researchers to obtain sufficient quantities of

crude toxin: to use in studies; to purify (Fish et al., 1968 and Fish and Lincoln, 1967; Leppla, 1982); and to analyze components of the toxin (Thorne, et al., 1960; Wilkie and Ward, 1967; Leppla, 1982, 1984).

Anthrax toxin is a complex consisting of three components (Smith and Stoner, 1967; Smith and Stanley, 1962; Lincoln and Fish, 1970). The holotoxin, or complete toxin, is biologically active causing death accompanied by severe pulmonary edema upon injection into test animals (Smith and Stoner,1967). The Fisher 344 male rat is particularly sensitive to the toxin (Beall et al., 1962; Haines et al., 1965) and may succumb within 40-50 min after injection of culture filtrates (Haines et al., 1965). The three toxin components are protecive antigen (PA), lethal factor (LF), and edema factor (EF). The PA is so named by virtue of the protective immune response which it elicits in the host when injected alone. Thus PA has been used as an immunizing agent (Belton and Strange, 1954). The LF component is lethal when combined with PA. The EF component induces local edema at the site of subcutaneous injection when in combination with PA. The components have been shown to be neither lethal nor edema-inducing separately (Haines et al.,1965). Because of this complexity, the mode of action of the holotoxin is more difficult to define than that of a simple toxin.

Much of the work with anthrax toxin has been conducted at Fort Detrick at Fredrick, Maryland in the U.S.

and in Porton, England. Because the research in both centers proceeded independently, the nomenclature for referring to the toxin components is not consistent. The American terms: edema factor (EF), protective antigen (PA), and lethal factor (LF) correspond respectively to factors I, II, and III in the British literature. The three toxin components have a molecular weight of 89,000, 85,000 and 83,000 daltons, respectively (Leppla, 1984). Although B. anthracis was shown to be toxigenic as early as 1954 (Smith and Keppie, 1954), interaction of the three components of the anthrax toxin and its mode of action upon cells has remained mostly undefined.

Test animals infected with B. anthracis have shown many physiological effects. As indicated by its pathogenesis, anthrax has cutaneous, pulmonary, and intestinal effects. Meningo-encephalitic involvement in human anthrax (Tahernia and Hashemi, 1972) and kidney pathology has also been noted (Nalin et al., 1977; Smith and Keppie, 1954). Whether the effects are primary or secondary is unclear, but toxin production in vivo is responsible for the effects (Fish and Lincoln, 1968).

Mechanism of Action of Anthrax Toxin

Most early work on the mechanism of action of anthrax toxin involved whole animal in vivo studies. With the advent and fine-tuning of cell and tissue culture techniques, the study of the effects of toxins at a cellular

level has been made possible. Species susceptibility can be more readily determined through use of tissue culture (Solotorovsky and Johnson, 1970; Dorland, 1982). Much of the current work with anthrax toxin has been influenced by Leppla. Leppla (1982, p. 3162) stated that no further studies on the mechanism of action of anthrax toxin are known to have been performed since 1967.

Study of the cellular interaction of the anthrax toxin components PA and EF by Leppla (1982 and 1984) has demonstrated that the EF component is an adenylate cyclase and in combination with PA results in a dramatic elevation of intracellular cAMP in chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Elevation of cAMP is also observed in polymorphonuclear neutrophils (PMN's) along with decreased phagocytic activity by the PMN (O'Brien et al., 1985). Oddly, PA and EF in tandem were shown by Wade et al.(1985) to enhance chemotaxis of PMN's. It has been hypothesized by Molnar and Altenbern (1963) and Leppla (1984) that PA binds to the cell's surface and acts as a mediator to allow EF and LF to enter the cell and express themselves in the cytoplasm.

Gross changes in cultured cells in response to the toxin vary with cell line. Leppla (1982) reported elongation in CHO cells in response to PA and EF. Bonventre (1965) reported no visible cytopathic effects in guinea pig spleen, mouse embryo primary explant, and buccal carcinoma cells.

Past research with other toxins has elucidated the presence of toxin-binding receptors in cultured cells and animal host tissue (Critchley et al., 1981; Boquet and Duflot, 1981; Dreyfus and Robertson, 1984; Yavin and Nathan, 1986). It is therefore likely that receptors for one or more components of the anthrax toxin also exist.

Techniques of Toxin Study

Toxin purification. In order to clearly identify the actions of any mixed or complex toxin it is essential to separate and purify the individual toxin components. Efforts to purify the PA, EF, and LF toxin components of *B. anathracis* began with Thorne et al. (1960). By utilizing a fritted glass filter they separated the toxin into two components. One component remained adsorbed to the filter while the PA component was contained in the filtrate. The adsorbed component, referred to as "filter factor", was eluted with alkaline buffers. The two components were shown to be toxic in combination, but not separately. Stanley and Smith (1961) later demonstrated that the filter factor described above was actually two components, namely EF and LF, which they termed factors I and III, respectively. Much of the early efforts to purify the components of anthrax toxin (see Lincoln and Fish, 1970 for review) failed to eliminate cross comtamination and based the levels of purity on serological and biological assays alone.

More recently Leppla (1982 & 1984) used ammonium sulfate precipitation and liquid chromatography methods to purify all three anthrax toxin components. The components described in Leppla's work (1982), however, were only 80% pure for EF and LF, and 90% or greater for PA. Leppla acknowledges, for instance, that the term "EF must be viewed as describing a functional and not a physical entity" (Leppla, 1982, p.3163), indicating that the level of purity allows some question as to the conclusions drawn from experimental results using those components.

One of the most promising methods to accomplish purification is the use of highly specific monoclonal antibodies coupled in affinity chromatography (Machuga et al., 1986). Initial purification procedures rely on other liquid chromatography techniques such as ion exchange and gel filtration. Still another method for producing pure toxin component has been attempted. Vodkin and Leppla (1983) successfully cloned the PA gene in E. coli and induced production of the PA component. Purification by this method, however, has not yet yielded PA at greater levels of purity than those previously reported.

Evaluation of toxin-cell interaction. As noted, the study of the effects of microbial toxins at a cellular level has been advanced by improving cell and tissue culture techniques. Recently, these techniques have allowed extensive study of the interaction of various toxins with cell membranes (Noda et al., 1981; Osborne

et al., 1982; Moynihan and Pappenheimer, 1981; Lin and Taniuchi, 1980; Donta et al., 1982). Studies with V. cholerae toxin and E. coli enterotoxin (Donta et al., 1982), C. tetani toxin (Lietzke and Unsicker, 1983; Critchley et al., 1985), and C. diphtheriae toxin (Mekada et al., 1982; Dorland, 1982) have been enhanced by the use of cell culture. Membrane preparations of animal tissues have also served to identify receptor sites for toxins. For example, tetanus toxin was found to bind to brain cell membranes (Rogers and Snyder, 1981) and cholera toxin bound to microvillus membranes (Bresson et al., 1984).

Once a given toxin was determined to be bound to a given cell type, a specific receptor could be searched for. It is now well established that cholera toxin is bound by the GM1 ganglioside (Critchley et al.,1982; Ledeen and Mellanby, 1977; Fishman et al., 1984). It has been shown that C. botulinum toxin also associates with gangliosides (Kitamura et al., 1980) and that diphtheria toxin binds to certain phospholipids in liposomes (Alving et al., 1980; Donovan et al., 1982). It has been recognized through such studies that different toxins may bind to the same or similar receptors (Tsuji et al., 1985; Simpson, 1984). Toxin processing by cells has also been elucidated by cell culture studies such as those done with diphtheria toxin (Dorland et al., 1979; Middlebrook et al., 1978).

Receptors have been solubilized and removed from cell membranes and successfully purified, as with the rat

intestinal receptors for E. coli heat-stable toxin (Dreyfus and Robertson, 1984). Identification and subsequent isolation and purification of receptors for bacterial toxins has allowed more detailed analysis of how the toxin interacts with cells to exert their effects. Diphtheria toxin has been one of the most intensively studied in this regard (Donovan et al., 1982; Zalman and Wisnieski, 1984; Kagan and Finkelstein, 1979; Hudson and Neville, 1985). Diphtheria toxin acts intracellularly which requires that the active portion of the toxin gain entry into the cell. In order to gain entry, diphtheria toxin interacts with the cell membrane by insertion of one toxin fragment into the cell membrane facilitating the entry of an enzymatic fragment of the toxin into the cytoplasm (Collier, 1975; Pappenheimer, 1977). Studies with diphtheria toxin receptor interactions also have demonstrated that different cell lines, as well as different species, seem to vary in the number of surface receptors for the toxin (Middlebrook et al., 1978). It is therefore evident that identification and characterization of a toxin receptor is helpful in elucidating the interaction of a toxin with a cell.

Research in the area of bacterial toxin receptors seems to be of great comtemporary interest (for a review see Eidels et al., 1983). One of the most utilized methods for detecting cell receptors for a given ligand is

to radiolabel the ligand with ^{125}I and assay for gamma radioactivity. This method has been used successfully for toxins, hormones, and other biological ligands such as ricin, a plant toxin (Nicolson et al., 1978), interferon (Aguet et al., 1982; Aguet and Blanchard, 1981), thrombin (Stein and Hoak, 1981), insulin (Katzen et al., 1981), tetanus toxin (Morris et al., 1980; Critchley et al., 1985; Rogers and Snyder, 1981; Lazarovici and Yavin, 1985), diphtheria toxin (Mekada et al., 1982), cholera toxin (Nielsen et al., 1984; Fishman, 1980; Bresson et al., 1984), botulinum toxin (Kitamra et al., 1980), and Staphlococcal leukocidin (Noda et al., 1981). Visualization of receptor activity or binding location has also been accomplished by utilizing ferritin (an electron dense protein) or colloidal gold. The gold may be either bound to the lectin (Loftus and Albrecht, 1983; Gershon et al., 1981) or conjugated to antibodies against the lectin. These conjugates are then visualized by electron microscopy for a qualitative assay. Immunofluorescence has also been useful (Lietzke and Unsicker, 1983).

All the above methods for detecting receptors on cell surfaces or membranes are indirect. These methods do, however, provide valuable information regarding such aspects of toxin-cell interactions as binding affinities, constants, saturability, reversibility, location of surface receptors, and receptor number per cell.

Initial Determination
of Receptor Existence

Receptor research has advanced with the discovery of not only toxins but also a wide variety of endogenous substances such as hormones, growth factors, interleukins, etc. all of which mediate their effects in the body through some form of receptor.

Binding of a given molecular species to a surface is not necessarily due to a specific receptor. Nonspecific binding can account for what may seem to be specific binding (Cuatrecasas and Hollenberg, 1975).

Cuatrecasas (1974) proposed specific criteria in order for the binding of a labelled ligand to be attributed to interaction with a receptor. Among the necessary criteria are: chemical specificity, saturability, high affinity, and reversibility.

Chemical specificity means that the ligand is bound by a receptor which chemically and/or physically distinguishes the ligand. Middlebrook et al. (1978) noted that unlabelled diphtheria toxin competes for receptor sites with the labelled toxin as does cross reactive mutant-195 (a toxin homlogue) and subunit "B" of the diphtheria toxin. This data, coupled with the observation that P. aeruginosa "exotoxin" A and abrin did not compete for the receptor, indicated that Middlebrook et al. (1978) demonstrated the specificity of the receptor.

Saturability of the receptor sites by a specific ligand can be demonstrated either by treating the receptor sites with increasing concentrations of the ligand over a specific time period or by treating the receptor sites with a set concentration of ligand over a variable period of time. At some point all the specific receptor sites will be occupied, thus achieving saturation and demonstrating a limited number of receptor sites.

Affinity can be demonstrated by calculation of an association constant. Scatchard (1949) analysis allows determination of an association constant for the receptor-ligand complex. Great or high affinity is evidence that the ligand is capable of maintaining association with the receptor of a cell, for example, for a period of time sufficiently long enough to elicit the intended response.

Reversibility can be shown if bound labelled ligand is removed by an excess of unlabelled ligand. This response indicates competition for the receptor site.

Binding Studies of Anthrax Toxin Components

The study of toxin binding kinetics looks at the quantitative and temporal features of toxin binding to receptors. The kinetics of the binding and interaction at a cellular level of anthrax toxin components has not been studied to the extent that other toxins have been studied. Prior to 1980, the work with anthrax toxin was confined mostly to *in vivo* experiments related to the study of how

the toxin acts. Gladstone (1946) used the protein-containing anthrax culture supernatant to invoke a protective immune response in animals. Smith and Keppie (1954) induced edema and lethality in test animals with injections of sterile plasma from guinea pigs infected with anthrax. With the subsequent identification of three distinct components of the anthrax toxin (Stanley and Smith, 1963), work was then directed toward purification of each component and determination of how each component functioned.

In early work it was shown that EF and LF appeared to compete for the available PA after intravenous injection into test animals (Stanley and Smith, 1961). Leppla (1982) demonstrated that EF acts as an adenylate cyclase to elevate cyclic AMP in cultured cells only in the presence of PA. Working with the toxin components with cultured cells, Leppla (1982) also demonstrated that LF blocks the action of EF on CHO cells, BHK-21 cells, and fetal Rhesus Lung (FRhL-2) cells, presumably by competing for the PA at the cell surface.

The apparent function of PA as a mediator for the passage of other biologically active component(s) is similar to the "A"/"B" subunit scheme for other bacterial toxins such as in diphtheria and cholera. In these cases, however, the subunits are covalently connected, whereas the anthrax toxin components are thought to be physically

independent of each other (Leppla, 1984). This aspect of the anthrax toxin has facilitated the separation/purification of the components.

An interesting aspect of Leppla's work (1982) from the standpoint of receptors is that the peak response of CHO cells to EF occurred at concentrations where EF and PA were both the same (about 1 ug/ml). This is consistent with a binding role for PA. Leppla (1982) also noted that the data indicated that the "essential components of the uptake system for EF are not consumed during EF action" (p. 3165). This observation implies that PA is not rapidly internalized and remains bound at the cell surface, at least in CHO cells, ready to mediate the binding or passage of EF or LF.

The available information points to the existence of receptors for PA on susceptible cells. Receptors have been identified for certain toxins such as the GM1-ganglioside for cholera and heat-labile toxins. It is possible, therefore, that PA binds to a similar receptor. With regard to characterizing the binding of PA, or any other of the anthrax toxin components, to cultured cells or tissue samples there is, to the author's knowledge, no published work. Any research attempting to characterize the binding of PA to cultured cells requires the PA to approach levels of purity beyond that reported in the literature thus far.

Research Proposal

It was the aim of this research to purify the anthrax toxin protective antigen (PA) component and to characterize its binding to a selected cell line, namely Madden-Darby bovine kidney (MDBK) cells. To accomplish this the subsequent outline was followed:

1) A toxigenic strain of Bacillus anthracis was cultured in vitro.

2) Biologically active holotoxin toxin was produced and identified.

3) The Protective Antigen (PA) component was purified to levels > 99%.

4) The PA was labelled with ^{125}I for use in a radio binding assay.

5) Distribution and localization of the labelled PA in vivo was determined after injection into a susceptible host.

6) A binding assay with the labelled PA in an in vitro cell culture system was developed.

7) The criteria for a receptor as outlined by Cuatrecasas (1974) was investigated on a selected cultured cell type.

8) It was determined if the receptor for the PA is proteinaceous by treatment with trypsin.

9) It was determined if the binding unit of cholera toxin could block the binding of PA, thus indicating the possibility of a ganglioside receptor.

MATERIALS AND METHODS

Toxin Production

Bacillus anthracis (Vollum 1B initially then, Sterne strain) vegetative cells were streaked on blood agar for isolation. Isolated colonies were transferred with a sterile inoculating loop to another blood agar plate and incubated at 37°C for 15-18 hr. The second plate was flooded with a sterile growth medium composed of Casamino Acids (Detroit, MI.) with chemical supplements as described by Haines et al. (1965), hereafter referred to as growth medium. The surface growth was suspended in liquid medium and then placed in a sterile tube for mixing. Aliquots were dispensed and frozen for subsequent use as inocula.

A blood agar plate was heavily streaked with cells from a thawed inoculum aliquot and incubated for 18 hr at 37°C. The plate was flooded with sterile growth medium and the colonies suspended and mixed to a uniform suspension. A sufficient volume of this mixture was transferred to about 15 ml sterile growth medium to give a reading of between 18-26 units on a Klett spectrophotometer. A 0.5 ml portion of this suspension was transferred to a Fernbach flask containing 460 ml growth medium and 5 ml sterile, 20% aqueous, glucose solution and was incubated at 37°C. After

4 hr incubation, 50 ml of sterile 9% sodium bicarbonate solution was added and the flask was returned to 37°C for an additional 18-20 h. After the complete incubation cycle the bacteria were pelleted by centrifugation at 10,000 x G for 30 min in sterile centrifuge bottles. Subsequently, sterile polyethyleneglycol 6,000 (PEG) (Union Carbide Corp., S. Charleston, W. VA.) was added directly to the supernatant to yield 0.01% PEG. The PEG-supernatant was subsequently filtered through a prefilter and a 0.22 μm membrane filter (Millipore, Bedford, MA.). Positive pressure was maintained with prepurified nitrogen. The sterile filtrate was collected in sterile flasks. This filtrate constituted the crude anthrax toxin. One ml of the sterile supernatant was plated on agar plates to check for contamination.

The crude toxin was routinely tested for antigenic activity by the Ouchterlony double diffusion method (Ouchterlony, 1948) and for biological activity by I.V. injection into Fisher 344 male rats (Haines et al., 1965) weighing between 225 and 300 g. Assay of EF was accomplished by subcutaneous injection into shaved backs of rabbits and visual observation for edema on the following day. The goat antisera to PA, LF, and whole cell B. anthracis used in the Ouchterlony assay were gifts from Dr. Stephen Leppla (United States Army Medical Research Institute of Infectious Disease, Ft. Detrick, MD.). The antisera against PA and LF, which contained hemolyzed red

blood cells were further "purified" by liquid chromatography with Affigel-Blue (BIO-RAD, Richmond, CA.).

The crude toxin was stored at -20°C or was put through additional concentration steps.

Toxin Concentration

Approximately 2 L of crude toxin were filtered overnight through a YM 10 or PM 30 Amicon filter (Danvers, MA.), with a 10,000 or 30,000 molecular weight exclusion limit respectively, utilizing a nitrogen gas positive pressure dialysis cell and holding tank. The final volume was about 50 ml. This solution constituted the concentrated toxin. This was assayed in the same manner as the crude toxin. The concentrated toxin was subsequently stored at -20°C until dialysis prior to additional purification.

Protective Antigen Purification

The concentrated toxin was thawed and dialyzed overnight in 0.002 M KH_2PO_4, 2 mM 2-mercaptoethanol (2-ME), and 0.05 M NaCl buffer pH 7.1 at about 4°C. Depending upon the viscosity of the dialyzed toxin (the more concentrated, the more viscous) the toxin was diluted so as to facilitate passage through the chromatography column. Routinely, approximately 40-50 ml of toxin solution was loaded onto a column of hydroxylapatite (Bio-Gel HTP, BIO-RAD, Richmond, CA.). The column was 2.5 cm in diameter, about 10 cm high, and used about 75 cm hydrostatic

pressure to give 0.1 ml/cm^2/min. After the toxin was loaded, the column was washed with starting buffer (2 mM KH_2PO_4 pH 7.1) with 2-3 column bed volumes. The eluent was monitored by spectrophotometry (254 nm), and when the absorbance returned to the baseline the elution gradient was started.

The toxin components were eluted from the HTP column with a buffer gradient of 0.002 M to 0.4 M KH_2PO_4 at pH 7.1, containing 2 mM 2-ME and 0.05 M NaCl in 500 ml total volume. The gradient was developed with a GM-1 gradient maker (Pharmacia, Uppsala, Sweden), and ran at the same flow rate as above or approximately 0.5 ml/min. The PA fractions, identified by Ouchterlony double diffusion immunoassay and electrophoresis were dialyzed overnight in 0.01 M Tris (hydroxymethyl) aminomethane (Tris) with 2 mM ethylene diamine tetracetate (EDTA) and 2 mM 2-ME, pH 8.1 (ion exchange chromatography starting buffer). These dialyzed fractions were then loaded onto an equilibrated DEAE (Cellex D, BIO-RAD, Richmond, CA.) column (1.4 cm in diameter and 6 cm high) washed with starting buffer with several void volume equivalents. The bound material was then eluted with a 0.0-0.3 M NaCl gradient, 500 ml total volume in starting buffer.

Electrophoresis of chromatography column fractions. Analysis of fractions from the HTP and DEAE columns was performed in the following manner. A sample from each

fraction was electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the technique of Laemmli (1970). After electrophoresis at 6 watts (constant power mode), the gel was stained with coomassie blue (R-250) for 1 hr and then destained overnight by diffusion in 40% methanol and 10% acetic acid. Silver staining (BIO-RAD) as well as coomassie blue staining was employed.

Determination of purity. A gel with a PA band to be assayed for purity was soaked in 1% glycerol and 10% acetic acid for 1 hr after destaining. The gel was transferred to a clean glass plate and an absorbance scan was performed on each lane of interest on a DU-8 Beckman spectrophotometer. The sensitivity was set at the highest setting with a minimum peak value of 0.001. The wavelength was set at 561 nm and the speed was 2 cm/min.

To determine antigenic purity, an aliquot of DEAE column fractions, crude toxin, and concentrated toxin were electrophoresed through agarose gel according to the Grabar-William's technique (outlined by LKB, Bromma, Sweden, application note 249) and then diffused against antibodies to PA and antiserum to B. anthracis whole cell.

To determine if the minor bands present in the PA fractions were breakdown products of the PA, the following experiment was performed. Protective antigen was run parallel in two lanes of a 10% SDS-PAGE gel, 1.5 mm thickness. After completion of the run, the gel was cut

lengthwise between the two lanes and one side was stained to determine the location of the PA band. The corresponding area was then cut out of the unstained half and mixed with SDS-PAGE dissociation mix and allowed to stand at room temperature for several days. The unstained PA band was then electrophoresed again and stained with coomassie blue.

The pure PA fractions were pooled, dialyzed, lyophilized and stored at -20°C.

Biological Activity of Pure PA

Purified toxin was assayed in the same fashion as the crude and concentrated toxin. The lyophilized PA was reconstituted with sterile triple distilled H_2O and tested with aliquots of LF (as identified by Ouchterlony analysis and SDS-PAGE) from the HTP fractions which showed no PA activity.

Protective Antigen Labelling

Lyophilized PA was reconstituted with triple distilled H_2O. The PA was labelled with ^{125}I according to the Pierce (Richmond, CA.) procedure using Iodobeads™. The Iodobeads consist of N-chloro-benzene-sulfonamide (sodium salt) covalently bound to polystyrene beads. The Iodobeads provide the oxidizing power to drive the ^{125}I labelling of tyrosine residues. Briefly, two Iodobeads were "loaded" with ^{125}I in a 0.2 ml volume of ^{125}I solution (New England Nuclear, Boston, MA.) containing 200 μCi

activity for 10 min. The beads were transferred to another reaction vial containing 2.0 ml of PA in solution at 160 µg/ml. The protein solution was allowed to react for 10 min, then it was removed from the reaction vial and held in an ice bath. Free ^{125}I was removed by gel filtration in a G-100 Sephadex™ (Pharmacia, Uppsala, Sweden) column. All fractions were assayed for gamma emission in a Packard 5650 auto-gamma machine, with 80% efficiency, to determine separation of free from bound ^{125}I. Concentration was adjusted to 30 ug/ml for PA. Determination was made using the BIO-RAD (Richmond, CA.) protein assay kit. Specific activity was 128,000 CPM/µg PA. The labelled protein (PA*) was stored at about 4°C for 40 hrs. until use.

The labelling of PA for use in vivo resulted in low-level specific activity. In order to obtain greater sensitivity for use in cell culture work the following labelling technique was employed. One mCi of ^{125}I was obtained from New England Nuclear (Boston, MA.). Bolton-Hunter reagent (with sulfo group) and Iodobeads were obtained from Pierce Chemical Company (Rockford, IL.). Three Iodobeads were added to 1 mCi ^{125}I in 200 ul of 25 mM NaH_2PO_4 buffer pH 7.6 and allowed to "load" for 10-15 min. Bolton-Hunter reagent (0.9 µg in 9 µl) was added and allowed to label for 7-10 min. Then 20-30 µg of PA in approximately 20 µl were added for an additional 50 min.

All reactions were carried out at room temperature. After reaction time was complete, the reaction mixture was placed on a G-50 Sephadex column, the Iodobeads rinsed with 100-200 µl buffer and the rinse also placed on the column. The column was eluted with about 5-10 ml of 25mM NaH_2PO_4 or 25 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. The fractions were collected and assayed for gamma emission. The fractions which contained the protein peak were pooled.

This labelling procedure was performed 8-10 times during the course of the study. The specific activity ranged from about 1.0 to 1.5 µCi/µg PA using the Bolton-Hunter reagent.

Determination of dissociation of ^{125}I from labelled PA. To determine the degree of dissociation of the ^{125}I over time from the labelled PA, the following experiment was performed: An aliquot of 0.1 ml of previously labelled PA was loaded onto an equilibrated G-50 fine Sephadex™ column (2.5 ml in a 3.5 ml syringe barrel). Equal-volume fractions were collected during the filtration until several column volumes of buffer had been collected. The fractions were then assayed for radioactivity. Before injection into test animals, the PA* solution was diluted with sterile 0.01 M Tris to achieve desired concentrations of PA*. Three amounts of PA* were used: 5 µg, 10 µg, and 20 µg.

Cell Line Selection and Culture

Prior to injection, each of three rats was anesthetized with 1 ml of a 10% solution of urethane by injection in the leg pouch on the inside of the thigh. Injection of the PA* with LF mixture was via the penile vein of the Fisher 344 male rats. All subjects were sacrificed within a 15-20 min time period and upon opening the chest cavity for dissection each was perfused to reduce the volume of blood in the organs of interest. As each organ was removed, it was rinsed in a saline solution, weighed, minced, and placed in a plastic tube for assay. The organs removed for assay were the brain, lungs, liver, heart, spleen, and kidney. Blood was also assayed from the subjects which received doses of 10 µg and 20 µg of PA*. The thyroid tissue was assayed from the animal which received 10 µg PA*.

Madin-Darby bovine kidney (MDBK) cells obtained from the American Type Culture Collection (Rockville, MD.) were grown as a monolayer to confluency in 75 cm^2 flasks in Eagle's Minimal Essential Medium (MEM) with 25 mM HEPES buffer, 10% fetal bovine serum (FBS), and penicillin/streptomycin (50 units/ml and 50 µg/ml respectively) at 37° C, and 5% CO_2. When the cell sheets reached confluency the cells were removed with trypsin and diluted 1:6 with growth medium and "seeded" onto 24 or 96-well plates (Costar, Cambridge, MA.). They were then incubated for

use the next day. Dilutions of 1:12 were seeded onto plates to be used 2 days after.

Estimation of
Cells per Well

The determination of the number of cells per confluent monolayer in the tissue culture wells was done in the following way. The cell sheets in 3 wells were rinsed with MEM and then treated with 100 µl of trypsin/EDTA at 0.15% and 0.1 respectively to remove the cells from the plastic well. The cells from each well were transferred to separate tubes and the wells were rinsed with 100 µl MEM. Each rinse was transferred to its respective tube. The contents of each tube was thoroughly mixed and the cell concentration was determined using a hemacytometer.

Protective Antigen
Binding Experiments

PA* binding assay. The PA* was diluted to the desired concentration in MEM, 25 mM HEPES with 10% FBS. The controls had 100-fold excess of unlabelled PA.

Confluent MDBK cells in 24-well or 96-well plates were rinsed twice with MEM without FBS or antibiotics, then PA* (with or without excess unlabelled PA) was added to each well and allowed to bind. For the 24-well plates 300 µl of treatment volume was used. For the 96-well plates 50 µl was used. After binding, the PA* solution was suctioned off of the monolayer and the well was rinsed 4 times with MEM. The monolayer was then treated with

trypsin/EDTA at 0.15% and 0.1% respectively at 37°C and the detached cells transferred to a plastic tube containing absorbent paper. Each well was then rinsed with 100 µl MEM, 25 mM HEPES and the rinse placed into its corresponding tube and all tubes assayed for gamma emission.

Determination of optimum excess level of cold PA. In order to determine the level of unlabelled PA to use during the binding experiments and to aid in determining the degree of specificity of PA* for the receptor, the following experiment was done. Monolayers of MDBK cells in 24 well plates were rinsed and treated with PA* at 500 ng/ml with parallel wells run with 10, 25, 50, 100, and 500 fold excesses of unlabelled PA for 1 hr at 37°C. The cell sheets were rinsed, removed, and counted as described above.

Effect of concentration of PA* on binding to MDBK cells. Monolayers of MDBK cells in 24-well or 96-well plates were rinsed and treated with increasing concentrations of PA* with parallel wells containing a 100-fold excess of unlabelled PA. All wells were incubated for 1 or 10 hr at 37°C and then removed, rinsed, and assayed.

Assessment of binding of PA* to MDBK cells over time. Monolayers of MDBK cells in 96-well plates were rinsed and treated with PA* in concentrations of 500, 1000, 2500, and 3000 ng/ml with parallel wells containing a 100-fold excess of unlabelled PA at 37°C. At timed intervals, one set of three wells for each concentration with its corre-

sponding parallel control series was removed from incubation and assayed for activity of bound PA*.

Reversibility of PA* binding. To determine if the binding of PA is a reversible event, 3-well sets of MDBK cells were treated for 6 hr with PA* at 2187 or 2500 ng/ml with corresponding controls. One set was assayed at this time and termed 100% binding at zero time. The treatment volume in the other sets were replaced with unlabelled PA at 2000 ng/ml. The cell sheets were then rinsed and assayed for bound activity at timed intervals.

Binding affinity of PA*. Utilizing the law of mass action, Cuatrecasas and Hollenberg (1976) suggested the following equation for determining the dissociation constant (K_D):

$$K_D = \frac{[H]\ [R]}{[HR]}$$

Where: H = hormone or ligand

R = receptor

HR = ligand-receptor complex

Another method for deriving the K_D was presented by Scatchard (1949). This is a graphic representation of the data which plots the ratio of bound/free ligand on the ordinate and the corresponding amount of bound ligand on the abscissa, producing a negative slope. The slope of the regression-fitted line is equal to $-1/K_D$. Westphal (1971) favors this method for evaluating binding data of the type presented in this research. The Scatchard analysis deter-

mines the maximum binding sites (abscissa intercept) as well as a visual representation of data that may suggest information concerning number of different receptors.

The K_D for PA* was compared to that of other toxins.

Partial Characterization of the PA Receptor

Effect of trypsin treatment. Confluent monolayers of MDBK cells in 96-well plates were treated with 0.15% trypsin plus 0.1% EDTA for 10, 15, and 20 seconds at 25°C. The cells were then rinsed 3 times with MEM with 20% FBS and incubated for 20 min. The cells were observed microscopically before use. The monolayers were rinsed and then treated with PA* at 3000 ng/ml. Untrypsinized cells were simultaneously treated with the same PA* with controls. The monolayers were treated 45 min, rinsed, and then assayed for bound activity. All experiments were performed at 37°C.

Blocking of PA* binding sites with cholera toxin subunit B. Confluent monolayers of MDBK cells in 96-well plates were treated with 0.0-, 0.1-, 1.0-, 10.0-, or 50.0-fold excesses (on a molar basis) of cholera toxin subunit B relative to final PA* concentration, for 105 min. After this time the PA* was added directly to the 50 µl cholera treatment volume to achieve 2500 ng PA*/ml and allowed to react for 2 additional hr. The wells were then assayed. All experiments were performed at 37°C.

RESULTS

Toxin Production and Assay

Biologically active culture supernatant (crude toxin) was obtained by culturing the anthrax bacillus and the crude toxin was concentrated. A 1 ml intravenous injection of the crude toxin into Fischer 344 rats resulted in lethality accompanied by severe pulmonary edema as evidenced by increased lung weight, compared to control animals, and fluid excretion from the nose. Time of death in test animals varied with each lot and ranged from about 60 to 90 min. The crude toxin, which was concentrated approximately 50-fold, also retained lethality when diluted back to the crude toxin level. Time to death of test animals injected with diluted toxin concentrate ranged from about 65 to 124 min. Table 1 gives an example of time to death with one lot of crude and concentrated toxin produced with the Vollum 1B strain. Lethality of toxin produced with the Sterne strain was essentially the same. The Ouchterlony double diffusion assay of crude and concentrated toxin showed lines of precipitation against anti-PA and anti-LF. Table 2 shows the Ouchterlony and edema assays with the dilute concentrated toxin of the same lot.

TABLE 1

TIME TO DEATH OF TEST ANIMALS
INJECTED WITH CRUDE OR CONCENTRATED TOXIN

Toxin preparation [a]	Time to death (min) [b]
Crude (flasks 1-3)	67
Crude (flasks 4-6)	78
Concentrate diluted 1:50	124

[a] Injection volume was 1 ml iv. One rat per preparation each weighing between 220-246 g.

[b] Data is from one representative lot of toxin harvest produced with B. anthracis Vollum 1B strain. Lethality with toxin from the Sterne strain was essentially the same.

TABLE 2

OUCHTERLONY AND EDEMA
ASSAY OF DILUTED TOXIN CONCENTRATE

Assay	Dilution of toxin	Test result [b]
Ouchterlony		
Anti-PA [a]	1:64	+
Anti-LF [a]	1:64	+
Edema test	1:32	+
	1:64	-

[a] PA = protective antigen. LF = lethal factor.

[b] Data is from one representative lot of toxin harvest from B. anthracis culture supernatant.

Protective Antigen Purification

Liquid chromatography. Fractions eluted from the HTP column as analyzed by SDS-PAGE revealed that PA which eluted with a peak at 150 ml was well separated from the bulk of the LF, which eluted with a peak at 210 ml (Fig. 1). The spectrophotometric profile at 254 nm also shows peaks at 90 and 130 ml. The first peak showed no protein with coomassie blue and higher absorbance at 260 nm than 280. The second peak (at 140 ml) proved to be a low molecular weight contaminant in addition to some PA.

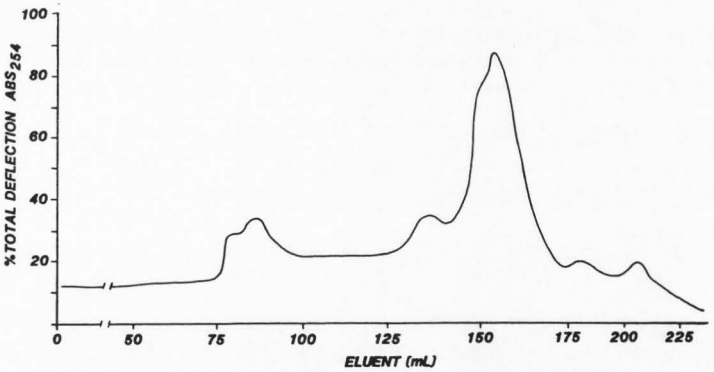

Fig. 1. Separation of B. anthracis toxin components on hydroxylapatite. Dialyzed, concentrated toxin was "loaded" onto a column (2.5 cm diameter and 10 cm high) of hydroxylapatite, then washed with several void volume equivalents of starting buffer. The column was eluted with a buffer gradient of 0.002 to 0.4 M KH_2PO_4, 2 mM 2-mercaptoethanol, and 0.05 M NaCl at pH 7.1 in a total volume of 500 ml. The eluent was monitored by in-line spectrophotometry at 254 nm and collected in 2 ml fractions. Protective antigen eluted primarily in the peak at 150 ml. Lethal factor eluted primarily in the smaller peak at 210 ml.

Analysis of the pooled PA fractions eluted from the subsequent DEAE column revealed that the PA eluted first at approximately 0.05 M NaCl. Initial fractions were free of contaminants with successive fractions containing two lower molecular weight peptides as shown by coomassie blue staining (Fig. 2). Silver staining in concert with the coomassie blue failed to reveal any additional contaminants of the PA fractions (Fig. 3).

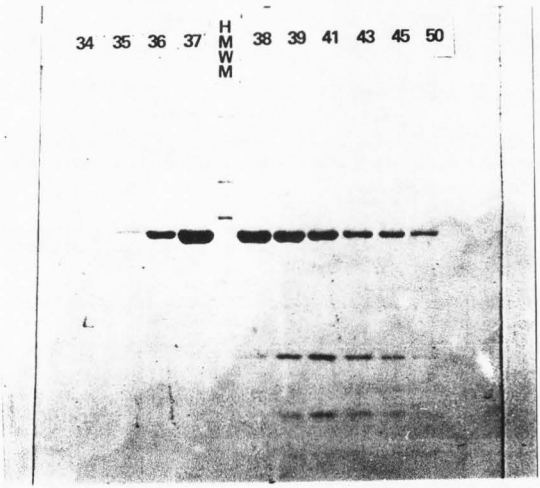

Fig. 2. SDS-PAGE of purified protective antigen (PA) stained with coomassie blue. Fractions eluted from DEAE column show PA without contaminating bands in fractions 36 and 37. Subsequent fractions (38,39, etc.) show additional minor lower molecular weight bands later shown to be breakdown products of PA. The 3 high molecular weight markers (HMWM) represent 200 K, 116.3 K, and 92.05 K daltons starting from the top.

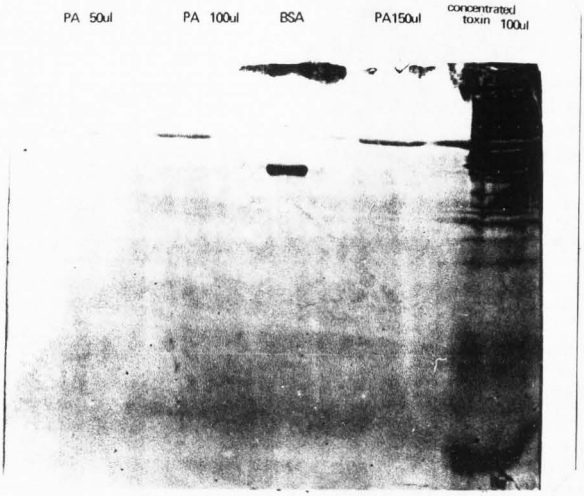

Fig. 3. SDS-PAGE of purified protective antigen (PA) stained with coomassie blue and silver staining. Purified PA, bovine serum albumin (BSA), and concentrated toxin were electrophoresed and then stained with coomassie blue and silver staining technique. Silver staining showed no additional contaminating bands in the PA samples.

Determination of purity. The scanning gel densitometry performed on the PA sample after the purification sequence showed only the PA band registering an absorbance (Fig. 4). With the sensitivity at the highest (most sensitive) setting and the minimum absorbance set at 0.001, the PA peak accounted for 100% of detectable bands. However, it was possible to detect with the eye two very faint lower molecular weight bands in the same lane with the PA. Taking this into account, the purity level of the PA was determined to be > 99%.

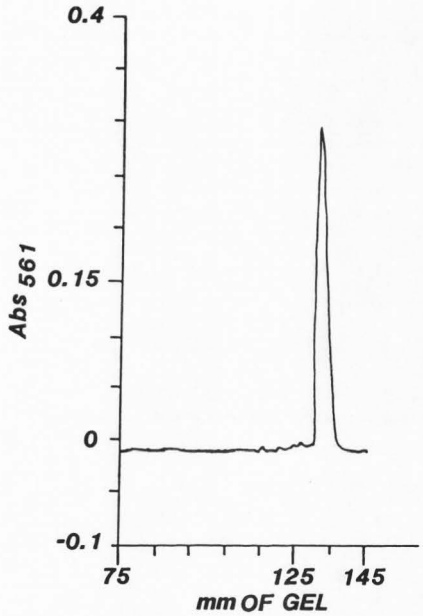

Fig. 4. Densitometric scan of purified PA preparation (approx. 8 µg protein) on SDS-PAGE stained with coomassie blue. An SDS-PAGE gel was mounted on a clean glass plate and was kept moist with 1% glycerol/10% acetic acid mix. The gel was then scanned at 561 nm in a Beckman DU-8 spectrophotometer to obtain the absorbance profile shown. The sensitivity was set at 1 (greatest sensitivity) with a minimum peak value setting of 0.001. The data obtained indicated that the PA peak (at about 135 mm) accounted for 100% of the protein in the purified PA preparation. Two very faint bands, however, were visible but failed to be detected at the 0.001 absorbance setting. Taking this into account, the purity level of the PA was determined to be > 99%.

Grabar-William's immunoelectrophoresis of purified PA showed only one immunoreactive band against both anti-PA and anti-B. anthracis antisera (Fig. 4).

Fig. 5. Grabar-Williams' immunoelectrophoresic analysis of B. anthracis toxin and protective antigen (PA). Purified PA was loaded into the center well (3), crude toxin into wells 1 & 4, and concentrated toxin into wells 2 & 5. After electrophoresis, anti-PA was loaded into channels 1 & 3 and anti-spore antiserum was loaded into channels 2 & 4. PA shows only one immunoreactive band against both antisera, whereas both the crude and concentrated toxin showed 2 antigenic species against the anti B. anthracis antiserum.

Demonstration of breakdown products. The unstained PA band cut from an SDS-PAGE gel and left at room temperature for several days was again electrophoresed by SDS-PAGE. The stained gel revealed two lower molecular weight peptides along with the PA band indicating that the two lower molecular weight peptides are derived from the PA.

Cell Line Selection

The *in vivo* distribution of PA* in a susceptible host, namely Fischer 344 male rats, upon intravenous injection of a PA*/LF mixture is shown in Figure 6. In the subject that received the lowest dose (5 ug PA*), the highest concentration of PA* was found in the kidney on a per gram tissue basis, followed by the liver, spleen, lung, and heart. In the rat dosed with 10 ug PA*, the highest concentration of PA* was found in the spleen, followed by the kidney, liver, lung, blood, and heart. In the animal receiving the 20 ug dose, the highest concentration of PA* was in the spleen, followed by the liver, kidney, lung, blood, heart, and brain. The thyroid showed next to the least activity the animal receiving the 10 ug PA* dose.

From these results and the implication of the kidney in the pathology of anthrax disease, it was decided to use an established kidney cell line from a susceptible host species for the following experiments.

Protective Antigen Binding Experiments

Labelling of PA for in-vitro binding studies. Upon completion of the gel filtration of the ^{125}I/PA mixture, aliquots of the collected fractions were assayed for radioactivity. Two well-defined peaks were observed (Fig. 7). The first peak represented the PA* and the second represented free ^{125}I. The PA* had a specific activity of

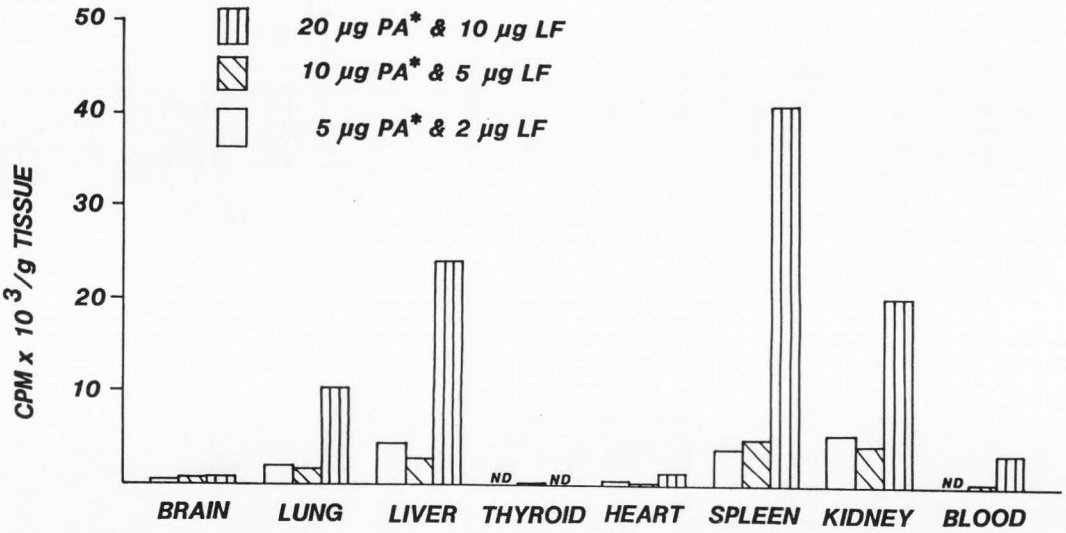

Fig. 6. In vivo distribution of ^{125}I-labelled protective antigen (PA*). The different amounts of PA* with the amounts of lethal factor (LF) shown were injected iv into 3 different Fischer 344 rats. The rats were sacrificed at 15 min, perfused, and the organs removed, rinsed, weighed, minced, and then assayed for radioactivity.

approximately 1 μCi/ug. The fractions representing the PA* were pooled for use in the binding experiments.

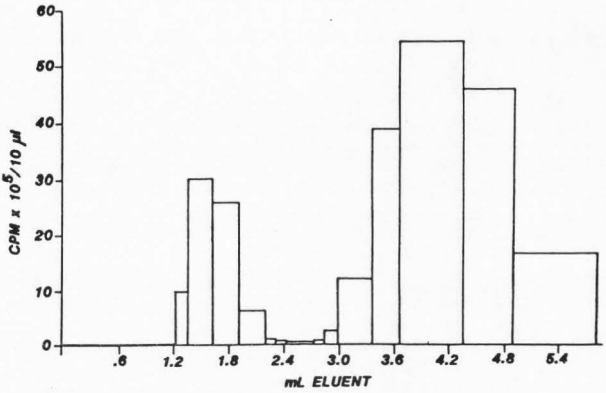

Fig. 7. Gel filtration of ^{125}I/labelled protective antigen (PA*) mixture. After radio-iodination of PA, the radioactive iodine/PA* mixture was chromatographed through a G-50 fine sephadex column. Fractions of eluent were collected in separate tubes and assayed for radioactivity. The first peak represents the PA* and the second peak represents free radioactive iodine.

Dissociation of ^{125}I and PA. Fractions collected from the gel filtration of PA* labelled 8 days earlier showed gamma activity in two well-separated peaks demonstrating the dissociation of the ^{125}I from the PA* (Fig. 8). During the time from day 0 to day 8 it was determined that approximately 20% of the total ^{125}I had dissociated from the PA*. A linear rate of dissociation was assumed and subsequently used in calculating specific activity for experiments requiring it.

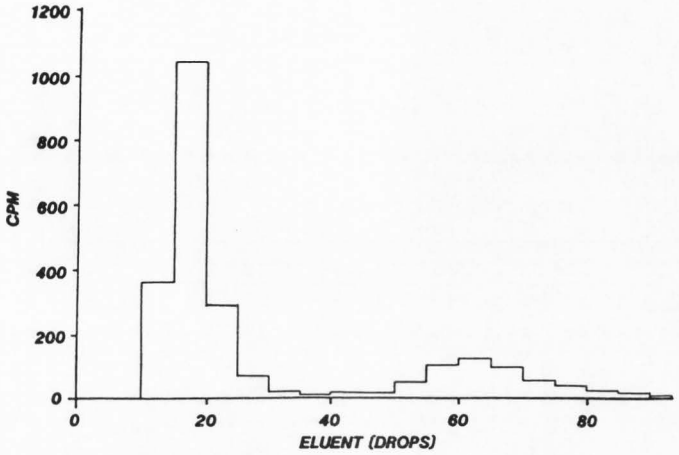

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Fig. 8. Dissociation of I and protective antigen (PA). Eight days after radiolabelling, an aliquot of PA* was gel filtered on a G-50 fine sephadex column. Fractions were collected in separate tubes and assayed for radioactivity. Two well-separated peaks are evidence of dissociation of radioiodine from labelled PA.

Effect of concentration of unlabelled PA on the binding of PA*. The total cell-associated radioactivity after a 1 hr exposure to PA* at 500 ng/ml was designated 100%. The same concentration of PA* in the presence of a 10-fold excess of unlabelled (cold) PA decreased the total binding of PA* to nearly 30% (Fig. 9).

Increasing the cold PA to a 100-fold excess decreased the total binding to just under 19%. The decrease in total binding reached 83.8% with a 500-fold excess of cold PA. A 100-fold excess of cold PA was used in all subsequent experiments to ensure minimal nonspecific binding.

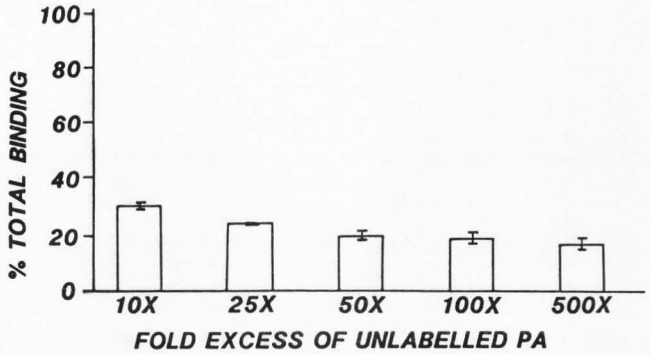

Fig. 9. Effect of excess unlabelled protective antigen (PA) on binding of PA* to MDBK cells. MDBK cells were treated with PA* at 500 ng/ml alone or with excess unlabelled PA for 1 hr. All treatments were then rinsed and assayed for radioactivity. Incubation at 37°C. Error bars represent standard error of the mean.

Binding of PA* at various concentrations. Initially, 24-well plates were used to demonstrate saturation by varying the concentration of PA*. When saturation was not observed at 2500 ng PA*/ml, 96-well plates were substituted for the 24-well plates since the available PA* was limited. Fifty microliters was used as a treatment volume rather than the 300 µl required by the 24-well plates. This volume reduction corresponded proportionately with the reduction in cell monolayer surface area. Saturation of binding sites on the MDBK cells was then attempted by increasing the high end of the concentration range to 5000 ng PA*/ml for 1 hr. The same experiment was repeated for 5 hr at 4°C and 37°C (Fig. 10).

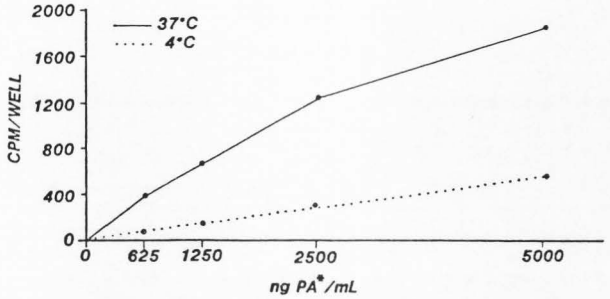

Fig. 10. Effect of concentration of labelled protective antigen (PA*) on its binding to MDBK cells. MDBK cells in triplicate wells were treated with PA* at various concentrations with corresponding controls for 5 hr at 37°C and 4°C. After this time the cell sheets were rinsed and assayed for radioactivity. Variance among wells representing total binding averaged 13% at 37°C and 24% at 4°C. Variance among control wells averaged 7% at 37°C and 74% at 4°C.

The binding experiment at 4°C appeared to give lower levels of cell associated radioactivity than that at 37°C.

At 5000 ng/ml it still did not appear that saturation was achieved in 5 hr. The treatment of cells over a 10 hr period with PA* concentrations as high as 5300 ng/ml demonstrated saturation at about 3752 ng/ml (Fig. 11). The number of binding sites was 72,000 per cell.

Binding of PA* over time. Initial experiments to determine binding of PA* at 2500ng/ml (Fig. 12) showed that saturation was being approached at about 12 hr. A rise in CPM/well occurred at 7 hr which was also noted in a subsequent experiment although less pronounced.

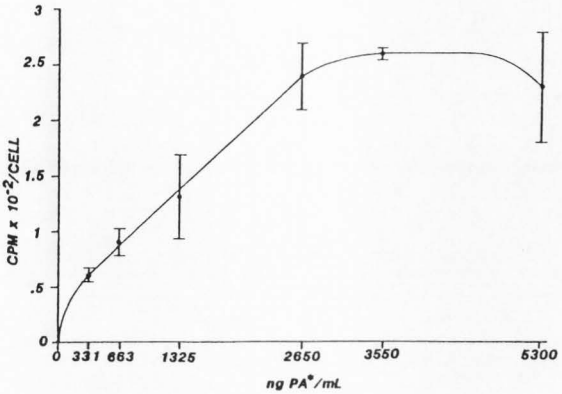

Fig. 11. Effect of concentration of labelled PA (PA*) on binding to MDBK cells. MDBK cells in triplicate wells were treated with PA* at varying concentrations with corresponding controls for 10 hr at 37°C. After this time the cell sheets were rinsed and assayed for radioactivity. Data is from 3 separate experiments (data points at 3550 and 5300 ng PA*/mL are from 2 experiments). Error bars represent standard error of the mean.

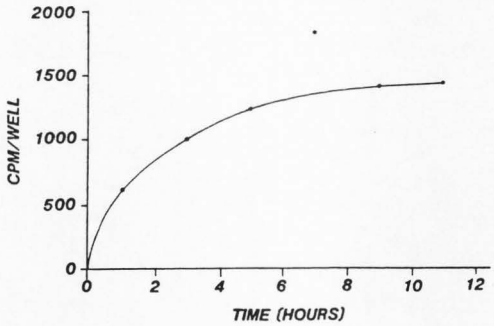

Fig. 12. Binding of labelled protective antigen (PA*) to MDBK cells over time (PA* at 2500 ng/ml). At timed intervals triplicate wells were rinsed and assayed for radioactivity along with 1 set of controls. Performed at 37°C. Variance in wells representing total binding averaged 5%. Variance in control wells averaged 9%. Note outlier at 7 hr.

In the following experiments cell-density-per-well counts were made at each assay time to account for the possibility that the rise noted in the curve at 7 hr (Fig. 12) could be due to cell division. Binding of PA* at 3000 ng/ml over time (Fig. 13) with accompanying cell counts failed to manifest the rise and showed saturation after 15 hr.

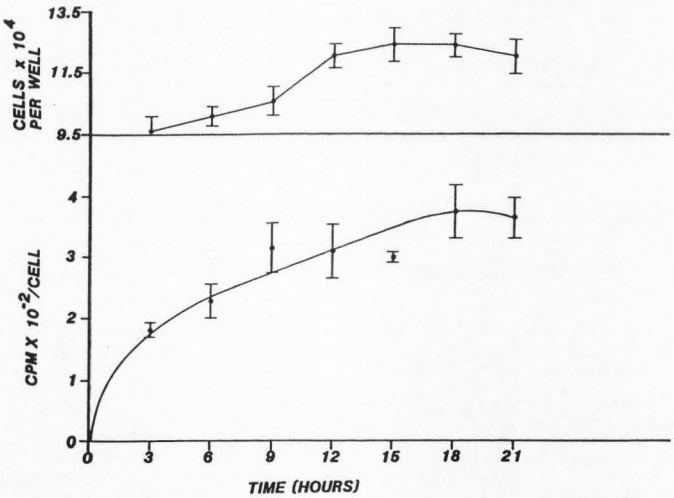

Fig. 13. Binding of labelled protective antigen (PA*) to MDBK cells over time (PA* at 3000 ng/ml). At timed intervals 1 set of 3 wells was rinsed and assayed for radioactivity along with 1 set of controls. Upper graph shows concentration of cells per well at same time intervals. Data is from 3 separate experiments at 37°C. Error bars represent standard error of the mean.

Reversibility. Cells treated with unlabelled PA after preloading with PA* for 6 hr resulted in an initial rapid loss of bound PA* in the first 15 min (Fig. 14) with a slower but linear rate of release for the remaining 2 hr of the experiment.

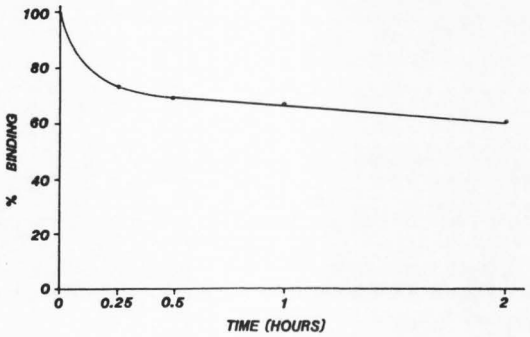

Fig. 14. Reversibility of labelled protective antigen (PA*) binding to MDBK cells over 2 hr. MDBK cells in triplicate wells were treated with PA* at 2500 ng/ml for 6 hr at which time one set of wells was assayed and designated 100% binding. The treatment volume in the other sets of wells was replaced with unlabelled PA at 2000 ng/ml and the wells were rinsed and assayed in triplicate at timed intervals. Incubation was at 37°C. Variance among wells representing total binding averaged 9%. Variance among controls wells averaged 18%.

Assays over longer periods of time under the same conditions (Fig. 15) demonstrated the same result with rapid initial release of PA* and then a slower rate of release over a 17 hr period.

Binding affinity of PA*. The Scatchard analysis of binding data of PA* to MBDK cells revealed a dissociation constant (K_D) of 17×10^{-9} M PA* (Figure 16).

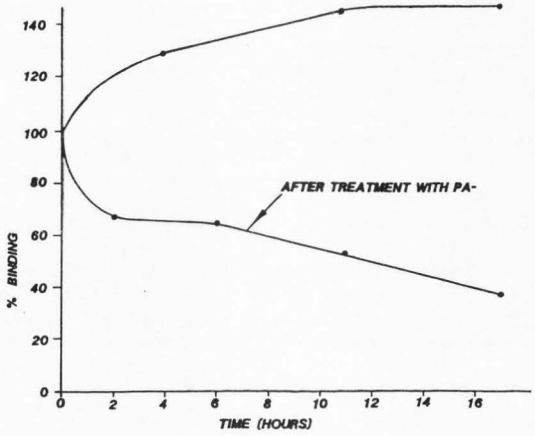

Fig. 15. Reversibility of labelled protective antigen (PA*) binding to MDBK cells over 17 hr. MDBK cells were treated with PA* at 2187 ng/ml for 6 hr at which time 1 set was rinsed and assayed. This was designated 100% binding. The treatment volume in the other sets of wells was replaced with unlabelled PA at 2000 ng/ml and the wells were rinsed and assayed in triplicate at timed intervals. Parallel sets of wells without the treatment volume replaced with unlabelled PA were also assayed at timed intervals and showed saturation at the 11 hr mark. Incubation was at 37°C. Variance among wells representing total binding averaged 13%. Variance among control wells averaged 17%.

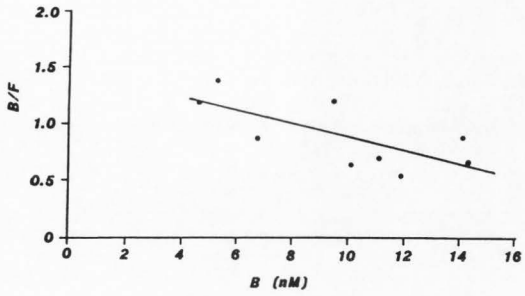

Fig. 16. Scatchard analysis. Binding data from saturation experiments was used to generate the points for the Scatchard analysis. Bound (B) toxin in nanomolar values is plotted on the abscissa and the bound/free (B/F) ratio is plotted on the ordinate. The line (-0.06 slope) was fitted by regression analysis (r = -.7).

Effect of Trypsin Treatment on Binding of PA*

Treatment of cells with trypsin at 0.15% and EDTA at 0.1% for more than 20 sec resulted in severe loss of the cell sheet due to detachment of the cells. Trypsin/EDTA was therefore limited to 20 sec or less. At 10 sec of trypsin/EDTA treatment the cells showed a "rounding-up" of the individual cells characteristic of trypsin treatment. This indicated that the protein molecules on the surface of the membrane responsible for attachment to the plastic well had been acted upon by the trypsin. The cell sheets utilized in the experiment sustained a loss of about 5-10% of the cells in 1 or 2 of the 3 wells in each set.

By one-way analysis of variance, the effect of the trypsin on the binding of PA* to MBDK cells was shown to be insignificant ($p>0.05$) (Fig. 17).

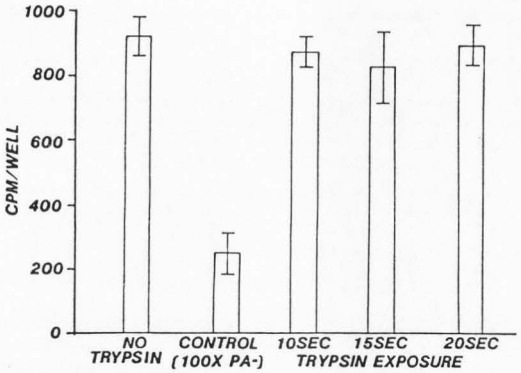

Fig. 17. Effect of trypsin treatment of MDBK cells on the binding of labelled protective antigen (PA*). MDBK cells in triplicate wells were treated with a solution of 0.15% trypsin and 0.1% EDTA (pH 7.0) for 10, 15, and 20 sec at 25°C. The cells were then rinsed twice with MEM containing 20% fetal bovine serum. After 20 min the cells were rinsed and treated with PA* at 3000 ng/ml. Parallel untrypsinized cells with controls were also treated with PA*. All wells were rinsed and assayed after incubation for 45 min at 37°C. PA- = unlabelled PA. Error bars represent standard error of the mean.

Blocking of PA* Binding with Cholera Toxin Subunit B

The binding of PA* to MBDK cells was shown to be blocked by the cholera toxin subunit B (Fig. 18). The degree of blocking appeared to be concentration dependent with about 10% binding reduction at a 0.1 molar ratio of cholera toxin subunit B and nearly 50% reduction at a 50 fold excess of subunit B.

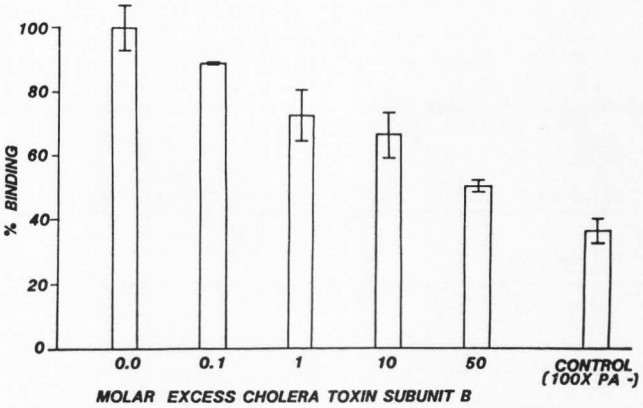

Fig. 18. Blocking effect of cholera toxin subunit B on binding of labelled protective antigen (PA*). MDBK cells in triplicate wells were treated with varying concentrations of cholera toxin subunit B or medium with or without unlabelled PA for 105 min. PA* was then added to each well to achieve 2500 ng/ml and then incubated for 2 hr. All cells were rinsed and assayed. Incubation was at 37°C. PA- = unlabelled PA. Error bars represent standard error of the mean.

DISCUSSION

It is well established that toxins bind to cell surface receptors in order to exert their effects. This research has attempted to obtain pure protective antigen component of the anthrax toxin and to characterize its binding interaction with an established cell line.

Toxin Production and Purification

The pathogenic B. anthracis Vollum 1B strain was used for toxin production initially. Biological activities of the toxin were successfully demonstrated. Lethality was demonstrated by intravenous injection of Fischer 344 male rats with sterile crude and concentrated toxin as well as purified PA and LF components. Subcutaneous injection of crude and concentrated toxin into rabbits was used to manifest the edema response. Times to death (TTD) were routinely as low as, or lower, than those reported by Haines et al. (1965) for crude toxin or by Ezzell et al. (1984) for purified components.

It was subsequently determined that the Sterne vaccine strain of B. anthracis produced toxin which possesed essentially the same degree of lethality for test animals. It is generally accepted that the avirulence exhibited by the Sterne strain is due to the lack of a capsule. For

safety considerations, therefore the majority of PA production for binding experiments was derived from B. anthracis Sterne strain.

In the early work with anthrax toxin, horse serum was added to the culture supernatant to prevent the adsorption of anthrax toxin onto the filters used (Haines et al., 1965). The variety of proteins in the serum would have considerably complicated the purification of PA for this research. I.G. Resnick of U.S. Army Dugway Proving Ground (personal communication) recommended the use of PEG to minimize toxin losses due to nonspecific binding. The use of PEG to minimize filter adsorption proved effective in this research as evidenced by the toxin yield and it did not add contaminating proteins to the culture supernatant. Subsequent dialysis easily removed the PEG.

Purification of the PA component beyond the 90% level has not been reported in the literature. Greater purity levels were considered necessary for the proposed binding studies in order to eliminate possible interference from contaminants. Efforts in this research succeeded in yielding purified PA at levels > 99% as revealed by SDS-PAGE with coomassie blue and silver staining. The purified PA maintained biological and antigenic activity as demonstrated by lethality in combination with LF and by immunoelectrophoresis against anti-PA antibodies and anti-whole cell antiserum.

The approach used to accomplish the PA purification differed from that used by Leppla (1982 & 1984) whose purification sequence involved 1) dilution of crude toxin, 2) batch adsorption to DEAE-cellulose, 3) elution followed by ammonium sulfate precipitation, 4) dialysis, 5) HTP chromatography with an elution gradient of 0.002 M to 0.5 M KH_2PO_4, 6) dialysis, and 7) DEAE Trisacryl-M™ (LKB, Bromma, Sweden) chromatography. In this research the sequence for purification was 1) concentration of crude toxin in an Amicon (Danvers, MA.) stirred concentrator cell, 2) dialysis, 3) HTP chromatography with an elution gradient of 0.002 M to 0.4 M KH_2PO_4, 4) dialysis (or gel filtration), and 5) DEAE-cellulose with Cellex-D chromatography.

The culture volume used also differed. Leppla (1982 & 1984) used a 20 L fermentor whereas only 1/2 L cultures in flasks were used in this research. Use of a smaller culture volume without agitation may minimize cell lysis which could add contaminating proteins.

Contamination by proteins contributed by lysis of older cells in the culture may also have been minimized by harvesting as early as possible during the recommended time period (Haines et al. 1965). Elimination of the centrifugation step, used to pellet the bacteria before filtration, also minimized cell lysis due to manipulation of the supernatant. Use of a pre-filter prevented the 0.22 um filter from clogging, thereby eliminating the

centrifugation step. As noted, this method showed no apparent reduction in toxin yield. Elimination of smaller molecular weight contaminants was also achieved by the concentration step in the Amicon cell with a 30,000 MW exclusion limit and by utilizing a 50,000 MW exclusion for the dialysis tubing.

Analysis of the fractions from HTP and DEAE chromatography consistently revealed 2 contaminants of about 30,000 and 50,000 MW which proportionately paralleled PA in amount in each fraction. Breakdown products of PA were suspected. Subsequent experiments demonstrated this suspicion to be true. Their combined approximate molecular weights and elution pattern were consistent with the assumption that they were derived from PA. This fact made it difficult to obtain purified PA without these contaminating bands. Again, shortening the culture time may minimize this problem.

Quickly processing the crude toxin from one step to the next without long intervening periods of storage or refrigeration is important in eliminating possible action on the toxin components by enzymes that may be present. Also, it was noted by Wilkie and Ward (1967) that biological activity of their anthrax toxin was lost in PO_4 buffers. However, this problem was not observed in the present study. Machuga et al. (1986) reported purification of the LF to the 99% level by immunosorbent chromato-

graphy. This technique may prove useful in the future purification of all three factors of the anthrax toxin. One concern, however, regarding this technique is that if the breakdown products of PA retained their antigenicity with respect to the antibodies used in the immunosorbent chromatography column, they would likely elute with the PA and be a continuing source of contamination.

The level of purity, as demonstrated by SDS-PAGE, achieved by Machuga et al. (1986) may be in question. It must be pointed out that the amount of LF run on the electrophoresis gel by Machuga et al. (1986) appeared to be just above the levels of detection by the staining method used (silver staining). If the level of detection were 0.25 µg, then 2.5 µg would have to run on the gel and be free of contaminants in order to be declared at $\geq$ 90% purity. This was apparently not the case with the gel they used to demonstrate purity.

In this research project the purified PA was electrophoresed at high and low concentrations in order to visualize both low level contaminants and masked bands near the PA respectively. The LF from the HTP column in this study appeared >90% homogeneous on SDS-PAGE and when further purified by ion-exchange chromatography,approached the same level of purity as the PA (99%). The LF was antigenically free of PA and was suitable for use in the lethality assay.

Protective Antigen Labelling

The use of ^{125}I in the labelling of ligands has found wide use in binding studies. This technique allowed for a more direct assay system with fewer steps and complications compared to radiologically labelled anti-PA or a fluorescence assay for example. The PA failed, however, to take up the label to a great enough specific activity (CPM/ug protein) for the sensitivity required in the binding studies. Therefore, it was decided to enhance the labelling process by use of the Bolton-Hunter reagent. This additional technique has been reported in a number of other binding studies (Morris et al., 1980; Kitamura et al., 1980; and Rogers and Snyder, 1981). With the Bolton-Hunter reagent, the PA was labelled to levels above 1 uCi/ug protein. This corresponded to levels commonly found in the literature and was suitable for use in the 96-well tissue culture dish assay. Higher levels of labelling may subject the ligand to deterimental effects from the isotope's energy of disintegration.

Dissociation of ^{125}I from the PA was observed over time. This phenomenon does not seem to be universally considered in binding studies utilizing labelled ligand. Failing to consider this occurrence could give misleading quantitative results.

The PA* was routinely utilized within 10 days of labelling and was kept on ice during that time.

In Vivo Study and Cell Line Selection

Wider use of cell culture in research has enhanced understanding of how toxins exert their effects (Solotorovsky and Johnson, 1970). Selection of the cell line for this study involved an experiment to determine which organs of a susceptible host might serve as targets for the anthrax toxin. Cattle and sheep which are naturally susceptible hosts to anthrax were impractical and too expensive to use. Although the Fischer 344 rat is relatively resistant to infection with *B. anthracis* (Stephen and Pietrowski, 1983), it is extremely sensitive to the anthrax toxin. This made the Fischer 344 rat a suitable choice for the *in vivo* study.

The *in vivo* distribution of PA* directed the decision as to what type of cultured cells would be used for the binding studies. Although the kidney and lung tissues showed less radioactivity after exposure to the labelled PA than the spleen or liver at the 20 µg level, the kidney showed the highest activity at the 5 µg level implying that PA may initially be selectively adsorbed by kidney tissue. The kidney and lung have been implicated in the lethality of anthrax infection (Nalin et al., 1977; Smith and Stoner, 1967; Smith and Keppie, 1954). Middlebrook et al. (1978) in studies with diphtheria toxin noted that of seven cell lines tested, the three most sensitive in their battery were derived from kidney tissue. They (Middlebrook

et al., 1978) also cited Solotrovsky and Gabliks' observation that among the most toxin sensitive cell lines were primary kidney cells. In view of these observations and the fact that cattle are a susceptible and primary host of anthrax, the MDBK cell line was chosen. It is seen in this project that receptors for PA are present on MDBK cells in significant numbers. This suggests a potential susceptibility of MDBK cells to either EF or LF. This possibility merits further investigation.

An interesting point regarding the in vivo study was that the blood contained very little of the radioactive PA* after 15 min indicating a rapid removal of the PA from circulation. This substantiates a similar observation by Molnar and Altenbern (1963).

Binding Assay

In preparation for performance of the in vitro testing, an assay system to monitor the presence of PA* was developed. The assay proved successful for the parameters investigated. The specific activity of 1 μCi/ug protein allowed rapid and simple quantification of nanogram quantities of PA* with statistical precision in the in vitro system. The assay had good reproducibility between triplicate wells with generally less than 15% variation and a high specific/non-specific ratio of cell-associated radioactivity. The cost of about $100.00 per 30 μg PA labelled is reasonable and provides enough PA* to conduct numerous

experiments at high concentrations of PA* (2000 - 5000 ng/ml).

Evaluation of Experimental Results for Receptor Criteria

The purification of PA to required levels and the development of a reliable assay system were critical steps to provide the tools for the binding studies performed. The criteria for identifying the receptor(s) for PA* on MDBK cells were investigated (i.e. specificity, saturability, reversibility, and affinity).

Specificity. It has been noted by Middlebrook et al. (1978) and Aguet et al. (1982) that competition between unlabelled ligand and labelled ligand for a receptor is a demonstration of receptor-ligand specificity. The binding of PA* was significantly reduced (approximately 70%) by the presence of a 10-fold excess of unlabelled PA in 1 hr of treatment, in 24-well plates (Fig. 9). These results are particularly significant in that the larger 24-well plates would accomodate greater numbers of possible non-specific binding sites for the PA*. It should also be noted that the 10% level of FBS in the treatment volumes not only aided in stabilizing the PA, but also could have blocked possible nonspecific binding.

Blocking of PA* binding by cholera toxin may also be considered evidence of the specificity of the binding of PA* to the MDBK cell receptor(s).

Saturability. The saturation of binding sites at 37° C with labelled PA at 3000 ng/ml over time (Fig. 12) as well as during the 10-hr saturation study at varying concentrations of PA* (Fig. 11) demonstrated that a limited number of binding sites are available on the MDBK cell surface. This also suggests that the PA remains bound and unprocessed or at least that the ^{125}I is not rapidly endocytosed with the PA being cleaved and excreted as is the case with ^{125}I-diphtheria toxin at 37°C (Middlebrook et al., 1978).

Calculations of receptor number per cell based on the saturation levels from the 10-hr concentration study (Fig. 11) and the time study (Fig. 12) showed about 72,000 and 114,000 receptors for PA per cell respectively. It must be borne in mind that the cell surface available for binding PA is highly reduced because cells are grown in monolayer rather than suspension. Therefore, the actual number of receptors per cell may be double the amount cited here. This number of binding sites assumes a PA to receptor ratio of one.

Ezzel et al. (1984) noted that the minimum amounts of PA and LF for lethality in Fischer 344 rats are 3 μg and 0.6 μg respectively. They cautiously suggest that a possible 5:1 ratio may exist between PA and LF with regard to pathological and/or receptor function. The prolonged time to saturation noted in Figs. 12 and 13 may be partly accounted for by the possibility of formation of PA* to

PA*-receptor aggregates. Aggregation of this type has been reported. Insulin, for example, has been shown to display this characteristic (Cuatrecasas and Hollenberg, 1975). Internalization of the PA* could also explain the apparent prolonged time to saturation particularly at 37° C. At this temperature the normal dynamic functions of the cell such as endocytosis would be fully operable. Middlebrook et al. (1978) have shown that internalization occurs with labelled diphtheria toxin. The graphic representation of their data shows a rise and then a significant fall in cell-associated radioactivity over time. The processing and excretion of the labelled A (active) subunit of the diphtheria toxin from cells may explain the decrease in cell-associated radioactivity noted as the cleaved toxin subunit would likely be incapable of reassociating with the cell. However, no such fall in cell-associated radioactiviy was noted with the PA* binding over time. Leppla (1982) observed that the delivery mechanism (presumably the PA-receptor complex) of EF to its site of action in the cell did not appear to be used up thus indicating the reusability of the bound PA for internalization of the EF. Gill (1978) observed that the "B" or binding subunits of toxins are probably not internalized. These observations suggest that internalization of PA, which functions as a binding subunit, probably did not contribute substantially to the prolonged saturation time noted in this research for PA binding to MDBK cells.

An observation worth noting in the 21-hr saturation study for PA binding (Fig. 13) is an apparent saturation at two stages (9 and 18 hr). The levelling off of radioactivity per cell after 9 hr occurs just prior to an increase in the cell count per well. A subsequent increase in radioactivity per cell was detected after 15 hr suggesting an increase in receptors per cell. This possible increase in receptors per cell is compatable with observations reported by Koulakoff et al. (1983) who noted that the ganglioside receptors on neuronal cells for tetanus toxin were expressed about 7 hr following the S phase of mitotic division. The apparent saturation of PA receptors at about 9 hr and subsequent binding increase may be indicative of delayed receptor expression. Delayed expression of receptors, then, is a process which must be considered in quantitative ligand binding studies.

Reversibility. The binding of PA* to MDBK cells was shown to be a reversible event in Figs. 14 and 15 with the rapid initial release of bound PA*. The slower rate of release that followed the initial drop in cell associated radioactivity may be due to a low dissociation rate following a time threshold. That is, after a certain amount of time, the PA* may be more firmly associated with (or perhaps imbedded into) the cell membrane. Another explanation for the slower rate of release of cell-associated radioactivity could be the release of internalized PA*. However, if this were so, it would seem that a sharper

drop in bound PA* should be observed.

Affinity. Data from the 10-hr saturation study (Fig. 11) was used to do Scatchard (1949) analysis. The binding constant (K_D) for PA* derived therefrom was 17×10^{-9} M. This relatively low constant is consistent with the greater length of time and higher concentrations required to obtain saturation. The K_D derived for PA* in this study was found to be much higher than that reported for diphtheria (Collier, 1975; Middlebrook et al., 1978), cholera (Bresson et al., 1984), tetanus (Rogers and Snyder, 1981), or enterotoxin of E. coli (Donta et al., 1982) as shown in Table 3. It should be noted that the dissociation constant for toxin binding may be temperature dependent (Sandvig et al., 1976).

TABLE 3

COMPARISON OF TOXIN DISSOCIATION CONSTANTS

Toxin	Cell type	Dissociation constant (K_D)
Protective antigen	MDBK	17.0×10^{-9} M
Tetanus[a]	mammalian brain	1.2×10^{-9} M
Cholera	rabbit intestine	1.2×10^{-9} M
enterotoxin E. coli	Y1 adrenal	0.6×10^{-9} M
Diphtheria	mammalian	10.0×10^{-9} M
Diphtheria[b]	Vero	1.1×10^{-9} M

[a] Performed at 0°C
[b] Performed at 4°C

It has been reported by Gill (1982) that a greater amount of anthrax toxin is required for lethality than that of other toxins such as those from C. botulinum, C. tetani, and Corynebacterium diphtheriae. If the K_D determined here for PA* is similar to that found in vivo, it could offer a reasonable explanation for this requirement. However, it is acknowledged that lethal dose is also dependent upon mode of action. For example, a neurotoxin such as that produced by C. botulinum will induce more life-threatening results than a toxin that causes diarrhea.

Based on available results, it appears that the binding of PA* is linear on the Scatchard plot suggesting a single receptor model. The fitted line for the Scatchard analysis (Fig. 16) had a correlation value (r) of -0.7. However, the possibility of a curvilinear relationship should not be overlooked which could indicate the existence of more than one receptor or the aggregation of PA* to already bound PA* as noted earlier. It needs to be considered that the affinity of PA* for MDBK cells may possibly be enhanced by such cofactors as divalent cations or perhaps even the presence of LF or EF. This would be a possibility for future investigation.

Other suggestions can be made for further evaluation of receptor criteria for PA*. Lin and Taniuchi (1980) reported use of chemicals which block receptor internalization and lysosomal processing but which still allow binding of cholera toxin. This approach might be of value

in assessing the degree to which internalization, if any, of PA* may have taken place. It was felt in this research that it was of greater value to conduct experiments at physiological temperatures in order to more closely simulate the *in vivo* situation. Although saturation studies performed at 4°C could have minimized possible internalization of PA*, the lower temperature may also have given misleading results in that the membrane fluidity of cells and hence the degree to which receptors may be displayed is greatly influenced by temperature (Sheeler and Bianchi, 1983).

Partial Characterization of Protective Antigen Receptor

It is well established that many toxins bind to gangliosides (Kitamura et al., 1980; Morris et al., 1980; Rogers and Snyder, 1981; Osborne et al., 1982; and Eidels et al., 1983). Cholera is one such toxin and its B subunit is available for purchase in purified form. It seemed a logical selection, therefore, for use in a blocking experiment against PA*. Cholera toxin subunit B was demonstrated to effectively block the binding of PA* to MDBK cells. The blocking was shown to be concentration dependent. This observation strongly suggests that at least one of the PA* receptors is the GM 1 ganglioside. It should not be discounted, however, that the cholera toxin-receptor complex may be located near the receptor for PA* and thereby stearically hinder the binding of PA*.

Trypsin treatment was shown to have no effect on the ability of the MDBK cells to bind to PA*. This would be expected if PA* receptors are gangliosides since gangliosides are not proteins. Cuatrecasas (1973) showed that trypsin had no effect on the binding of cholera toxin to liver cells. Yavin and Nathan (1986), however, demonstrated trypsin sensitivity in the binding of tetanus toxin to nerve cells, whose receptors are also gangliosides (Kitamura et al., 1980). Yavin and Nathan (1986) concluded that the trypsin sensitivity was due to the sensitivity of a protein closely associated with the ganglioside receptor and which may serve to contribute greater specificity to the receptor. The observation that trypsin treatment had no effect on PA* binding suggests that there is no protein associated with the receptor which is critical for PA* binding.

Practical Applications

The purification of PA beyond the 99% level enhances the capability of producing high affinity antibodies, both polyclonal and monoclonal.

Development of the assay system used in this research to quantitate the presence of PA* should prove useful in future research with the anthrax toxin components.

Any study which contributes to the understanding of the nature of bacterial toxins and how they exert their effects is of value. With understanding of how something

works comes the potential for making that thing useful. Great interest recently has stimulated work in the area of hybrid molecules for potential use in medicine or research (Colombatti et al., 1983; Blythman et al., 1981; Krolick et al., 1980; Moolten et al., 1982; Gilliland et al., 1980; Olsnes, 1981; and Roth and Maddux, 1983). A hybrid molecule might, for example, have the active subunit of a toxin coupled to an antibody against a cancer cell for use in cancer therapy. Obviously specificity, stability of the molecule, and the capability of delivering the toxic portion intracellularly are primary considerations. Protective antigen provides another potential binding subunit that, with modification, could deliver toxic molecules to specific target cells.

CONCLUSIONS

From the experimental data obtained in this research the following conclusions can be made:

1) Use of the Sterne strain of B. anthracis yields toxin comparable in lethality to that of the Vollum 1 B strain and minimizes the biological hazard associated with the culturing of B. anthracis

2) Higher yields of PA can be obtained by use of PEG to minimize nonspecific binding of the toxin to the filter during filtration. Use of PEG also minimizes contamination by serum proteins which were previously used to block nonspecific binding.

3) PA can be purified to levels > 99%.

4) The radioassay system which has been developed is suitable for PA* binding studies.

5) Binding of PA* to MDBK cells exhibits specificity, saturation, reversibility and moderate affinity. These results verify the presence of a specific receptor(s) for PA* on MDBK cells. Specificity was demonstrated by competition of unlabelled PA, cholera toxin subunit B, and the serum proteins in the 10% fetal bovine serum of the treatment medium. Saturation of the potential binding sites was shown by two methods: over time at a set concentration of PA* and by varying the concentration of PA* with a

set treatment time. Reversibility was demonstrated by the fact that about 25% of bound PA* was removable within 15 min of treatment with unlabelled PA. Affinity, although not as high as that reported for other toxins, was demonstrated for PA* with a K_D of 17×10^{-9} M.

6) It is likely that the receptor for PA* on MDBK cells is a ganglioside as indicated by cholera toxin subunit B blocking in conjunction with lack of trypsin sensitivity.

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Professional Experience:	Microbiologist, Dugway Proving Ground Half of time during cooperative education program with Utah State University Dugway, Utah (1979-1986)
	Assistant Instructor, Utah State University Logan, Utah (1984)
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