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RABBIT ERYTHROCYTE BAND 3:

A RECEPTOR FOR STAPHYLOCOCCAL ALPHA TOXIN

by

INDAR MAHARAJ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

WINDSOR, ONTARIO

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This work is dedicated to my parents, brothers
and sisters.

ABSTRACT

Enzymes known to specifically cleave the band 3 component of rabbit erythrocyte membrane were found to reduce both the hemolytic sensitivity to and the binding of alpha toxin of Staphylococcus aureus. Lectins which bind specifically to band 3 also inhibited the toxin. Lectins which do not bind specifically to band 3 have little or no effect. Purified band 3, conventionally isolated by affinity chromatography on a Concanavalin A column was homogeneous, had a molecular weight of 100,000 and inhibited the activity of alpha toxin. The preparation was itself nonhemolytic and did not induce aggregation of rabbit erythrocytes. Antibodies to the receptor and band 3 were indistinguishable indicating that band 3 and the receptor were serologically identical.

Flavin derivatives reduced activity of the toxin. Inhibition by these compounds were not due to their binding on to the erythrocyte membranes.

Anti-alpha toxin coupled to CNBr-agarose over a wide pH range, but the immunoabsorbent was most efficient when the antibody was coupled in the pH range 4.5-6.5. The toxin bound best to the insoluble antibody at slightly alkaline pH. A comparison of two eluents glycine-HCl and urea, indicated the glycine-HCl eluted more of the adsorbed toxin.

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LIST OF ABBREVIATION

AB-ab		Antibinding antibody
ATP		Adenosine Triphosphate
CACE		Con A Coated Erythrocytes
Con A		Concanavalin A
DBA		<u>Dolichos biflorous</u> Agglutinin
DIDS		4,4'-diisothiocyano -2,2'-Stilbene disulfonic acid.
FMN		Flavin Mononucleotide
LCA		<u>Lens. culinaris</u> Agglutinin
LGCE		Lysine Glutaraldehyde Coated Erythrocytes
PBS		Phosphate Buffered Saline
RBC		Red Blood Cell
RCA		<u>Ricinus communis</u> Agglutinin
TAME		Tosylarginine methylester
TLCK		N-p-Tosyl-L-Lysine Chloromethyl Ketone
TPCK		L-l-Tosylamide -2-Phenylethyl Chloromethyl Ketone'
TVA		<u>Triticum vulgaris</u> Agglutinin
UEA		<u>Ulex europeus</u> Agglutinin

INTRODUCTION.

Alpha toxin (alpha hemolysin) is one of several toxic proteins produced by Staphylococcus aureus (Arbuthnott, 1970). The toxin is injurious to several types of cells. These include erythrocytes, platelets and leucocytes (Bernheimer and Scharwtz 1963; Manohar, et al. ., 1967; Gladstone, 1966). Of all susceptible erythrocytes, rabbit erythrocytes are the most sensitive whereas human erythrocytes are the most resistant (Cooper, et al., 1966). Muscle cells are sensitive to alpha hemolysin and the toxin is known to induce spastic paralysis in smooth muscles (Cassidy, et al., 1974). Some cells in tissue culture are also very susceptible (Jeljazewicz, 1970). The toxin induces dermonecrosis when injected subcutaneously in rabbits and is lethal to all common laboratory animals injected intravenously in small doses (Wiseman, 1975).

In 1888, de Christmas demonstrated a toxic principle in culture-supernatant of staphylococci which induced inflammatory reactions in experimental animals (McCartney and Arbuthnott, 1978). Several years later, broth cultures of staphylococci were observed to be hemolytic to rabbit erythrocytes (Wiseman, 1975). Since that time, researchers have sought to delineate the role of alpha toxin in cytotoxicity and pathogenesis.

Regretably, relatively little is known about the mode of action of the toxin. Bernheimer (1976), lamented, "Its impossible to specify the nature of the functional lesion respons-

ible for the loss of the normal permeability properties of cells exposed to alpha toxin." He further speculated that ~~The~~ insensitivity of some cells relative to others may be due to the absence or difference in distribution of the membrane lipids and proteins. McCartney and Arbuthnott (1978), echoed a similar note recently. They stated that the specific receptor for alpha toxin has not been identified and that the intramembrane events following interactions of the toxin with the receptor have yet to be specified.

The concept of a receptor site (toxin binding site) is not novel. The idea was advanced several years ago (Marucci, 1963ab; Cooper, et al., 1964a; Madoff, et al., 1964). These authors have suggested the existence of a membrane component which is capable of interacting with the toxin before lysis is induced in the target cell. More recently the subject was investigated by three groups working independently (Barei and Fackrell, 1979; Cassidy and Harshman, 1976; Kato, et al., 1975; Kato and Naiki, 1976). Despite the efforts of these groups the nature of the receptor and therefore the mode of action of the toxin remain very elusive.

This investigation is essentially devoted to the isolation and identification of the receptor component to alpha toxin on the rabbit erythrocyte membrane.

LITERATURE REVIEW

Staphylococcus aureus releases four immunologically distinct hemolysins when grown in artificial media. These include alpha toxin, beta toxin, gamma toxin and delta toxin (Elek and Levy 1950; Fackrell and Wiseman, 1976). The possession of protein A and the elaboration of coagulases and alpha toxin have distinguished pathogenic Staphylococcus aureus from S. saprophyticus and S. epidermidis (Baird-Parker, 1974).

Physical Characteristics

The molecular weight of the toxin varies from 10,000 - 45,000 (Wiseman, 1975). The variations are due in part to the method of weight determination and method of purification. In addition, Dalen (1975b) has produced evidence indicating that degradation of the toxin by extracellular staphylococcal proteases is responsible for the variations observed.

The toxin is reported to exist in different forms. Bernheimer and Schwartz (1963) showed that purified alpha hemolysin when examined by analytical ultracentrifugation was found to consist of two components: a larger, slower moving peak having a sedimentation velocity of 3S, and a smaller, faster moving peak having a sedimentation velocity of about 12S. The 3S component was toxic, whereas the 12S component was not. A component with a sedimentation coefficient of 3.1S was also reported for purified alpha toxin (Lominski, et al., 1963).

The authors also noticed the presence of a heavy component (16S) of low hemolytic potency in their purified preparation and suggested it may be an inactive polymer.

At least three different forms of staphylococcal alpha toxin have been shown to exist: a soluble 3S active toxin, a soluble inactive 12-16S toxin and an insoluble aggregate (Arbuthnott et al., 1967). Aggregation of biologically active alpha toxin to an insoluble biologically inactive form could be induced by heating the toxin at 60°C or allowing the toxin to age. Coulter (1966) observed the formation of a non-hemolytic threadlike precipitate when the toxin was allowed to stand for three days at 0°C. Fackrell and Wiseman (1976) obtained a 1.4S hemolysin when freshly prepared; but several days standing increased the value to 3.0S. The aggregate however can be dissociated in 8 M urea to yield a product very similar, if not identical, to the native toxin (Bernheimer, 1968).

The different forms of the toxin are also reflected the heterogeneity of the pI values. Wadstrom (1968) demonstrated that alpha toxin could be separated into three components with different isoelectric points ranging from 6.5-9.2. The main component of pI 8.5 had 80% of the hemolytic activity. Refocusing of each of the components in the urea showed a similar banding pattern. From this it was inferred that the three components were identical but represented different forms of each other.

Alpha toxin from five strains of S. aureus, including Wood 46, were electrophoresed by Goode and Baldwin (1974). The isoelectric point of the five toxins was about 8.6. The authors observed that the toxin migrated as three peaks in the ultracentrifuge which indicated heterogeneity. But in contrast to Wadstrom, refocusing of each peak did not yield multiple forms. Mc Niven et al., (1972) found a main component of pI 8.5 which accounted for about 90% of the hemolytic activity, and three minor components with pI ranging from 5.0-9.2. A pI value of 8.5 for alpha toxin has been confirmed by other authors (Fackrell and Wiseman, 1976).

Chemical Properties

Purified alpha toxin is a protein. The amino acid composition has been studied by several groups (Bernheimer and Schwartz, 1963; Coulter, 1966; Six and Harshman, 1973ab; Fackrell and Wiseman, 1976). In general, these investigators all agree that the toxin contains no unusual amino acids. It appears enriched with respect to dicarboxylic amino acids but lacks cysteine residues. The lack of cysteine residues indicates the absence of disulfide bonds.

The toxin carries a high positive charge. Coulter (1966) suggested that the positive charge may be due to the presence of lysine, arginine and histidine residues. He also suggested that the amination of aspartic and glutamic acid residues may be a contributory factor.

N-terminal amino acid analyses have been reported but are at variance. Coulter's (1966) evidence indicated that the N-terminal amino acids were histidine and arginine: a fact suggesting the presence of two polypeptide chains. Six and Harshman (1973b) have reported alanine for the N-terminal and lysine for the C-terminal. None of the above findings has been confirmed by Wiseman and Caird (1970). They found the N-terminal was histidine. While the controversy of the N-terminal remains unresolved, it is generally agreed that the difference in N-terminal is due to proteolytic degradation of the toxin during preparation (Harshman, 1979; Dalen, 1976b; Wiseman, 1975).

Biological Effects

Alpha toxin has been described as the most potent of the several toxic products released by pathogenic staphylococci (Arbuthnott, 1970). Erythrocytes sensitive to the toxin show various degrees of susceptibility. The degree of sensitivity depends on the animal from which they are derived (Bernheimer, 1968) which in turn depends on the number of receptors per erythrocyte (Barei and Fackrell, 1979).

Not only are erythrocytes sensitive to the toxin, but platelets treated with the toxin show gross alteration of morphology and release substantial quantities of their contents (Bernheimer and Schwartz, 1965). The lethal effect of alpha toxin on blood platelets has been reported by Manohar

et al., (1967). Extensive damage to rabbit leucocytes by the toxin both in vivo and in vitro has been observed by several groups (Kumar and Lindorfer, 1962; Kumar et al., 1962 Szmigielski, et al., 1967).

Alpha toxin is lethal for all common laboratory animals when injected intravenously (Arbuthnott, 1970; Harshman, 1979). The mechanism of death is obscure. Although it has been shown that the toxin causes contraction of smooth muscles of blood vessels slowly and irreversibly (Wurzel et al., 1966; Cassidy et al., 1974), Wurzel has suggested that it is unlikely that the cause of death is due to generalized vasoconstriction since the toxin can also paralyse the striated muscles (Lominski et al., 1962; Cassidy and Harshman, 1979). In addition, the cytolytic effects of the toxin should be considered. Recent studies by Cassidy and Harshman (1979) have suggested that the diaphragm may be affected also, thus, inducing respiratory distress. Owing to the generalized effect of the toxin, the cause of lethality is difficult to identify. Subcutaneous injection of the toxin causes extensive necrosis. Thal and Egner (1961) suggested that necrosis is due to the action of the toxin on the smooth muscle of the blood vessels. This induces constriction of the vessels which is accompanied by ischemia. The resultant effect is the formation of a necrotic lesion.

Most cell lines tested are susceptible to the toxin.

These include Ehrlich ascites cells, KB cells, chicken embryo cells, Hela cells and rabbit, human, calf and monkey kidney cells (Wiseman, 1975). The effects of the toxin on these cell lines are diverse. KB cells treated with the toxin show a reduction in cellular dehydrogenase activity, glucose uptake and lactic acid production (Szmigieiski, et al., 1967).

Galanti et al., (1968), have observed that the toxin induces an instantaneous decrease in ATP content in cell cultures of rabbit kidney and chick embryo; while Arbuthnott and Symmington (1969) found that the toxin triggered an immediate increase in the rate of succinate oxidation in mouse ascites tumour cells. It was suggested that all cytopathic effects of the toxin may be due to the action of the toxin on the cell membranes resulting in the lysis of the cell (Madoff, et al., 1963).

Apart from animal cells, Bernheimer (1966) has observed that spheroplasts of certain species of bacteria including S. aureus and pleuropneumonia-like organisms (PPLO) are sensitive to alpha toxin.

Biosynthesis.

The mechanism regulating the biosynthesis of alpha toxin remains unresolved. Gladstone (1938), who obtained alpha toxin in a completely synthetic medium, found that arginine, proline and glycine stimulated toxin production but not histidine.

Dalen (1973ab) showed that the addition of histidine, serine and glycine enhanced production of the toxin, but that arginine and proline were of no consequence. Since the effect of histidine in inducing toxin production was immediate while that of serine and glycine was delayed, Dalen suggested that histidine might be an inducer of early toxin synthesis, while the stimulating effect of serine and glycine might be related to their role as histidine precursors. The stimulating effect of histidinol is also due to its function as the immediate precursor of histidine. Dalen (1973c) showed that the production of toxin declined significantly when Wood 46 cultures were incubated under conditions favouring optimum activity of histidinase.

The beneficial effect of CO₂ was investigated by Burnet (1930). He found that the aureus variant of S. aureus incubated in broth for 3-5 days produced appreciable quantities of hemolysin in an atmosphere containing 10-20% CO₂. In contrast, toxin production by the albus variant of S. aureus was inhibited by CO₂. Cultures incubated in as much as 50% CO₂ have given satisfactory yields (Manohar et al., 1966).

Studies currently in progress in this laboratory have shown that incubation of our working cultures of S. aureus strain Wood 46 with 10% CO₂ does enhance the toxin titres by 32 fold. However, under the same conditions, the same concentration of CO₂ was found to inhibit toxin production on

S. aureus strain Wood 46 (10832) obtained from ATCC (Lo, Chamney and Fackrell, unpublished observations).

Dalen (1973ab) has suggested that the effect of CO₂ in increasing toxin yield is related to its role as a precursor of histidine, but the exact mechanism has not been elucidated. Claims of CO₂ stimulating toxin yields by its buffering capacity have been refuted. Control of pH by other means have not produced the same results (Wiseman, 1975). Thus the puzzle of CO₂ in enhancing toxin production remains unsolved.

Although the factors which influence the production of toxin have not been clarified, arbitrary methods have been devised which yield high titres of purified toxin.

Mode of Action

Despite the devastating toxic effect of the hemolysin its mode of action has not been elucidated. In an attempt to understand the mechanism, the kinetics of the interaction of alpha toxin and rabbit erythrocytes has been studied by several groups (Marucci, 1963ab; Madoff, et al., 1964; Lominski and Arbuthnott, 1962; Cassidy and Harshman, 1976; Barei and Fackrell, 1979). The kinetics of the toxin-erythrocyte interaction has been characterized by the production of a sigmoidal curve. Marucci has divided the curve arbitrarily into three regions to describe the activity of the toxin: a

lag period, a period of accelerated lysis and a terminal period of creeping lysis. He postulated that at least two sequential steps are necessary for the inducement of lysis.

The first step, corresponding to the lag period, requires the interaction of the toxin with the erythrocytes. The second step, corresponding to the period of rapid lysis, which leads to the release of hemoglobin is an intrinsic reaction of the damaged cell and is not dependent on the activity of the toxin. The first step is reversible by anti-toxin but not the second. Cells that have proceeded to the second step have already been damaged and are doomed to progress to lysis. Such cells are said to be committed.

Lominski and Arbuthnott (1962), also recognized three sequential phases similar to those of Marucci: a lag phase during which little or no hemolysis occurs, followed by a period of rapid hemolysis and a final phase during which the rate of the reaction decreases. The kinetic studies of Madoff et al., (1964) and those of Cassidy and Harshman (1976b) are in agreement with those presented above. In addition, both groups have demonstrated that the toxin causes a rapid release of potassium ions followed by the release of hemoglobin from the damaged erythrocytes. Cassidy and Harshman have dissected the kinetics of the toxin-erythrocyte interaction into three sequential events: 1) binding of free toxin 2) an induction of

foci of membrane injury leading to the release of potassium and 3) the eventual colloidal osmotic lysis of the cells as revealed by the leakage of hemoglobin. It should be noted that events 1 and 2 postulated by Cassidy and Harshman are comparable to the lag or prelytic phase of the other authors.

Several proposals have been put forward concerning the interaction of alpha toxin with erythrocyte membranes and its subsequent induction of hemolysis. It has been postulated the native toxin is released as an inactive protease (pro-toxin) which became activated by membrane bound proteolytic enzymes. It is the activated form of the toxin which interacts with membrane proteins thereby inducing lysis (Wiseman and Caird, 1970; Wiseman, 1975; Wiseman et al., 1975). The evidence adduced in support of this theory is as follows: (1) the interaction of the toxin with erythrocyte and erythrocyte ghosts, releases quantitative amounts of material containing nitrogen which absorbs at 280 nm and reacts with Folin phenol reagent, (2) a decrease in nitrogen content was observed in ghosts exposed to the toxin, (3) inactivated toxin (protoxin) was unable to hydrolyse tosylarginine methylester (TAME), whereas toxin activated by incubation with trypsin coupled to carboxymethylcellulose not only hydrolysed the substrate (TAME) but showed a greater affinity for the substrate. This increased affinity was translated into an increase in the specific activity of the toxin.

Freer et al., (1973), could not substantiate the evidence of a protease activation mechanism for alpha toxin. Instead, they have proposed that disruption of susceptible biomembranes is due to the surface activity of the toxin: that is, disruption may be explained solely by direct interaction of the toxin with membrane lipids. Of interest is the fact that this proposal is based on the study of the interaction of the toxin with artificial membranes. A similar concept was introduced by Weissmann et al., (1966). Their data indicated that alpha toxin initiated the release of chromate anions and glucose from artificial phospholipid vesicles. Buckelew and Colacicco (1971) also found that alpha toxin disrupts lipid monolayers and natural membranes, from which they concluded that the surface activity of the toxin played a significant part in its action upon membranes. McCartney and Arbuthnott (1978) recently cautioned that the surface activity theory may not fully explain the hemolytic action of the toxin.

Although the findings that alpha toxin disrupts liposomes have been confirmed by Cassidy et al., (1974), they have questioned the validity of applying information derived from artificial membranes to that of natural membranes (Cassidy and Harshman, 1976). It has been clearly established that erythrocytes of different species vary in their sensitivity to the toxin. However, no variation in sensitivity was found in liposomes derived from membrane lipid extracted from erythrocytes of different animal species. These studies have

no doubt undermined the strength of the proposal made by Freer et al., (1973) and thus the force of the surface activity theory is considerably weakened.

The existence of a specific membrane component capable of binding the toxin has been suggested by several authors (Marucci, 1963a; Wiseman and Caird, 1972; Klainer, et al., 1964; Cooper et al., 1964ab; Klainer et al., 1972). Klainer, et al., (1964) demonstrated the presence of the toxin on the cell surface and suggested that the initial toxin-erythrocyte interaction is probably a surface phenomenon. A specific glycoprotein in the erythrocyte membrane was postulated as the binding site for the toxin by Kato et al., (1975). For unexplained reasons, the same group later postulated that a Glc-Nac ganglioside was the receptor for alpha toxin (Kato and Naiki, 1976). This observation has not been confirmed (McCartney and Arbuthnott, 1978). Cassidy and Harshman (1973, 1976b) after probing the erythrocyte membranes of several different species concluded that the primary binding site of alpha toxin on biomembranes is a surface membrane protein. They proposed the existence of a specific receptor substance for alpha toxin in membranes. The number of receptor sites (substances) for alpha toxin on rabbit erythrocytes is about 5,000 while no receptor sites were found on human erythrocytes (Cassidy and Harshman, 1976). The authors showed that Pronase[®]

treatment of erythrocytes resulted in a decreased number of binding sites. Barei and Fackrell (1979), on the other hand estimated 125,000 receptor sites on rabbit erythrocytes but none on human erythrocytes. A number of 37,000 receptors were postulated to represent a threshold below which hemolytic sensitivity is not observed. A correlation was established between receptor number of erythrocytes derived from different animal species and sensitivity to the toxin.

MATERIALS AND METHODS

BIOLOGICAL METHODS

1. Cultures.

Staphylococcus aureus strain Wood 46 (Burnet, 1929) met the criteria for S. aureus outlined by Baird-Parker (1974). Stock cultures were preserved by lyophilization in a sterile skimmed milk medium. Working cultures were sub-cultured once a month on 1.5% (w/v) agar slants supplemented with Dolman-Wilson (DW) medium (Dolman and Wilson, 1940) and incubated at 37°C for 24 h. These were then stored at 4°C. Checks for purity were carried out regularly.

2. Toxin Production and Purification.

i) Production.

Alpha toxin was produced by the method of Wiseman et al., (1975). Essentially, this method involved inoculating one litre sterile flasks containing 500 mL of DW medium and incubating them in a shaker-incubator at 37°C, under 10% CO₂ for about 36 to 48 h. The organisms were pelleted by centrifugation and the supernatant recovered.

ii) Purification.

The toxin was purified by a modification of the method of Wiseman et al., (1975). Crude hemolysin was precipitated by treating the culture supernatant with 35% cold methanol (-20°C) at pH 4. The precipitate was centrifuged (12,000g, 15 min) and the pellet homogenized in phosphate buffered saline (PBS).

The homogenate was fractionated with 40% (v/v) saturated ammonium sulfate and centrifuged (12,000g, 15 min). The pellet formed was discarded and the supernatant fractionated with 60% (v/v) saturated ammonium sulfate. The resulting precipitate (purified toxin) was redissolved by over night dialysis against several changes of PBS, and then clarified by centrifugation at 30,000g for 1 h.

The alternative method of toxin production by Dalen (1976c) was generally used because it was rapid and simple and considerable purification was achieved. The technique involved heating the culture supernatant (60°C) until a flocculent nonhemolytic suspension was observed. The material was centrifuged (20,000g, 30 min.), and the pellet was washed twice with borate buffer (0.03 M, pH 8.6). The pelleted inactive toxin was reactivated and solubilized by homogenization in the same buffer with 8 M urea. Undissolved material was removed by centrifugation (20,000g, 1 h). The toxin preparation was stored in urea to maintain its stability and diluted prior to use.

3. Toxoid Production.

Toxoid was produced by heat inactivating purified toxin for 30 min. at 60°C (Arbuthnott et al., 1973).

4. Erythrocyte Preparation.

Rabbit red blood cells were the only type of erythrocytes used in this study. Erythrocytes were obtained by puncturing

the marginal ear vein of the animal. To prevent coagulation the blood was collected in heparinized tubes or defibrinated by shaking with glass beads. The blood was then filtered through several layers of cheese cloth, centrifuged (600g, 5 min) and the buffy coat removed. After three washes in PBS the packed cells were adjusted to a 2% (v/v) suspension in the same buffer. It was stored at 4°C when not in use.

5. Erythrocyte Ghost and Band 3 Preparation.

Erythrocyte ghosts were prepared generally according to the method of Dodge et al., (1963), except that the first two washes in milliosmolar buffer were done at 12,000g for 15 min. ~~The buffer~~ included 1mM ethylenediaminetetra-acetic acid (EDTA). Treatment of erythrocyte membranes with EDTA releases into solution bands 1,2 and 5 (Fairbanks et al., 1971), leaving the rest of the membrane proteins intact (Findlay, 1974). The ghosts were solubilized with 0.5% (v/v) Triton X-100 which selectively releases bands 3,4,2,6 and the bands stainable by Periodic Acid Schiff (PAS): namely, PAS 1 and PAS 2. These bands are all released in a lipid-free form (Steck, 1974). The solubilized membranes were centrifuged (20,000g, 40 min) at 4°C. The supernatant was retained and the Triton X-100 eliminated by adsorption onto Amberlite XAD-2 beads (Sigma) according to the method of Cheetam (1979).

The solubilized material, free of Triton X-100, was used as a source of crude receptor.

Purified band 3 obtained by passing crude receptor through a Concanavalin A (Con A) affinity column. The specifically bound material was eluted with phosphate buffer (0.2 M, pH 7.2) supplemented with 0.1 M NaCl, 10 mM MgCl and 0.1 M α -methyl-D-mannopyranoside (see section on Con A affinity chromatography).

6. Hemolytic Titration (HU_{50}).

Hemolytic titrations were performed in Microtitre[®] plates (Cooke Lab. Products) with U-shaped wells in accordance with the method of Lo and Fackrell (1979). In brief, this method entailed serially diluting 50 μ L of toxin in 50 μ L of PBS. One hundred μ L of 2% erythrocytes was then added to each dilution of toxin. The mixture was brought up to 200 μ L with PBS and allowed to incubate for 1 h at 37°C. The unlysed cells were separated from the lysed cells by centrifugation (600g, 5 min). The supernatant was diluted 1:3 with PBS and the hemoglobin content determined spectrophotometrically (541 nm, 1 cm light path). The series of wells containing the 50% end point were plotted against the $\log_2$ of the toxin dilution. One hemolytic unit (HU_{50}) was therefore defined as the reciprocal of that toxin dilution required to give 50% hemolysis.

7. Kinetic Hemolytic Titration.

The kinetic hemolytic assay has the distinct advantage of detecting and amplifying small changes in the sensitivity of the erythrocytes during lysis. Such changes would be reflected as differences in the hemolytic profiles of the different systems. These changes may otherwise be difficult to detect if the system is allowed to reach equilibrium as is the case with the HU₅₀ system. The assay was therefore used as a confirmatory test, independent of the HU₅₀ system. It involved adjusting 50 uL of diluted erythrocytes to an absorbance of about 1.5 (650 nm, 1 cm light path) in a final reaction volume of 300 uL. The system invariably included 50 uL of toxin and PBS or the test material. The extent of hemolytic activity was monitored by a decrease in absorbance over a period of about 15 minutes.

8. Adsorbent Preparation.

i) Lysine Glutaraldehyde Coated Erythrocytes (LGCE)

LGCE was prepared by the method of Lo and Fackrell (1979). Erythrocytes were coated with toxoid. The bound toxoid molecules were covalently linked to the erythrocyte membrane with glutaraldehyde. Free aldehyde groups were subsequently neutralized with lysine.

ii) Concanavalin A Coated Erythrocytes (CACE).

Twenty mL of 2% erythrocytes were incubated with Concanavalin A (Con A) (250 ug/mL) for 20 min. at RT. The cells were washed several times with PBS to eliminate all unbound

Con A.

9. Antisera Production.

i) Anti-Band 3.

Fifty white rats were cared for in accordance with the principles of Care of Experimental Animals - A Guide For Canada. When the rats were three months old, they were bled by cardiac puncture for preimmune sera. The sera were pooled. Each animal was then injected intraperitoneally with 0.5 mL of a 2% (v/v) suspension of ghosts in PBS. One week later the injections were repeated. On the third week, the animals were bled by cardiac puncture and the antisera pooled. The pooled antiserum was split into two 25 mL fractions. One fraction was allowed to incubate with 0.2 mL of packed CACE for 15 minutes at RT. The mixture was then checked for any changes in antibody titer which was monitored by a hemagglutination assay. The adsorption procedure was repeated until no further change in hemagglutination titre was observed. Thus all populations of antibodies directed to antigenic determinants on the membrane, except those blocked by Con A, would be adsorbed. The unadsorbed antibody population would therefore represent anti-band 3, since band 3 on the erythrocyte membrane is the receptor for Con A. (Findlay, 1974; Sharom et al., 1977). This anti-band 3 preparation was purified to IgG and concentrated according to the method of Campbell et al., (1970).

ii) Anti Receptor.

The anti-receptor method is based on the assumption that

alpha toxoid like the toxin binds to and blocks the receptor site.

The other 25 mL of antisera was adsorbed with 1 mL of packed LGCE until no decrease, as judged by hemagglutination assay, in antibody titre was observed by further adsorptions. Based on the assumption made above, all populations of antibodies directed to antigenic determinants on the membrane would be adsorbed leaving anti-receptor antibodies in the supernatant. The anti-receptor antibodies were subsequently purified to IgG and concentrated as described above.

iii) Anti-alpha toxin.

Purified alpha toxin, obtained by the method of Bock et al., (1976), was converted to toxoid and used in the production of antitoxin as described by Lo (1979).

10. Hemagglutination Assay.

These tests were carried out in micro-titre plates. A serial dilution of 50 uL of antisera in 50 uL of PBS was prepared. To these dilutions, 50 uL of erythrocytes (1%, v/v) were added. The reaction mixture was allowed to incubate for 1 h. at RT. The plates were read visually for agglutination and the titre expressed as the reciprocal of the antisera dilution in the last well showing agglutination. This test has been standardized (Lo, 1979).

11. Enzyme Digestion of Erythrocytes.

Erythrocytes (4%, v/v) in PBS were incubated with Pronase

(0.1 mg/mL) (Calbiochem) at room temperature for 7 h. with periodic shaking. Samples were removed at fixed time intervals, washed twice in PBS to get rid of residual Pronase, adjusted to a 2% suspension and titrated against the toxin to determine any change in sensitivity. The hemolytic titres of control erythrocytes processed similarly but without the enzyme compared to those of treated cells were expressed as % inhibition.

Several other enzymes were tested for their effect on the sensitivity of the erythrocytes to alpha toxin. These included lipase, β -galactosidase, β -glucosidase and sialidase (Sigma). Two proteases, trypsin and α -chymotrypsin, were tested similarly. However, the trypsin was pretreated with L-1-tosylamide -2- phenylethyl chloromethyl ketone (TPCK) to eliminate traces of chymotrypsin activity; whereas α -chymotrypsin was pretreated with N-a-p-tosyl -L-lysine chloromethyl ketone (TLCK) to eliminate traces of trypsin activity. Erythrocytes were pre-incubated with the above enzymes for varying periods of time. Changes in susceptibility of the erythrocytes due to exposure to the enzymes were assessed by comparison to control erythrocytes.

PHYSICAL AND CHEMICAL METHODS

1. Buffer Preparation.

Sodium phosphate buffer, 0.01 M, pH 7.2, was prepared according to the principles of Gomori (1955), and supplemented with 0.85% (w/v) NaCl. This was referred to as phosphate buffered saline (PBS).

2. Immunoaffinity Chromatography.

Sepharose 4B[®] (Pharmacia) was activated with CNBr (Sigma) at a concentration of 0.3 mg/mL and immediately coupled to anti-alpha antibody (5 mg/mL) according to the principles outlined by Cuatrecasas (1970). The coupled Sepharose[®] (Agarose) was packed in a 10 mL column and equilibrated with adsorption buffer that was used for binding the toxin onto the column.

Crude toxin (0.9 mg) was added to the column and after overnight incubation at 4°C to ensure maximum adsorption, the column was flushed with more adsorption buffer to remove non-specific protein. Adsorbed toxin was removed by elution with glycine-HCl buffer (0.2 M, pH 2). Two mL fractions were collected and immediately neutralized with 50 uL of 0.2 M NaOH. The protein concentration was estimated spectrophotometrically ($E_{280}^{1\%} = 13.5$) (Fackrell and Wiseman, 1976).

3. Con A Affinity Chromatography.

Thirty mg of Con A was dissolved in 10 mL of citrate buffer (0.2 M, pH 6.5) and coupled onto 15 mL of sepharose which had been activated by 0.3 mg/mL of CNBr in accordance with the principles of Cuatrecasas (1970). The Con A-sepharose

RESULTS

derivative was packed in a column (15 X 1 cm) and washed with several volumes of phosphate buffer (0.02 M, pH 7.2) containing 0.1 M NaCl, 10 mM MgCl₂. This was referred to as the equilibration or wash buffer. The eluent buffer, which was used to desorb specifically bound material, consisted of the same buffer supplemented with 0.1 M α -methyl-D-mannopyranoside. The column was run at 4°C. To obtain pure band 3, crude receptor was applied to the column in excess. The unbound material was flushed out with several volumes of equilibration buffer. The bound protein was removed by desorption with eluent buffer and collected at a flow rate of 1 mL per hour. The eluted material was dialysed against several volumes of PBS overnight at 4°C, frozen and concentrated by lyophilization.

4. Protein Determination.

Protein concentration was determined by either the method of Lowry et al., (1951) or Bradford (1976) with bovine serum albumin (BSA) as standard. The protein concentration for alpha toxin was determined spectrophotometrically using an extinction coefficient of 13.5 (1 cm light path).

5. Molecular Weight Determination.

Molecular weight determination was performed according to the method of Laemmli (1970). Determinations were carried out on 7.5% slab gels supplemented with 1% sodium dodecyl

sulfate (SDS). The standards were BSA (MW 66,000), α -amylase (MW 48,000), carboxypeptidase A (MW 34,000), trypsin (MW 24,000), β -lactalbumin (MW 18,400) and lysozyme (MW 14,300). All standards were obtained from Sigma. Fifty μ L (1mg/mL) of each standard and 150 μ L (0.3 mg/mL) of band 3 preparation were applied. The gels were run at RT for about 2.5 h (120 mV, 25 mA). The gels were then stained overnight in Coomassie Brilliant Blue R (0.25%, w/v) and were subsequently destained in a solution of 5% (v/v) acetic acid and 7% (v/v) methanol.

CHAPTER 1

EFFECT OF ENZYMES ON THE HEMOLYTIC SENSITIVITY OF ERYTHROCYTES

Previous reports (Kato et al., 1975; Cassidy and Harshman, 1976b) have agreed that rabbit erythrocytes treated with Pronase increased their resistance to alpha toxin. This event was accompanied by a corresponding decrease in binding of the toxin onto the erythrocyte membrane. The authors, however, disagreed on the effect of trypsin. Kato et al., (1975) claimed that in trypsinized erythrocytes, sensitivity was reduced by about 80%. Cassidy and Harshman (1976) were unable to substantiate this claim. They found no change in cells exposed to trypsin. Since enzymes have proved useful tools for investigating the structure and function of membranes the need arose to reproduce some of these critical enzyme experiments.

Erythrocytes were incubated with Pronase for 7 h at RT. They were washed with PBS twice to get rid of the enzyme. With time, cells treated with Pronase increased their resistance to hemolysis by the toxin. The hemolytic titre was reduced from 140 HU to 40 HU. A concomitant decrease in binding of fluorescently labelled toxoid on the erythrocyte membrane was observed (Fig. 1). Overnight digestion proved futile as the cells became extremely fragile and most lysed spontaneously during the washing procedure. Consequently Pronase digestion was limited to 7 h.

Fig.1

Effect of Pronase on Erythrocyte Sensitivity.

- Reduction in hemolytic titre after exposure to the enzyme.
- O-O Binding of fluorescent toxoid is decreased after the cells have been treated with the enzyme.

The data suggested that the enzyme was most probably degrading the binding site of the toxin. As a result of the activity of Pronase, both binding of the toxin and hemolytic sensitivity of the erythrocytes were substantially reduced. Since band 3 and glycophorin which is also known as sialoglycoprotein, major glycoprotein or PAS 1 and 2, are the only polypeptides on the erythrocyte surface sensitive to the enzyme (Bender et al., 1971) these polypeptides became targets of close scrutiny as possible candidates for receptor function. The next step involved selective enzymatic cleavage of the above polypeptides and observing for alteration in sensitivity of the cells to the lysin. Trypsin was selected for this purpose.

Erythrocytes were split into two portions. To one, TPCK-trypsin (0.2 mg/mL) was added; to the other, used as a control, PBS was added. These were allowed to stand for 6 h at RT, with periodic shaking. Two other fractions were treated similarly and allowed to incubate overnight. After the cells were washed twice with PBS, they were tested for changes in hemolytic sensitivity both by kinetic and HU assays. The kinetic assay measures the rate of lysis whereas the HU test reflects the final equilibrium of the lytic reaction. The data (Fig. 2) indicated no change in sensitivity in cells treated with the enzyme for 6 h. Similarly, cells exposed to the enzyme overnight (at least 15 h) revealed no difference

Fig. 2

Effect of Trypsin on the Hemolytic Sensitivity of Erythrocytes.

No difference in sensitivity is observed between cells exposed to the enzyme and control erythrocytes.

▼-▼ Cells treated with trypsin.

O-O Control erythrocytes.

in sensitivity to the toxin as judged by kinetic and HU assays.

Significant is the fact that trypsin selectively cleaves glycophorin and not the band 3 polypeptide (Bretscher, 1973; Cabantchik and Rothstein, 1974b; Steck et al., 1976). The data not only suggested that glycophorin is not involved in the binding of alpha toxin but also suggested that band 3 had a receptor function.

Since α -chymotrypsin selectively degrades band 3 (Steck et al., 1976; Marchesi et al., 1976) it became crucial to determine its effect on the hemolytic sensitivity of the erythrocytes. Overnight exposure to TLCK- α -chymotrypsin (0.1 mg/mL) reduced the hemolytic titre from 180 to 130 HU (Fig. 3). The increased resistance of the erythrocytes was also confirmed by kinetic assay (Fig. 4). When the concentration of the enzyme was doubled (0.2 mg/mL), for the same time period, there seemed to be a corresponding increase in the resistance of the cells to the toxin. The hemolytic activity was reduced from 101 to 50 HU: a decrease of about 50%. This finding supported suspicions of band 3 being the receptor.

To test the effect of membrane sugar residues on the binding and hemolytic activity of the toxin several glycosidases and sialidase were used.

Erythrocytes were incubated with sialidase (0.2 units/mL) overnight at RT and washed with PBS. Erythrocytes treated with sialidase increased their resistance to the hemolytic activity of the toxin (Fig. 5). Fifty seven percent inhibition

Fig. 3

Effect of α -chymotrypsin on the hemolytic sensitivity of erythrocytes.

●-● Control: untreated erythrocytes.

O-O Erythrocytes exposed to α -chymotrypsin overnight.

Fig. 4

Kinetic hemolytic assay showing the effect of α -chymotrypsin on the hemolytic sensitivity of erythrocytes.

O-O Erythrocyte control: erythrocytes and PBS.

●-● Toxin control: erythrocytes, toxin and PBS.

▼-▼ Erythrocytes exposed to the enzyme plus toxin and PBS.

Fig. 5

The effect of sialidase on the hemolytic sensitivity of erythrocytes.

O-O Control: Untreated cells and toxin.

▼-▼ Cells treated with sialidase and challenged with toxin.

was observed. This inhibition was confirmed by kinetic assay (Fig. 6). The kinetic profile of the cells treated with sialidase is unusual. Although the change in the slope of the curve during the lytic phase is very similar to the control a definite divergence is observed during the phase of creeping lysis. The significance of the difference, however, in the shape of the curve is not readily apparent, but it should be noted the unusual divergence observed would have been in an HU assay. The finding implies that sialic acid residues in situ contribute to the hemolytic activity of the toxin. The loss of the negatively charged sialic acid residues due to the activity of the enzyme may have reduced the net negative charge on the erythrocytes thus interfering with the interaction of the erythrocytes and the positively charged toxin. This decreased interaction would result in decreased binding of the toxin and therefore reduced hemolysis is observed.

Erythrocytes treated with β -galactosidase (0.2 mg/mL) showed no change in hemolytic sensitivity. Fig. 7 shows that no deviation occurred between those cells exposed to the enzyme for 4 h and the untreated cells. Overnight digestion produced no discernible changes in the hemolytic patterns as assessed by HU (Fig. 8) and kinetic assay (Fig. 9). Initially, these results would imply that galactose residues are of little significance to the lytic function of the toxin.

Fig. 6

Kinetic assay on the effect of sialidase on the sensitivity of erythrocytes.

O-O Erythrocyte control: erythrocytes and PBS.

●-● Toxin control: untreated erythrocytes, toxin and PBS.

▼-▼ Erythrocytes exposed to sialidase plus toxin and PBS.

Fig. 7

The effect of β -galactosidase on erythrocyte sensitivity to alpha toxin.

▼-▼ Control erythrocytes: untreated erythrocytes and toxin.

O-O Erythrocytes treated with the enzyme and challenged with toxin.

Fig. 8

The effect of β -galactosidase on the hemolytic sensitivity of erythrocytes after overnight digestion.

O-O Erythrocyte control: untreated erythrocytes and toxin.

●-● Erythrocytes treated overnight with the enzyme and challenged with toxin.

Fig. 9

Kinetic assay on the hemolytic sensitivity of erythrocytes after overnight digestion with β -galactosidase.

O-O Erythrocyte Control: erythrocytes and PBS.

●-● Toxin Control: untreated erythrocytes, toxin and PBS.

V-V Erythrocytes treated with enzyme plus toxin and PBS.

But this may not necessarily be so, as the galactose residues may be protected in situ. β -galactosidase hydrolyses the $\beta(1-4)$ linkages of lactose to release glucose and galactose. Whether these components are directly linked by $\beta(1-4)$ bonds on band 3 is not known. Only trace amounts of glucose (Marchesi et al., 1976) are found on band 3. This would suggest the existence of few, if any, susceptible $\beta(1-4)$ linkages; the hydrolysis of which, if significant to the hemolytic activity of the toxin, may not be readily be detectable.

Treatment of erythrocytes with glucosidase (0.2 mg/mL) varied from 5 h to overnight. The cells were then washed twice with PBS and assayed for changes in hemolytic sensitivity. Fig. 10 shows no change in hemolytic sensitivity in the cells exposed to the enzyme for 5 h. Extended periods of digestion (at least 15 h) did not alter the hemolytic titre of the toxin (Fig. 11). Kinetic assay on cells digested overnight (Fig. 12) confirmed no irregularity in the hemolytic profile of these cells. β -glucosidase hydrolyses the $\beta(1-4)$ linkages joining glucose units found on band 3. Whether these residues are linked together by $\beta(1-4)$ links is unknown. The data may not necessarily reflect the importance of the glucose residues to the hemolytic activity of the toxin.

Lipase (0.2 mg/mL) digestion of erythrocytes for 3 h revealed no detectable difference between the cells treated

Fig. 10

The effect of β -glucosidase on hemolytic sensitivity of erythrocytes after 5 h. of digestion.

O-O Erythrocyte Control: untreated erythrocytes and toxin.

●-● Erythrocytes treated with enzyme and challenged with toxin.

Fig. 11

The effect of overnight digestion by β -glucosidase on the hemolytic sensitivity of erythrocytes.

O-O Erythrocyte Control: untreated erythrocytes and toxin.

●-● Erythrocytes treated with the enzyme plus toxin.

Fig. 12

Kinetic hemolytic assay on the sensitivity of cells exposed to β -glucosidase overnight.

O-O Erythrocyte Control: Erythrocyte and PBS.

●-● Toxin Control: Untreated cells, toxin and PBS.

V-V Cells exposed to the enzyme plus toxin and PBS.

with lipase and cells not exposed to the enzyme. Fig 13 shows a hemolytic titre of about 1024 HU for both treated and control erythrocytes. A profile on the kinetics (Fig. 14) of the toxin-erythrocyte interaction indicated that lipase has no effect on the receptor component.

Overnight digestion with lipase was attempted. However prolonged digestion rendered the erythrocytes too fragile to wash. The data suggest that the binding site may not be lipid in nature.

In summary, it was observed that those proteases capable of degrading band 3, namely Pronase and α -chymotrypsin, were also capable of reducing the hemolytic sensitivity of the erythrocytes. On the other hand, those which do not, have no effect on the erythrocyte sensitivity. Sialidase treatment increased the resistance of the cells; while glycosidases and lipase were found to have no effect.

This information led to the hypothesis that the transmembrane protein band 3, is the specific receptor for alpha toxin. To test this hypothesis lectins of known specificities were tested to determine their effect on the activity of the toxin. The rationale is that compounds which bind onto band 3 should, but not necessarily, inhibit binding of the toxin molecules; those which do not bind should not inhibit.

Fig. 13

The effect of lipase on the hemolytic sensitivity of erythrocytes after 3 h. of digestion.

O-O Erythrocyte Control: undigested erythrocytes and toxin.

V-V Cells treated with the enzyme and toxin.

Fig. 14

Kinetic assay on the hemolytic sensitivity of erythrocytes after treatment with lipase for 3 h.

O-O Erythrocyte Control: erythrocytes and PBS.

●-● Toxin Control: untreated erythrocytes, toxin and PBS.

V-V Erythrocytes treated with the enzyme plus toxin and PBS.

CHAPTER 2

THE RECEPTOR AND BAND 3 ARE IDENTICAL

To determine the effect of lectins with known binding specificities on the toxin, several concentrations of each lectin were preincubated with erythrocytes (2%) for 10 minutes at RT. Each mixture was challenged with various dilutions of toxin in a chess-board type titration. The hemolytic titres obtained were compared to control erythrocytes treated similarly but without the lectin.

Con A binds specifically to band 3 (Nicholson, 1976; Marchesi et al., 1976). Preincubation of the erythrocytes with Con A was found to severely restrict the activity of the toxin. Fig 15 indicates that, at a concentration of Con A of 64 ug/mL, the lytic activity of the toxin was almost abolished. The lytic function of the toxin, however increased as the Con A concentration decreased. No significant inhibition was observed at less than 2 ug/mL, thus suggesting that the protection of the erythrocytes afforded by the lectin was a specific event. The data further indicate that the binding of Con A to band 3 renders the erythrocytes resistant to the toxin.

Lens culinaris agglutinin (LCA) like Con A is specific for the sugar α -D-mannose (Sharon and Lis, 1972) and binds specifically to band 3. However, some binding of this agglutinin onto glycophorin has been reported (Findlay, 1974). Fig 16 indicates that as the concentration of this lectin is increased its inhibitory effect on the toxin is correspondingly increased.

Fig. 15

Inhibition of the hemolytic activity of alpha toxin by
Concanavalin A. M.W. 55,000.

Fig. 16

Inhibition of alpha toxin by Lens culinaris agglutinin.

M.W. 42,000.

The relationship between the concentration of LCA and % inhibition appears to be linear up to about 64 ug/mL; beyond this point the inhibition plateaus.

Ricinus communis agglutinin type I (RCA I) is specific for the sugar D-galactose. Receptors of this lectin are located on the band 3 component (Adair and Kornfield, 1974). When RCA I was preincubated with erythrocytes and subsequently challenged with toxin, severe inhibition of the toxin was observed: Fig. 17 indicates better than 80% inhibition. Beyond 4 ug/mL toxin inhibition appeared to be directly proportional to lectin concentration.

Similarly, when erythrocytes were preincubated with the Ricinus communis agglutinin type II (RCA II) and subsequently challenged with toxin a dramatic decrease in titre of the toxin was observed. At about 128 ug/mL the hemolytic titre was reduced by about 80% (Fig. 18). The protection afforded by RCA II, compares favourably with RCA I. It is interesting to note that RCA II also has receptors on band 3 (Adair and Kornfield, 1974), but unlike RCA I the specific inhibitor of this lectin is N-acetylgalactosamine.

The protective profiles described by both lectins, RCA I and RCA II, are different at the concentrations tested. Whether this difference reflects different mechanisms of protection, or whether the observation is significant is

Fig. 17

Inhibition of alpha toxin by Ricinus communis agglutinin
type I.M.W. 120,000.

Fig. 18

Inhibition of alpha toxin by Ricinus communis agglutinin
type II. M.W. 60,000.

uncertain. It should be noted however that RCA II also binds to glycophorin (Wong and Roses, 1979).

Since those lectins having a specific affinity for band 3 were capable of neutralizing the effect of the toxin, it could be predicted that lectins lacking this specificity would have no drastic restrictions on the hemolytic activity of the toxin. Such anticipation would be consistent with the hypothesis that band 3 is the receptor protein for alpha toxin. Triticum vulgaris agglutinin (TVA) also called wheat germ agglutinin binds specifically and predominantly to glycophorin (Kahane et al., 1976; Marchesi et al., 1976); however binding of this lectin to band 3 has also been reported (Findlay, 1974). When TVA was preincubated with erythrocytes and challenged with toxin, the protection conferred by this lectin was considerably lower than those lectins, which bind specifically to band 3 (Fig. 19). The limited protection observed, is presumably due to the nonspecific binding of the lectin onto the band 3 protein.

Dolichos biflorous agglutinin (DBA), sometimes referred to as lectin A1, is specific for the blood group A antigens (Sharon and Lis, 1972). Since these antigens are located on the glycophorin component (Nicholson, 1976), this would suggest the presence of receptors on this component specific for DBA. Fig. 20 indicates that the amount of inhibition is about 35% at a lectin concentration of 128 ug/mL. The amount of inhibition rendered by this lectin is substantially lower than those lectins

Fig. 19

Inhibition of alpha toxin by Triticum vulgaris agglutinin-
M.W. 26,000. This lectin is specific for glycophorin.

Fig. 20.

Inhibition of alpha toxin by Dolichos biflorous agglutinin-
M.W. 140,000. This lectin is specific for glycophorin.

specific for band 3. The protection offered by DBA may be due to limited binding by the lectin on band 3. This is not unexpected since the lectin is specific for the sugar α -D-N-acetylgalactosamine; traces of which is present on band 3. Whether DBA also has receptors on band 3 is unknown.

Ulex europaeus agglutinin (UEA) is also referred to as lectin H because it agglutinates blood groups containing the H substance. This lectin was found to have no protective effect on erythrocytes challenged with alpha toxin. UEA has a specificity for the sugar L-fucose (Sharon and Lis, 1972). However, only trace amounts of this sugar was found on band 3 (Furthmayr et al., 1976). This relative absence may result in very little or no binding of the lectin onto the postulated receptor. Therefore, binding (if any) of UEA onto band 3 was insufficient to inhibit toxin activity. The data also suggest that the inhibition of the toxin is not due to the presence of lectin protein in the assay system.

To directly compare the inhibitory effect of all the lectins on the toxin, the lectins were all tested under identical conditions. Table 1 presents a summary of the protective spectrum of the lectins on erythrocytes when challenged with the same toxin preparation and tested under identical conditions. At an arbitrary lectin concentration of 32 ug/mL it may be observed that the best protection is offered by the band 3

TABLE 1

Effect of lectins on the hemolytic sensitivity of erythrocytes.

Lectin Concentration (ug/mL)	% Inhibition						
	Con A	RCA I	RCA II	LCA	DBA	UEA	TVA
250	90	90	92	64	46	0	24
125	90	92	86	56	30	0	12
64	90	90	84	45	24	0	2
32	82	52	46	44	20	0	0
16	78	32	32	42	14	0	0
8	72	26	30	40	12	0	0
8	66	14	22	16	8	0	0
2	52	12	12	20	6	2	2
1	20	8	12	14	2	0	0
0	0	0	0	12	0	0	0

specific lectins: Con A being most protective. Little or no protection was observed by those lectins, DBA and TVA, which do not bind predominantly to band 3.

All the lectins examined, except UEA, induced agglutination. This phenomenon however was not responsible for the protection observed. Firstly, at concentrations of lectin where there was no agglutination, protection was observed. Secondly, TVA which does induce agglutination afforded little protection. Finally, all cells, whether agglutinated or not, were hemolysed when exposed to sufficiently high concentrations of the toxin.

The data indicated that lectins with a specific affinity for band 3, as shown in human erythrocytes have a conspicuous inhibitory effect on the activity of the toxin. It became necessary therefore to establish whether this effect was non-specific: that is, due to protein-protein interaction of the toxin and lectins; or whether it was a specific event due to the blocking of receptor by the lectins. Since lectins bind to sugar residues and inhibit the toxin, it would suggest that sugars are probably essential to the binding of the toxin. Also, sialidase was found to reduce hemolytic activity of the toxin. Thus implying that sialic acid residues are important to the hemolytic function of the toxin. Sugars were therefore tested to find out if they block the hemolytic activity of the toxin. Seventeen sugars, mostly those found on the erythrocyte membrane, were tested (Table 2) using a serial dilution of toxin.

TABLE 2

The effect of sugars on the hemolytic activity of alpha toxin

* Sugars	% Inhibition
D-fucose	0
L-fucose	0
D-arabinose	0
L-arabinose	0
Glucose	4
N-acetylglucosamine	0
D-mannose	2
α -D-methylmannopyranoside	0
Mannitol	0
Galactose	0
N-acetylgalactosamine	0
Lactose	0
Cellobiose	0
Mellibiose	1
D-fructose	5
Ribitol	0
Sialic acid	0

* The final concentration of the sugars was 250 ug/mL.

If any noticeable changes were detected a chess-board type titration was carried out to investigate further. Of the seventeen sugars tested (Table 2) none showed any significant effect on the hemolytic titre of the toxin. The data imply that binding of the lectins to the sugar components sterically blocks the binding of the toxin to the specific receptor site, thereby limiting binding and reducing hemolysis in the process.

The possibility of protein-protein interaction between the lectins and toxin was tested. Con A was selected as representative of the lectins because it offered most protection to the erythrocytes and can be specifically inhibited two readily available sugars, glucose and mannose. Fig. 21 shows that when either glucose or mannose was incorporated into the assay system the protection offered by Con A was substantially reduced. This indicated that the sugars were inhibiting the Con A and preventing it from binding to the receptor. The data also suggest that the lectin was rendering the receptor site physically inaccessible to the toxin rather than neutralizing its effect by direct interaction, thus ruling out the possibility of protein-protein interaction.

Affinity chromatography, using lectins as the ligand, has been extensively used to isolate band 3 from crude extracts of erythrocyte membranes (Findlay, 1974; Adair and Kornfield, 1974;

Fig. 21

Prevention of Con A inhibition by glucose and mannose.

Reagents were added in the following sequence:

V-V Toxin plus erythrocytes

O-O Toxin plus Con A and erythrocytes

V-V Toxin plus mannose, Con A and erythrocytes

●-● Toxin plus glucose Con A and erythrocytes

The final concentration of reagents were: erythrocytes 2% (v/v);

Con A 12.5 ug/mL; mannose 125 ug/mL and glucose 125 ug/mL.

Furthmayr et al., 1976; Wong and Roses, 1979). If Con A which binds to band 3 inhibits the toxin, then band 3 isolated by Con A affinity chromatography should be a potent inhibitor of the toxin. The next step therefore involved isolation of band 3. Before proceeding to isolate band 3, it was necessary to test for the presence of an inhibitory substance in an extract of solubilized membranes. Such a test would indicate the presence of receptor material in the crude membrane extract. Erythrocyte ghosts were solubilized with Triton X-100. The Triton X-100 was subsequently removed because it is hemolytic at low concentrations, and interferes with protein determination.

Several dilutions of solubilized membranes, free of Triton X-100, (crude receptor) were preincubated with toxin for 1 h. at RT. The effect of the crude receptor on the toxin was assessed by kinetic assay (Fig. 22). The data show that the crude receptor restricts the lytic activity of the toxin. In addition, the effect of the crude receptor material on the toxin was concentration dependent indicating the occurrence of a specific event. The data further suggest that the receptor is most probably present in the crude preparation. It should be noted that the extracted proteins only consisted of bands 3,4, 2,6 and the PAS bands 1 and 2 (Steck, 1974).

Having established the presence of inhibitory material (probably receptor) in the crude extract of ghosts, a Con A affinity column was prepared to isolate band 3. The

Fig. 22

Inhibition of alpha toxin by erythrocyte membrane solubilized with 0.5% Triton X-100.

- Erythrocyte Control: Erythrocytes and PBS.
- Toxin Control: Toxin and erythrocytes.
- ▼ Toxin, crude receptor (diluted 1:2) and erythrocytes.
- Toxin, crude receptor (diluted 1:4) and erythrocytes.
- ▽ Toxin, crude receptor (diluted 1:8) and erythrocytes.

The system consisted of 50 uL toxin and 50 uL erythrocytes.

This was made up to 300 uL with either PBS or crude receptor.

The toxin was preincubated with the receptor for 1 h. at RT.

procedure was carried out essentially as described by Findlay, (1974). Crude receptor was added to the column (15 X 1 cm) in excess. The unbound material (Fig. 23) was recovered with equilibrating or wash buffer. The specifically bound material was desorbed with Con A eluent buffer containing α -D-methyl mannopyranoside. Material from the first peak represented unbound material whereas the second peak represented band 3 or pure receptor.

Purified band 3 (40 ug) was preincubated with alpha toxin and its inhibitory effect on the toxin monitored by HU assay. The data (Fig. 24) indicate that the activity of the toxin was checked when band 3 was included in the system. The hemolytic titre decreased from 70 HU to 12 HU: a drop of over 80%.

Fig. 25 shows that as the receptor was diluted its protective effect decreased accordingly. Not only does this indicate the occurrence of a very specific and predictable event but also indicates that the protective effect of the receptor is concentration dependent.

Kinetic assay involving the receptor preincubated with the toxin (1 h, RT) also indicated that the hemolytic activity of the toxin was drastically curtailed by the receptor (Fig. 26). The presence of the receptor appeared to both increase the lag period and alter the slope of the curve. The inhibitory effect of band 3 on the toxin does fulfill the primary criterion

Fig. 23.

Con A Affinity Chromatography. Elution profile for crude receptor. The first peak represents the material unretarded by the column. The second peak represents material specifically eluted by the eluent buffer: band 3 or pure receptor.

Fig. 24

Inhibition of alpha toxin by band 3.

O-O Control: erythrocytes and toxin.

●-● Erythrocytes plus toxin and receptor.

The toxin was preincubated with 40 ug (4×10^{-4} umoles) of band 3 for 1 h at RT. The toxin titre was reduced from 70 to 12 HU.

Fig. 25

The effect of serial dilution of receptor on alpha toxin.
The receptor (20 ug) was serially diluted in PBS and a
constant amount of toxin (1 HU) and erythrocytes added.

Fig. 26

Inhibitory effect of band 3 on hemolysis.

O-O Erythrocyte suspension in PBS.

●-● Toxin control: toxin and erythrocytes.

V-V Inhibition of hemolysis by 20 ug of purified band 3.

▼-▼ Inhibition of hemolysis by 40 ug of purified band 3.

of a receptor.

Band 3 purified by affinity chromatography was judged homogeneous both by polyacrylamide gel electrophoresis and SDS- gel electrophoresis. Electrophoresis of 30 ug of purified band 3 indicated the preparation was pure (Fig. 27C). It is assumed that at this concentration any protein contaminants would be obvious. Fig. 27B shows those bands selectively solubilized with Triton X-100. Because the PAS bands stain poorly with Coomassie Blue these bands are not seen in this preparation. In general, it should be noted that proteins with a relatively high content of carbohydrates stain poorly with Coomassie Blue. Thus the amount of material stained does not reflect the amount applied (Adair and Kornfield, 1974). Fig. 27A shows most of the major bands of the erythrocyte membrane stainable with Coomassie Blue. Notably absent from this preparation are the spectrin bands I and II, apparently lost during the preparation. The ghosts were solubilized in sample buffer according to Fairbanks et al., (1971).

The molecular weight determination for the purified band 3 was done on SDS-slab gels. The molecular weight of 100,000 (Fig. 28) agrees with that of Vimr et al., (1976) for rabbit erythrocyte band 3.

If band 3 and the receptor were in fact the same entity then antibodies to band 3 and the receptor should have identical properties. These two populations of antibodies should conceivably protect erythrocytes from the toxin.

Fig. 27

SDS-polyacrylamide gel electrophoresis on: (A) Erythrocyte ghosts (40 ug) solubilized by the method of Fairbanks et al., (1971). (B) ghosts solubilized with 0.5% Triton X-100. (C) Band 3 (30 ug) purified by affinity chromatography.

Band 3

A

B

C

Fig. 28

Standard curve for the molecular weight determination of band 3 on SDS-polyacrylamide gel.

Fifty μL of each standard (1 mg/mL) and 150 μL of band 3 (0.3 $\mu\text{g/mL}$) were applied.

Antibodies raised in rats to rabbit erythrocyte ghosts were used as a source of anti-receptor and anti-band 3. Preimmune sera were obtained from the animals and pooled. The pool of preimmune sera gave a hemagglutination titre of less than 1:1 with uncoated erythrocytes. After immunization the titre increased to 128. When a fraction of the immune serum was adsorbed with Con A coated erythrocytes (CACE) the titre decreased to 8 (Table 3). These residual antibodies were not removed by repeated adsorptions. These unadsorbed antibodies represented anti-band 3.

An identical drop in titre, from 128 to 8, was observed when antiserum was adsorbed with toxoid coated erythrocytes (LGCE). Once again the residual antibodies were not removed by repeated adsorptions. This aliquot of antibodies represented antireceptor. Attempts to further reduce the antibody titres by cross-adsorptions had no effect (Table 3). A 25 mL aliquot of anti-band 3 was cross-adsorbed several times with 1 mL of packed LGCE (15 min, RT). After each cross-adsorption the hemagglutination was monitored using uncoated erythrocytes. No change in agglutination titre was detected.

Similarly 25 mL of anti-receptor was cross-adsorbed with 0.2 mL of packed CACE. Even after repeated adsorptions the hemagglutination titre remained constant at 8 (Table 3).

Both anti-receptor and anti-band 3 were purified to IgG and concentrated. When the anti-receptor antibodies were

TABLE 3

Hemagglutination titres of anti-rabbit erythrocyte membrane after each adsorption.

No. of Adsorptions with	CACE	LGCE
0	128	128
1	32	64
2	8	8
3	8	8
4	8	8

No. of Cross Adsorptions with	LGCE	CACE
1	8	8
2	8	8
3	8	8
4	8	8

included in an HU assay the hemolytic titre decreased from 75 HU to 40 HU. Similarly when the anti-band 3 antibodies were included in the assay an almost identical drop in hemolytic titre was observed: the titre decreased from 73 HU to 38 HU a decrease of about 48%.

Since these two populations of antibodies had thus far exhibited identical properties, the anti-band 3 antibodies were selected for use in the next kinetic inhibition assay. The figure 29 shows a prolonged lag period and a reduction in the slope of the curves when the antibodies were incorporated into the system. This observation indicated that the anti-band 3 antibodies were inhibiting the activity of the toxin. As the antibodies were diluted the protective effect was reduced, suggesting a concentration dependency.

These experiments suggest that Con A and toxoid block the same sites on the erythrocyte membrane and thus the receptor and band 3 are serologically identical.

FIG. 29

Inhibitory effect of anti-band 3.

- O-O Erythrocyte control: erythrocytes in PBS.
- V-V Toxin Control: erythrocytes, toxin and PBS.
- Erythrocytes, anti-band 3 (256) and PBS.
- ▼-▼ Erythrocytes, anti-band 3 (128) and PBS.
- Erythrocytes, anti-band 3 (64) and PBS.

The system consisted of 50 uL of toxin, 50 uL of erythrocytes made up to 300 uL with either PBS or anti-band 3. The antibodies were preincubated with the erythrocytes for 1 h. before being challenged with the toxin.

CHAPTER 3

INHIBITION OF ALPHA TOXIN BY FLAVIN DERIVATIVES

It has been reported that flavin mononucleotide (FMN) and riboflavin are potent inhibitors of alpha toxin. Also, that the inhibition is brought about by binding of these compounds onto the erythrocyte membrane (Kato et al., 1975).

It was decided to confirm these observations and attempt to determine the functional group(s) of these compounds responsible for the inhibition of the toxin.

Varying concentrations of FMN were preincubated with erythrocytes (15 min, RT) and challenged with several dilutions of toxin. The data indicate a linear relationship between FMN concentration and inhibition up to about 6,500nM (Fig. 30). Beyond that FMN concentration, the curve levels off indicating that inhibition may be obtained optimally at a concentration of 6,500 nM (6.5 μ M). Inhibition by the nucleotide is also presented in kinetic assay (Fig. 31). The data confirms that FMN inhibits the toxin. It also shows that inhibition of the toxin by FMN is concentration dependent and that FMN at a concentration as low as 1000 nM (1 μ M) can substantially reduce toxin activity.

When erythrocytes were pretreated with riboflavin (15 min, RT) and then challenged with toxin an inhibitory effect similar to that of FMN was observed. The data (Fig. 32) indicate that,

Fig. 30

Effect of varying concentrations of FMN on alpha toxin.

The arrow indicates the optimum concentration of FMN

(6,500 nM) required to neutralize the activity of the toxin.

Fig. 31

Inhibition of alpha toxin by FMN.

O-O Erythrocyte Control: erythrocytes in PBS.

●-● Toxin Control: erythrocytes, toxin and PBS.

V-V Erythrocytes, FMN (4,000 nM) and PBS.

D-D Erythrocytes, FMN (2,000 nM) and PBS.

■-■ Erythrocytes, FMN (1,000 nM) and PBS.

The erythrocytes were preincubated with the FMN for
15 min. at RT.

Fig. 32

Inhibition of the hemolytic of alpha toxin by riboflavin.
The arrow indicate the optimum concentration of riboflavin
required to inhibit the activity of the toxin.

at relatively low concentrations (6,500 nM) of riboflavin, toxin activity was almost completely neutralized. Again a direct proportionality was observed between the extent of inhibition and riboflavin concentration: the optimum being 6,500 nM.

Flavin was represented by lumiflavin (Sigma). This compound lacks the ribitol and ribitol phosphate moieties present on riboflavin and FMN respectively. Lumiflavin was used to determine the functional group responsible for the inhibition of the toxin by FMN and riboflavin. When erythrocytes were preincubated with lumiflavin (15 min, RT) and challenged with toxin the protective ability of this compound was found to be noticeably lower than either riboflavin or FMN. Fig. 33 shows that at a concentration as high as 40,000 nM (40 μ M) less than 50% inhibition was observed. By comparison, a concentration of 6,500 nM for FMN and riboflavin produced approximately 90% inhibition.

Initially, these results would imply that the ribitol moiety, absent on lumiflavin but present on riboflavin and FMN, is the functional inhibitory group. This suggestion is however premature especially when one considers that ribitol by itself has no effect on the activity of the toxin (Table 2). Clearly more work is required in this area in order to establish unequivocally the functional inhibitory group(s).

Fig. 33

Effect of lumiflavin on the hemolytic activity of alpha toxin.

The concentrations of lumiflavin ranged from 625 nM to 40,000 nM.

The arrow indicates 40,000 nM of lumiflavin.

Since FMN is an anion, the prospect of FMN binding onto band 3 and inhibiting the toxin as a result, appeared an especially attractive hypothesis. To test this possibility, it was necessary first to establish whether FMN was binding onto the erythrocyte membrane before attempting to determine whether the compound bound specifically to band 3.

FMN was selected to represent this group because of its ready solubility in PBS. To determine if FMN were binding to the membrane, 1 mL of erythrocytes (4%) was incubated in a test tube with 1 mL of FMN (8,000 nM). The mixture was allowed to incubate for 1 h at RT. A control tube of PBS and FMN (4,000 nM, final concentration) was prepared similarly. At the end of incubation both tubes were centrifuged (600 g, 5 min). The supernatant of the tube with the erythrocytes was carefully removed and checked for any decrease in protective ability. The results were compared to the control. Since the FMN concentration (4,000 nM) was not excessive (ref. Fig. 30) one would expect that incubation of FMN with erythrocytes would result in a decrease in the FMN concentration if it were binding to the membranes. The data (Fig. 34) show no decrease in protection in the FMN supernatant in which the erythrocytes were suspended. This observation would indicate no decrease in the FMN concentration, suggesting therefore that the FMN was not binding onto the erythrocyte membrane.

To confirm the preceding observation a 'wash experiment'

Fig. 34

The effect of erythrocytes on FMN concentration.

O-O Supernatant of FMN (8,000 nM) and erythrocytes (2%).

●-● FMN (8,000 nM) and PBS.

The erythrocytes were preincubated with the FMN for 1 h at RT. Final concentrations of FMN and erythrocytes are 4,000 nM and 2% respectively.

was carried out. It involved washing erythrocytes pretreated with FMN and monitoring any changes in hemolytic sensitivity. One mL of FMN (4,000 nM, final concentration) was added to 1 mL erythrocytes (2%, final concentration) and allowed to equilibrate for 1 h. at RT. These cells were washed three times with PBS to eliminate all unbound FMN. A control tube with erythrocytes was treated similarly except that 1 mL of PBS was added in place of the FMN. The washed cells were reconstituted to a 2% suspension. Both treated and untreated cells were titrated against serially diluted toxin and their hemolytic titres compared. The cells treated with FMN yielded a hemolytic titre of 78 HU. The untreated erythrocytes had a titre of 61 HU. The data indicated that the protective effect of the FMN was lost by washing. From these experiments it may be inferred that the FMN-receptor bond is very unstable or that the FMN does not bind to the erythrocyte membranes. If the latter is the case, it would imply that the toxin is being directly inactivated by the FMN. Therefore in experiments of this nature removal of unbound or unreacted compounds is imperative if confusing or misleading interpretations are to be avoided. The results of this experiment eliminated the need to test for binding of FMN onto band 3.

To determine the stability of the toxin-receptor bond a 'split titration' was attempted. This involved two sequential

hemolytic titrations in which the lysate from the first titration was used as a source of toxin for the second. The system included 50 uL of toxin serially diluted with PBS and 100 ~~uL~~ of erythrocytes (2%). The final reaction volume of 200 uL was made up with PBS. The mixture was incubated for exactly 1 h at 37°C. This assay was done in triplicate. After incubation the mixture was centrifuged (600 g, 5 min) to pellet the unlysed erythrocytes. Fifty uL aliquots of supernatant from the first well above was serially diluted in PBS in a second hemolytic titration. This titration was treated exactly as described for the first. Since the lysate from the first titration was used as the source of toxin for the second, then the background hemoglobin was increased. To correct for this background hemoglobin, 50 uL of lysate from the first well of the first titration was serially diluted in PBS. The volume was made up to 200 uL with PBS. These were then diluted 1:3 with PBS and used as corresponding blanks.

A comparison of the two titrations (Fig. 35) shows no discernible difference in hemolytic titres between the first and second titrations. The data suggest that the toxin was not consumed during the lytic process. As the lytic capacity of the toxin remained undiminished it would imply that the toxin is being regenerated. The data not only reflect the enzyme-like property of the toxin but, more importantly, indicate that the toxin-receptor bond is unstable. Whether this is an indication of the instability of the FMN-receptor bond is highly speculative at this time.

Fig. 35

Regeneration of alpha toxin.

V-V Serially diluted toxin incubated with erythrocytes.

O-O Toxin derived from the lysate of the previous hemolytic titration and incubated with erythrocytes.

Both titrations were incubated at 37°C for 1 h.

CHAPTER 4

IMMUNO-AFFINITY OF STAPHYLOCOCCAL ALPHA TOXIN

Affinity chromatography has been applied to the purification of antigens and their cross-reacting materials (Murphy et al., 1973), and toxins (Heatley, 1976; Hughes et al., 1974; Sjoberg and Holmgrew, 1973). The technique has been used to isolate receptors (Adair and Kornfield, 1974; Findlay, 1974). It has been used in this laboratory to characterize staphylococcal mutants and their toxins. The method was developed specifically, with the intention of isolating the antigenic fragments of staphylococcal alpha toxin but was applied to the study of the receptor.

Sepharose 4B was activated and coupled with antibody as previously described. Toxin adsorbed by the affinity column was removed by elution buffer. A typical profile of an immuno-affinity column is shown (Fig. 36). The first peak contains unadsorbed material while the second peak represents specifically bound alpha toxin. All fractions from the second peak with an absorbance greater than 0.01 at 280 nm were pooled and the total protein content calculated ($E_{280}^{1\%} = 13.5$).

Table 4 summarises the data from a series of experiments designed to measure the effect of pH on the coupling of the IgG fraction of the anti-alpha toxin to CNBr-agarose and the pH dependence of toxin binding to the insoluble antibody. Eight columns of antibody agarose were constructed that

Fig. 36

Elution profile for crude alpha toxin adsorbed onto antibody agarose. The arrow indicated the addition of elution buffer. The elution buffer was glycine-HCl (0.2 M, pH 2).

differed only in the buffer used to couple antibody to the CNBr-agarose. After equilibration with the appropriate buffer crude toxin was added. Specifically bound alpha toxin was recovered as shown in Fig. (36). Each column was tested with several adsorption buffers and the average recoveries expressed per mL of insoluble antibody. Clearly, acetate, at pH 4.5 is the most efficient buffer for coupling antibody to the activated agarose. The recovery of 83 ug/mL is 28% higher than the next closest value. Invariably, the values at acidic pH were higher than those at alkaline pH, implying that acidic conditions favour the coupling of antibody to the CNBr-agarose. The data do not suggest that more antibodies coupled at the lower pH, only that more reactive sites are available after coupling.

A plot of average yield versus pH (Fig. 37) shows an inverse relationship between pH and coupling efficiency. As the pH of the coupling is increased a steady decrease in recovery is observed. A comparative study of acetate and citrate coupling buffers at pH 5.5 shows an average yield of 60 ug/mL when citrate buffer was used as the solvent; but only 55 ug/mL when acetate was used (Table 4). There was little observable difference between the effect of phosphate and citrate coupling buffers at pH 6.5, since the yields are 50 and 51 ug/mL respectively. A marked difference is

Fig 37

The effect of pH on the ability of alpha toxin to bind to antibody agarose.

observed between borate and phosphate buffers. Hence acetate pH 4.5 was retained as the best coupling buffer. A comparison of the buffers used in binding the toxin onto the immuno-adsorbent column indicated a steady increase in toxin recovery from pH 4.5-6.5, after which the recoveries decline (Table 4). Low binding in the acidic pH range is probably due to the protonation of the amino groups of both the insoluble antibody and the antigen.

The effectiveness of the binding buffers determined by comparison of the three overlapping pH's indicated that (a) citrate is a better binding buffer than acetate at pH 5.5 (b) phosphate buffer is also better for binding than borate buffer at pH 7.6. The binding buffer of choice is potassium phosphate, pH 6.5.

To determine the optimum antibody concentration required for coupling, the processes of activation, coupling, binding and elution were carried out as described initially. This time, however, the activated agarose was split into two fractions. After coupling, the antibody concentrations were 11.1 and 25 mg/mL respectively. The recovery of toxin from these columns was 448 and 668 ug/mL respectively. The coupling and binding buffers were acetate pH 4.5 and phosphate pH 6.5 as determined previously.

The table (5) shows that no linear correlation exists between the product yield and the amount of antibody coupled.

Table 4 Effect of Coupling and Adsorption Buffers on Toxin Recovery

Adsorption Buffer	Coupling Buffers						Average of Adsorption Buffers
	Acetate pH	Citrate pH	Phosphate pH	Borate pH			
	4.5	5.5	6.5	7.6	8.5		
Acetate pH	4.5	35*	20	13	18	30	22
	5.5	34	29	26	21	18	20
Citrate pH	5.5	63	33	46	47	42	44
	6.5	72	63	104	87	84	67
Phosphate pH	6.5	160	81	81	72	69	75
	7.6	160	89	70	42	68	69
Borate pH	7.6	54	53	75	57	56	49
	8.5	87	73	66	64	57	64
Average for Coupling Buffers	83	55	60	51	50	48	29

*Toxin Recovery per ml of insoluble antibody (ug)

TABLE 5

The effect of antibody concentration on the ability of antibody agarose to bind alpha toxin.

Antibody	Toxin Eluted
mg/mL agarose	ug/mL agarose antibody
5	160
11.1	448
25	668

A comparison of eluent buffers, glycine-HCl (0.2 M) and urea was performed with two columns with antibody concentrations of 25 ug/mL of agarose each. The results indicated that glycine-HCl eluted 58% more toxin than did urea.

In summary, sodium acetate buffer pH 4.5 was shown to be best suited for coupling of the anti-alpha toxin onto the activated agarose, that potassium phosphate pH 4.5 was most favourable for binding the alpha toxin to the immunoadsorbent, that glycine-HCl was a better eluent than urea and that the optimal concentration of anti-alpha antibody was about 25 mg/mL of agarose.

Application of immuno-affinity chromatography.

Antibinding antibodies (AB-ab) are antibodies directed against alpha toxin and are capable of neutralizing the toxin by prevention of binding of the toxin onto membranes (Lo and Fackrell, 1979). Although this definition suggests that the antibinding antibodies prevent binding of the toxin to the receptor in situ this may not hold true for a system involving the isolated receptor.

This laboratory has demonstrated that CNBr-agarose coupled to AB-ab, was capable of binding to alpha toxin. Conceivably, these agarose antibodies (immunoabsorbent) may be applied to probing the existence of a complex consisting of the toxoid/toxin, its receptor and antibinding antibodies. Information on the existence of such a complex could be applied to the characterization of antibinding antibodies and provide an insight into the nature of the binding sites of the participating components. The following experiments were designed to probe the existence of a receptor-toxoid complex capable of binding to the antibinding antibodies.

Basically, insolubilized AB-ab were preincubated with either toxoid, crude receptor or a mixture of receptor and toxoid. If these components bind to the immunoabsorbent they reduce the number of binding sites available to the toxin. This effect may be monitored by HU assay. In the absence of these competing components maximum binding of toxin to the immunoabsorbent is obtained. The net result is a decrease in

the final concentration of toxin. The hemolytic titre obtained by incubating the toxin with the AB-ab in the absence of competing substances, was therefore arbitrarily set to zero % of toxin activity. The 100% reference point was set by incubating plain agarose beads with the toxin.

To 100 uL of 10% (v/v) AB-ab agarose beads 200 uL of PBS was added. The mixture was allowed to incubate (30 min, RT) and washed twice with PBS. Two hundred uL of toxin was added to the beads and the mixture allowed to incubate further for 1 h at RT. After centrifugation (600 g, 5 min), the supernatant was tested for hemolytic activity by HU assay. The data (Fig 38) show a titre of 4 HU. This value was set to zero % of toxin activity. On the other hand, 100 uL of uncoupled agarose (10%) incubated with the same volumes of PBS and toxin exactly as described above, yielded a titre of 64 HU. This titre represented 100% of toxin activity.

When 100 uL of crude receptor, diluted to 200 uL with PBS, was preincubated with the antibinding beads (30 min, RT) and treated as above, a hemolytic titre of 5.7 HU was observed. If however, the receptor was replaced by 100 uL of toxoid (diluted to 200 uL with PBS) and processed similarly the titre increased to about 10.5 HU. The data would suggest that the binding sites on the AB-ab are occupied by the toxoid, hence fewer toxin molecules are able to bind to the immunoadsorbent. This effect was thus translated into an increased

Fig. 38

The effect of inhibitors on the binding of alpha toxin to AB-ab agarose.

●-● AB-ab agarose plus PBS and toxin.

▼-▼ AB-ab agarose plus crude receptor and toxin.

∇-∇ AB-ab agarose plus toxoid and toxin.

□-□ AB-ab agarose plus preincubated toxoid and receptor plus toxin.

O-O Controls: Uncoupled agarose beads were preincubated separately with PBS, crude receptor, toxoid and a mixture of toxoid and receptor. These were processed similarly and in parallel to the corresponding tests described above.

hemolytic titre. Crude receptor (100 uL) was preincubated with 100 uL of toxoid (30 min, RT). When this mixture was incubated with the AB-ab beads and processed, the titre of the toxin was found to increase to about 18.4 HU. This increase in titre suggests that the toxoid-receptor complex is capable of binding to the immunoabsorbent thus reducing binding of the toxin. This is reflected in an increase in hemolytic titre by about 400%.

Controls for these experiments were carried out in parallel and under identical conditions to those of the tests except that 10% plain agarose was substituted for the AB-ab agarose beads. Since the beads are inert the toxin titres for all the control experiments corresponded to about 64 HU or 100% toxin activity.

Altogether the data does argue for the formation of a toxoid-receptor complex capable of binding to AB-ab. It also suggests the existence of two binding sites on the toxoid: one capable of binding to the antibody and another capable of binding to the receptor component. The complexity of the situation however may have been understated. For example, what would be the effect of an unstable toxoid-receptor complex when incubated with the immunoabsorbent? Evidently, more work is required in this area before a definitive conclusion could be reached.

The existence of a toxoid-receptor complex does allow a more penetrating scrutiny into the binding mechanism between the participating components. A stable toxoid-receptor

complex can be used to isolate a population of antibody quite different from the antibinding and indirect hemagglutinating antibodies previously isolated (Lo and Fackrell, 1979).

DISCUSSION

Evidence presented by Cassidy and Harshman (1976) demonstrated that toxin binding and hemolytic sensitivity were considerably reduced when cells, preincubated with Pronase, were challenged with alpha toxin. Moreover, they reported that no change in hemolytic sensitivity was observed in cells digested with trypsin. Kato et al., (1975) however, had reported a dramatic decrease in hemolytic sensitivity with this enzyme. Robson Wright et al., (1968) also reported inhibition of hemolysis in erythrocytes incubated with aromatic polysulfonic acids. Although these authors concluded that the polysulfonic acids inhibited hemolysis by inactivating the toxin per se, they conceded that interaction of the acids and the erythrocytes may play a role.

Strikingly common to all the treatments outlined above, apart from reducing hemolytic sensitivity, is the fact that they only affect band 3 and/or glycophorin. Pronase has been shown to cleave only two polypeptides on the erythrocyte membrane. They are band 3 and glycophorin (Bender et al., 1971; Bretscher, 1973; Cabantchik and Rothstein, 1974b). Trypsin on the other hand has been shown to specifically degrade glycophorin (Steck et al., 1976; Bretscher, 1973; Cabantchik and Rothstein, 1974b), leaving band 3 and other polypeptides of the erythrocyte membrane intact.

It has been convincingly demonstrated that band 3 is an anion transport channel and aromatic sulfonic acids and their

congeners have common binding sites on the band 3 trans-membrane protein (Cabantchik and Rothstein, 1974ab; Barzilay et al., 1979; Barzilay and Cabantchik, 1979; Marchesi et al., 1976; Steck, 1978).

Taken together the data clearly implicate either band 3 or glycophorin in the receptor function. Further, the fact that a number as high as 125,000 receptors per rabbit erythrocyte has recently been generated by this laboratory (Barei and Fackrell, 1979) would clearly suggest the involvement of a major membrane polypeptide.

The data indicate that the proteases which degrade band 3 do increase the resistance of the erythrocytes to the toxin. On the other hand, those which do not degrade band 3 do not affect hemolytic activity. Digestion of erythrocytes with Pronase have been shown to both reduce the binding of toxoid to the cell membranes and reduced hemolytic sensitivity of the cells to alpha toxin (Fig. 1). This finding confirms the observation made by Cassidy and Harshman, (1976). It should be noted that Pronase degrades only glycophorin and band 3 (Bretscher, 1973; Bender et al., 1971). α -Chymotrypsin which selectively cleaves band 3 (Liu and Palek, 1979), was also found to increase erythrocyte resistance to the toxin (Fig. 3, 4). Cassidy and Harshman, (1976) have also observed a reduction in the binding of toxin to erythrocytes preincubated with α -chymotrypsin. By contrast, trypsin which cleaves glycophorin, but not band 3, does not alter the hemolytic sensitivity of

erythrocytes (Fig. 2). Together the data suggest that band 3 is probably the receptor for alpha toxin.

Sialic acid, glucose and galactose are all present on band 3 (Furthmayr, et al., 1976). Sialidase, but not glucosidase and galactosidase altered the hemolytic sensitivity of erythrocytes to the toxin (Fig. 5,6). However, sialic acid alone up to a concentration of 250 ug/mL had no inhibitory effect the toxin. Sialic acid residues are negatively charged. They confer a net negative charge on the cell membrane. The action of the sialidase may have reduced this negative charge on the membrane thus limiting the interaction between the positively charged toxin and the negatively charged cell membrane. In addition, the reduction of the negative charge on the membrane may have encouraged cell-cell interaction thus sterically excluding the toxin.

Further evidence implicating band 3 as the receptor for alpha toxin was derived from the studies with lectins. As indicated (Table 1, Fig. 15, 16, 17, 18) erythrocytes coated with Con A, LCA, RCA (type I and II) all exhibited a tremendous resistance to lysis by the toxin. Common to these lectins, is the fact that they all bind specifically to band 3 (Findlay, 1974; Sharom et al., 1977; Marchesi et al., 1976; Adair and Kornfield, 1974).

TVA and DBA (Fig. 19, 20) offered comparatively little protection. The limited protection offered by TVA is consistent

with the finding that the lectin binds specifically and predominantly to glycophorin (Kahane et al., 1976) and only to a small extent to band 3 (Findlay, 1974). The relatively small degree of protection offered by DBA (lectin A I) is not unexpected. The lectin is specific for the group A antigens which are located on the glycophorin component (Nicholson, 1976). Binding of DBA to glycophorin would therefore be anticipated. Whether receptors for this lectin also exist on band 3 is unknown. This is not unreasonable to assume since this lectin has a specificity for the sugar N-acetyl-galactosamine which is present on band 3 (Furthmayr et al., 1976).

UEA (lectin H) exhibited no detectable protective ability. The lectin has a specificity for L-fucose (Sharon and Lis, 1972). However, only trace amounts of this sugar are found on band 3 (Marchesi et al., 1976; Furthmayr et al., 1976). This relative absence of fucose may have resulted in very little or no binding of the lectin onto the postulated receptor. Therefore, binding (if any) of UEA onto band 3 was insufficient to inhibit toxin activity. The lack of inhibitory activity by this lectin also demonstrates that protection of erythrocytes by the lectins is not due to protein-protein interaction between the toxin and the lectin.

Failure of the rat antisera, raised to rabbit erythrocyte ghosts, to show a reduction in hemagglutination titre (Table 3)

when cross-adsorbed by CACE or LGCE suggests that toxoid and the lectin block the same receptor sites. This observation is strengthened by the finding that antibodies left after cross-adsorptions with LGCE or CACE share identical protective characteristics (Fig. 29). The data strongly suggest that the receptor and band 3 are serologically identical.

Band 3 obtained conventionally from the Con A affinity column was apparently pure as a single band was observed on SDS-polyacrylamide slab gels (Fig. 27). The diffuse nature of the band with trailing edge is characteristic of band 3. The band 3 material was itself nonhemolytic and induced no aggregation when incubated with erythrocytes. The molecular weight of 100,000 for band 3 agrees with that of Vimr et al., (1976) for band 3 of rabbit erythrocyte membranes. The co-contaminants in band 3 preparations, described by Findlay, (1974) were not observed. The removal of Triton X-100 from our solubilized membranes is the major difference in the preparative methods. The Triton X-100 had to be effectively removed because low concentrations were hemolytic.

Kato et al., (1975) postulated that a flavin mononucleotide (FMN) binding glycoprotein, which is sensitive to Pronase, on the erythrocyte membrane was the receptor for alpha toxin. The observation of a Pronase sensitive glycoprotein being the receptor is consistent with the findings presented thus far; moreover it is consistent with the postulate that band 3 is

the receptor for alpha toxin. Also of interest is the fact that the same workers (Kato and Naiki, 1976) later postulated that a ganglioside, extracted from human erythrocytes, was the receptor for alpha toxin: a finding which could not be confirmed (McCartney and Arbuthnott, 1978). Band 3 is an ubiquitous component of red cell membranes and about 5×10^5 copies of this receptor per erythrocyte is found in human RBC (Bretscher; 1973). Presumably, there is a similar number of band 3 on rabbit erythrocytes. Yet human erythrocytes are resistant to lysis by the toxin whereas rabbit erythrocytes are very sensitive. Initially these observations would argue against band 3 being the receptor for alpha toxin. However, Con A which causes spontaneous agglutination of rabbit red cells, does not agglutinate human erythrocytes (Sharom et al., 1977). It is believed that the Con A receptor (band 3) on human is masked.

Barei and Fackrell, (1979) have reported that no specific binding of alpha toxin to human erythrocytes has been detected. It appears that while band 3 is present on in human red cells it is incapable of binding either Con A or alpha toxin, probably for similar reasons.

It is well established that lysis of erythrocytes by alpha toxin is preceded by a release of K^+ ions. Recently, Cassidy and Harshman (1976) were able to obtain K^+ release without lysis

by incubation of rabbit erythrocytes at 4°C. It is interesting that band 3 functions as an anion transport channel (Marchesi et al., 1976; Barzilay et al., 1979; Barzilay and Cabantchik, 1979; Cabantchik et al., 1978) and that exposure of band 3 to Pronase induces K⁺ release (Bender et al., 1971). It is therefore not unreasonable to suggest that K⁺ release by alpha toxin is the result of toxin-receptor interaction.

The actual number of receptors per cell remains in doubt. Cassidy and Harshman (1976), reported 5,000 receptors per cell, whereas Barei and Frackrell (1979) has estimated as many 125,000 per cell. In either case the reported number of receptors is well below the minimum 500,000 copies of band 3 per cell reported by Bretscher (1973).

It has been shown that the action of sialidase on the erythrocytes results in reduced hemolytic activity and presumably reduced binding of the toxin. It is also known that erythrocytes spontaneously release sialic acid residues during the processes of maturation and aging. Whether this loss is uniform among copies of band 3 (receptor) is not known. Presumably the heterogeneity established with respect to the differential loss of sialic acid residues would affect binding of the toxoid/toxin to the receptor component.

Apart from the presumed differences in the sialic acid composition of band 3, there is also some doubt as to the

homogeneity of band 3: some authorities (Wong and Roses, 1979) believe that band 3 may be an aggregation of several co-migrating heterogeneous proteins. These factors may also help to explain the discrepancy between the actual amount of receptors and those observed.

The interaction of alpha toxin with band 3 can now be examined in detail to establish the precise mechanism of binding and hemolysis. It will therefore be instructive to determine whether alpha toxin or toxoid inhibits anion transport. It has been demonstrated (Robson Wright et al., 1968) that aromatic polysulfonic acids inhibit the activity of the toxin. It may be useful to determine whether compounds which bind to band 3 and/or those which inhibit anion transport, competitively inhibit the binding and lytic activity of the toxin. In this respect 4, 4'-diisothiocyano 2, 2'-stilbene sulfonic acid (DIDS) which binds specifically to band 3 and inhibit anion transport, may be useful.

Wong and Roses (1979) have demonstrated that band 3 derived from erythrocytes of patients with myotonic muscular dystrophy exhibit decreased autophosphorylation. This phenomenon, the authors suggested, may be due to an alteration in the conformation of band 3. If this change is sufficient to affect the binding of the toxin to erythrocytes, then confirmation of this hypothesis may be easily obtained by comparing changes in hemolytic patterns of normal and muscular dystrophic human, rabbits or rats.

Affinity chromatography with staphylococcal alpha toxin has indicated that acetate buffer, at pH 4.5, is the most efficient buffer for coupling the antibody to the activated agarose (Table 4). The data indicate that recovery values at acidic pH were higher than alkaline pH, implying that acidic conditions favour the coupling of antibody to the CNBr-agarose. The data do not suggest that more antibody couples at the lower pH, only that more reactive sites are available after coupling. This observation may be due to one of several factors. CNBr hydrolysis occurs rapidly under alkaline conditions, therefore under the acidic conditions more active CNBr-agarose may be available. Also, the pH dependency of coupling is due to the reaction between the unprotonated form of the free amino groups and the activated agarose (Cuatrecasas, 1970). Consequently proteins, usually rich in lysyl residues, have several points of anchorage when coupled at alkaline pH. The multiple binding of the antibody to the matrix could either distort the antibody or sterically block the reactive sites. The higher number of reactive sites available after coupling of the antibody at low pH values may be because CNBr is not hydrolysed as rapidly as it is under alkaline conditions. However, the observation that all of the columns coupled a similar amount of antibody protein suggest that there is sufficient active CNBr-agarose at all pH values. Thus at

alkaline pH, low toxin binding due to multiple binding of antibody to the matrix is more probable than rapid CNBr hydrolysis.

The plot of average yield versus pH (Fig. 37) shows that as the pH of the coupling is increased a steady decrease in toxin recovery is observed. The data indicate that acetate buffer, pH 4.5, was the best coupling buffer. This is in disagreement with other authors (Porath and Kristiansen, 1975) who suggest that coupling at a pH lower than 6.5 is unpredictable, but it supports the view that a methodology be developed for each system.

Table 4 indicates a steady increase in the binding of the toxin to the immunoadsorbent from pH 4.5 - 6.5 after which the values decline. Low binding in the acidic pH range is probably due to the protonation of the amino groups of both the insoluble antibody and the antigen. Since the toxin carries a high positive charge, possibly owing to the presence of large amount of glutamine and asparagine (Fackrell and Wiseman, 1976), and since the IgG molecule is also positively charged, it seems that both molecules repel each other at low pH. However, as the isoelectric point of the molecules is approached the magnitude of the repulsive forces is diminished, thus resulting in better binding. The optimum binding observed in the pH range of 6.5-8.5 is in conformity with this suggestion. The data is also in

harmony with the finding of Kleindschmidt and Boyer, (1952). They demonstrated that the stability of the antigen-antibody complex decreased with decreasing pH; that the optimum antigen antibody interaction occurred in the pH range 6.25 - 8.45; and that inhibition of precipitation of the complexes occurs beyond this range.

Table 5 shows no linear correlation between product yield and the amount of antibody coupled. The optimum apparently is about 25 mg/mL. Stage and Mannik (1974) have found that coupling occurs as a function of protein concentration up to about 60 mg/mL agarose. This however, is no reflection of the binding capacity, as a high degree of substitution does not ensure a quality immunoabsorbent (Porath and Kristiansen, 1975).

SUMMARY

The conclusions derived from the preceding study are as follows: (1) Pronase and α -chymotrypsin which degrade band 3 were also found to reduce binding and hemolytic activity of the toxin. Trypsin, which selectively cleaves glycophorin, had no effect on hemolytic activity. Sialidase was found to inhibit toxin activity but β -galactosidase, β -glucosidase and lipase were ineffective.

(2) Con A, LCA, RCA I and RCA II bind specifically to band 3. These lectins almost completely abolished the activity of the toxin. UEA, DBA and TVA have no specific affinity for band 3. These lectins had little or no effect on the hemolytic profile of the toxin.

(3) Sugars had no inhibitory effect on the toxin activity.

(4) Band 3, isolated conventionally by Con A affinity chromatography, was homogeneous, had a molecular weight of 100,000 and inhibited the activity of the toxin. The preparation was itself nonhemolytic and did not induce agglutination of the erythrocytes.

(5) The receptor and band 3 were serologically identical. The above findings indicated that band 3 was the receptor for staphylococcal alpha toxin.

(6) Flavin derivatives were found to restrict the hemolytic activity of the toxin. This restriction was not due to the binding of these compounds to the erythrocyte membranes.

(7) Affinity chromatography of staphylococcal alpha toxin showed that: (i) anti-alpha toxin coupled to agarose was most efficient when the antibody was coupled in the pH range 4.5-6.5. (ii) the toxin bound best to the insoluble antibody at a slightly alkaline pH and (iii) glycine-HCl was a better eluent buffer than urea.

(8) Evidence was obtained for the existence of a toxoid-receptor complex capable of binding to AB-ab.

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