THE IMMUNOLOGY OF STAPHYLOCOCCAL ALPHA TOXIN.

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THE IMMUNOLOGY OF STAPHYLOCOCCAL ALPHA TOXIN

by

CHING YEE LO

A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGY

WINDSOR, ONTARIO
NOVEMBER, 1983
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802740
To Teresita
ABSTRACT

There are two types of antibodies to the alpha toxin of *Staphylococcus aureus*: the antibinding antibodies (AB-Ab) and the indirect hemagglutinating antibodies (IHA-Ab). Based on the results of fluorescence polarization immunoassay and precipitin reactions, the minimal number of antigenic determinants for alpha toxin is 3: one IHA determinant and two antibinding determinants. The two types of antibodies were characterized in terms of antibody responses, neutralization, immunoglobulin classes, and complement fixation *in vitro* and *in vivo*.

The antibody responses in rabbits immunized with alpha toxoid were readily demonstrable by the indirect hemagglutination test and by the complement fixation test but not by the neutralization test. The preimmune status of the animal appears to influence the course of the AB-Ab response. The proportion of the two populations of antibodies fluctuates among the different rabbits and with time in the same animal. I Ab appear to contribute a greater part of the neutralization capacity to whole sera. The neutralization capacity observed in antiserum is less than the sum of the neutralization capacities of the individual populations of antibodies because one toxin can bind more than one antibody. The binding of any one molecule of antibody (AB-Ab or IHA-Ab) is sufficient to neutralize a molecule of the toxin. No non-neutralizing antibody to the toxin was detected. Neutralizing antibodies are found in IgG, IgM and IgA classes.

A serologic survey revealed that 72% of normal human sera were positive for complement-fixing antitoxins and 95% of these
sera were positive for IHA-Ab. The fixation of complement by membrane-bound immune complexes does not result in lysis of the carrier erythrocytes. Both IHA-Ab and AB-Ab induced the reverse Arthus type of skin reaction in rabbit.

This investigation revealed that the protective value of IHA-Ab is superior to that of AB-Ab.
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LIST OF ABBREVIATIONS

AB-Ab  anti-binding antibodies
CP     complement fixation
ELISA  enzyme-linked immunosorbent assay
Fab    fragment antigen-binding
FP     fluorescence polarization
IHA    indirect hemagglutination
IHA-Ab indirect hemagglutinating antibodies
IgA    immunoglobulin class A
IgG    immunoglobulin class G
IgM    immunoglobulin class M
LGCE   lysine-washed glutaraldehyde-fixed toxoid-coated erythrocytes
PBS    phosphate buffered saline
QPT    quantitative precipitin test
S.E.   standard error

UNITS
C      degree(s) Centigrade
CH_{50} 50% hemolytic unit(s) of complement
x g     times unit of gravity
h      hour(s)
HU     hemolytic unit(s) of staphylococcal alpha toxin
min    minute(s)
N     neutralization unit(s)
N_{AB} neutralization unit(s) of AB-Ab
N_{IHA} neutralization unit(s) of IHA-Ab
N_{w}  neutralization unit(s) of whole serum
INTRODUCTION
According to the 8th edition of Bergey's manual of determinative bacteriology, alpha toxin is among the most significant traits associated with pathogenic strains of staphylococci (Baird-Parker 1974). Arbuthnott (1970) considered that "of the several toxic and potentially toxic products of pathogenic staphylococci, the most potent is the alpha toxin."

Despite much study, the role of the alpha toxin of *Staphylococcus aureus* in the pathogenesis of infection and the protective role of anti-alpha toxin remains unclear. These problems may not be resolved until such time as the toxin is both chemically and antigenically characterized. While considerable data on the physical and chemical characteristics of the toxin are available (Wiseman 1975, Rogolsky 1979), information regarding its antigenic characteristics is so limited that even the class of immunoglobulins to which the antitoxin belongs (Felsenfeld and Felsenfeld 1966) is unconfirmed. Numerous attempts to determine the value of anti-alpha toxin either in protection against the onset of infection or in the alteration of the course of the disease, give conflicting results. These studies were reviewed by Elek (1959) and Ekstedt (1972). Differences in the methods of immunization and challenge, as well as host variation, may account for part of the conflict. However, the fundamental differences in the various antitoxins have been neglected. Arbuthnott (1970) suggested that "a further detailed study of the nature of the immunoglobulins produced in experimental immunization with alpha toxoid would seem to be required."

Previously, we discovered two populations of antitoxins in the
immune sera of rabbits and pig (Lo and Fackrell 1979). One population is called the anti-binding antibodies (AB-Ab) because these antibodies prevent the binding of the toxin to erythrocyte membranes. The other population is called the indirect hemagglutinating antibodies (IHA-Ab) because these antibodies neutralize alpha toxin on erythrocytes and thereby bring about an indirect hemagglutination reaction. These findings not only shed new light on the complexity of neutralization, but also differentiated the hemolytic reaction of the toxin into 2 immunologically distinguishable steps: binding and destruction. Furthermore, the immunologic knowledge concerning the toxin was instrumental in stimulating advances in two areas in this laboratory. Firstly, a specific receptor for alpha toxin on erythrocyte membranes was implicated. This implication led to the measurement (Barei and Fackrell 1979) and the subsequent identification (Maharaj and Fackrell 1980) of the receptor. Secondly, cross-reactive materials produced by various mutants were immunologically identified and classified (Machowski-Syrowik 1980).

Thus, the study on the immunology of alpha toxin proved to be invaluable in the past. The present study was undertaken in order to establish a foundation upon which the antigenic anatomy of alpha toxin can be built. The present study concentrated on three objectives:
1. to measure the number of antigenic determinants on alpha toxin.
2. to characterize the different populations of antibodies.
3. to evaluate the role of complement in the toxic activities of the toxin.
LITERATURE REVIEW
Discovery

Around the turn of the century, medical investigators (De Christmas 1888; Leber 1888; Brieger and Fraenkel 1890; Rodet and Courmont 1892; Van de Velde 1894; Neisser and Wechsberg 1901; Kraus and Pribram 1906) noticed that staphylococcal products displayed toxicities such as hemolysis, dermonecrosis and lethality for some animals. In 1929 Burnet showed that antisera from rabbits injected with crude bacterial filtrate abolished all of the three toxic manifestations (Burnet 1929, 1931). He put forth the hypothesis that "...the one toxin is responsible for the three characteristic activities of staphylococcal filtrates..." (Burnet 1931). His view was substantiated when the purified toxin was shown to exhibit hemolytic, dermonecrotic, lethal and leucocidal activities (Kumer and Lindorfer 1962; Kumer et al. 1962). Similar findings were reproduced independently at about the same time (Bernheimer and Schwartz 1963; Goshi et al. 1963, Jackson 1963; Lominski et al. 1963). Finally, this hypothesis was reinforced by studies on mutants. The majority of mutants obtained by ultraviolet irradiation lost all three toxic activities, presumably as a result of a single mutational event (McClatchy and Rosenblum 1966a). In this laboratory, the study on mutants resulted in the same conclusion. Thirty six staphylococcal mutants were obtained, all of which produced materials that cross-reacted with antibodies to alpha toxin. All of these cross-reactive materials were nonhemolytic and nonlethal for mice (Machowski-Syrowik 1980).

Around the 1930's and the 40's four types of staphylococcal hemolysins were recognized. Bigger, Boland and O'Meara (1927)
described a "hot-cold" hemolysin that was subsequently found to be relatively active on sheep erythrocytes (Bigger 1933). The hot cold reaction is such that hemolysis is significantly increased if the incubation at 37 C is followed by a period of holding at 4 C. In 1935, Glenny and Stevens distinguished immunologically, 2 staphylococcal hemolysins, and found that erythrocytes of rabbit were more sensitive to the one hemolysin while erythrocytes of sheep were more sensitive to the other. They designated the former hemolysin alpha- and the latter hemolysin beta-, a terminology adopted ever since. In the following year, Morgan and Graydon (1936) reported two antigenically different hemolysins which they termed alpha 1 and alpha 2. Two years later, Smith and Price proposed the existence of gamma lysin which was subsequently confirmed by Mark (1951) who demonstrated immunologically that delta lysin is distinct from alpha, beta and gamma lysin, and is identical to alpha 2. The four hemolysins have been reviewed (Wiseman 1975; Rogolsky 1979; Freer and Arbuthnott 1983).

Based on the above record, it is clear that specific antisera played a crucial role in the recognition of not only the different staphylococcal hemolysins but also the wide spectrum of biological activities of the alpha hemolysin.

Terminology

Alpha hemolysin is also known as alpha-lysin (Freer and Arbuthnott 1976). Aside from its erythrocyte-lysing activity, this protein is lethal, dermonecrotic, destroys many types of cells, and damages blood platelets (Bernheimer 1965; Arbuthnott
1970; Wiseman 1975). Thus, the term "hemolysin" fails to
describe the broad spectrum of biological activities. In the
search of a better term than hemolysin, Bernheimer (1974)
proposed the term "cytolytic toxins", which were defined as
bacterial products capable of causing physical dissolution of a
variety of cells in vitro. Since low concentrations of the toxin
caused permeability changes without cell lysis (Thelestam and
Mollby 1975a, b), McCartney and Arbuthnott (1978) introduced the
term "membrane-damaging toxins". Rogolsky (1979) suggested that
"the term hemolysin...should be retired with due recognition of
its past distinction". The term alpha toxin is generally
preferred over the term alpha hemolysin by current investigators
(Dalen 1976b, c, d; Watanabe and Kato 1978; Harshman 1979;
Thelestam and Mollby 1979; Maharaj and Packrell 1980; Fussle et
al. 1981; Bhakdi et al. 1981, Lo et al. 1982, Freer and
Arbuthnott 1983), except by some workers in England (Kehoe et al.
1983; Fairweather et al. 1983).

Production and Purification

Numerous methods for the production and purification of
alpha toxin have been vigorously compared and reviewed by Wiseman
(1975). Since then, two relatively simple methods of
purification were reported: one took advantage of the aggregation
of the toxin by heat (Dalen 1976c), and the other involved
adsorption chromatography using controlled pore glass (Bock et
al. 1976). Details of these two methods will be described in
Materials and Methods.
Physicochemical Characteristics

Purified alpha toxin is a protein. Several reports on the amino acid composition (Bernheimer and Schwartz 1963; Coulter 1966; Six and Harshman 1973; Packrell and Wiseman 1976a) are in reasonable agreement. The toxin is positively charged and migrates towards the cathode (Coulter 1966). There has been no evidence of disulfide bonds or sulfhydryl groups in this protein.

Various reports on the molecular weights of alpha toxin, ranging from 10 to 45 kilodaltons, have been summarized (Wiseman 1975). Although the difference in the methods employed may be important, the apparent discrepancies in molecular weight (M.W.) may be attributed to the proteolytic degradation of the toxin in crude preparations. Dalen (1976b) found that in culture, alpha toxin (M.W. 39,000 daltons) was degraded by extracellular proteases to three stable intermediates with M.W. 27,500, 23,500 and 12,000 daltons respectively. This finding could also explain why on different occasions, histidine and arginine (Coulter 1966), histidine (Wiseman and Caird 1970; Packrell and Wiseman 1976a) or alanine (Six and Harshman 1973a,b; Watanabe and Kato 1978) have been ascribed to be the N-terminal amino acid. The partial amino acid sequence of fragments of the toxin is known (Kato and Watanabe 1980).

Alpha toxin occurs in multiple forms. Bernheimer and Schwartz (1963) subjected purified alpha toxin to sucrose density gradient electrophoresis and found 4 peaks which were biologically similar but electrophoretically distinct. In isoelectric focusing, although 80% or more of the hemolytic activity was recovered in a major component with pI 8.5 ± 1 (Wadstrom 1968;
McNiven et al. 1972; Goode and Baldwin 1974; Packrell and Wiseman 1976b), 3 minor components, all of which were biologically active, were located at other pH's (Wadstrom 1968; McNiven et al. 1972). When each of the components were refocused, the same pattern of 4 bands appeared (Wadstrom 1968).

Multiple forms of the toxin could have arisen through aggregation. Arrhenius (1907) was the first to report that brief heating at 60 C converted alpha toxin into nonhemolytic aggregates which became hemolytic again following heating at 100 C. Coulter (1966) found that aggregates, which precipitated upon storage of purified alpha toxin in physiological buffer at 4 C, were nonhemolytic but immunogenic.

Multiple forms of alpha toxin were also observed in sedimentation studies. The sedimentation coefficient of alpha toxin changes from 1.4S, when freshly prepared, to 2.8S after 3 days of standing at 4C in physiological buffer (Packrell and Wiseman 1976b). Preparations of purified toxin actually contained 70 to 90% of 3S molecules and 10 to 30% of 12S forms (Arbuthnott et al. 1973; Bernheimer 1974). These 12S forms were indistinguishable by electron microscopy from those 12S forms obtained by heating at 60 C: both forms were seen as rings which contained 6 subunits arranged hexagonally (Arbuthnott et al. 1973). According to Arbuthnott and coworkers (1973) 12S forms are nonhemolytic and nonlethal, but Bernheimer (1974) found that the hemolytic activity varied considerably among the different preparations of 12S forms. Fussle et al. (1981), who chromatographed alpha toxin (purified by Behringwerke, Marburg,
W. Germany) over Sephacryl-S 300 (Pharmacia, Sweden), obtained preparations containing 98% of 3.3S toxin. The hemolytic forms with S value between 2.8 and 3.3 has been referred to as the "native toxin" (Fussle et al. 1981) as opposed to the 12S forms. Wiseman (1975) reasoned that the inactivation of alpha toxin at 60°C and the subsequent reactivation at 100°C, or by 8 M urea (Arbuthnott et al. 1967; Dalen 1976c) may be the result of interconversion between the 3S and the 12S forms, and that the insoluble precipitates may be the result of further aggregation of the 12S forms.

Biosynthesis

Little is known about the biosynthesis of alpha toxin. Arginine, glycine and proline were found to be essential in toxin production (Dalen 1973c; Gladstone 1938). Parker et al. (1926) increased production by incubating cultures under 10% CO₂ in air. Burnet (1930) found that 20-40% CO₂ and 0.3% agar enhanced the yield of toxin in his "Wood 46" strain but not in the "Wood Albus" variants. The use of CO₂ was followed by others (Kumar and Lindorfer 1962; Lominski and Arbuthnott 1962; Robinson and Thatcher 1963; Marucci 1963a; Coulter 1966; Wiseman et al 1975). Dalen (1973a,b) suggested that the stimulating effect of CO₂, serine and glycine, were related to their role as precursors of histidine. However, he found that, unlike tetanus toxin (Miller et al. 1960), alpha toxin production was not directly correlated with the intracellular level of free histidine (Dalen 1973b).

Alpha toxin is produced during the logarithmic phase of growth. It is an exotoxin because it is released by intact cells
(Duncan and Cho 1971). It constituted 2% of the dry weight of the cell (Bernheimer and Schwartz 1963; Duncan and Cho 1971) and was localized solely on the inner surface of the cytoplasmic membrane prior to release (Coulter and Mukherjee 1971).

The genetic control of alpha toxin biosynthesis was examined by McClatchy and Rosenblum (1966a,b). They obtained 2 groups of mutants. One group produced immunologic cross-reactive material with varying degrees of dermonecrotic and lethal activities. The other group produced no cross-reactive materials and is usually fibrinolysin negative. The close association between the production of fibrinolysin and alpha toxin was confirmed (Wheller 1975, Brown and Pattee 1980). Furthermore, transductional analysis demonstrated genetic recombination between members from the two groups but not between members within each group. Based on these findings, they postulated that at least two genetic loci, a regulatory and a structural gene, were involved in the biosynthesis of alpha toxin.

There is some evidence that alpha toxin may be under plasmid control. Witte (1976) found a high spontaneous rate of loss of the alpha toxin marker in clinical strains of S. aureus. There are 3 genetic linkage groups for the chromosome of S. aureus and the chromosomal determinant for alpha toxin production was found in linkage group 3 (Pattee et al. 1978). In strain Ps53, this determinant was adjacent to the determinant of penicillinase production (Brown and Pattee 1978). The absence of plasmids in the toxin-producing strain 8325 argued against plasmid involvement in toxin production (Brown and Pattee 1980). Recently, the determinant for alpha toxin from strain Wood 46 has
been cloned in *E. Coli* K-12 (Kehoe et al. 1983), *B. subtilis*, and other strains of *S. aureus* (Fairweather et al. 1983).

**Biological Activities**

The 5 effects (hemolytic, dermonecrotic, lethal, platelet-damaging, and cytotoxic) were described in the International Encyclopedia of Pharmacology and Therapeutics (1971), as well as by others (Wiseman 1975; Jeljaszewicz 1972, Jeljaszewicz et al. 1978; Freer and Arbuthnott 1976; Rogolsky 1979).

The diversity of cell types susceptible to alpha toxin implied a common denominator among the different target cells. Since all susceptible cells have, in common, a cell membrane which is the primary contact with the toxin, studies focused on the interaction between alpha toxin and membranes. Various methods used in these studies included fluorescein-labelled antitoxin (Kaplan 1972), fluorescein-labelled anti-immunoglobulin (Klainer et al. 1964), $^{125}$I-labelled toxin (Cassidy and Harshman 1973, 1976), fluorescein-labelled toxoid (Barei and Packrell 1979), adsorption of the toxin with membranes (ghost) (Wiseman et al. 1975), and electron microscopy (Freer et al. 1968, 1973).

All these studies showed that alpha toxin interacts with membranes. Furthermore, membrane-bound alpha toxin was recovered and identified as such (Fussle et al. 1981). Most investigators considered the cell membrane to be the primary site of attack by alpha toxin, and erythrocytes are the favourite model as target cells.

Two fragments were obtained by mild tryptic digestion of alpha toxin: The 20 kilodalton fragment was nontoxic and tended
to aggregate, but the 17 kilodalton fragment retained its lethality for mice (Watanabe and Kato 1978, Kato 1979).

Hemolytic Events

In the 60's, studies on the kinetics of hemolysis (Lominski and Arbuthnott 1962; Marucci 1963a,b; Cooper et al. 1964a,b; Madoff et al. 1964; Arbuthnott 1970) revealed a sigmoidal curve that indicated three stages in the time course of hemolysis: a prelytic lag, a lytic phase, and then a tailing off or slow down in the rate of hemolysis. In the prelytic lag, loss of selective permeability of the membrane was indicated by the leakage of potassium ions. The lytic phase represented a time of rapid release of hemoglobin from the damaged erythrocytes (Lominski and Arbuthnott 1962, Cooper et al. 1964a,b). Marucci (1963b) identified two steps in the hemolytic process. The first step involved the reaction of alpha toxin with the erythrocyte. The second step, which led to the release of hemoglobin, was an intrinsic reaction of the damaged cell and took place without further participation of the toxin. The first step could be stopped by antitoxin but not the second step, at which point, the cells were "committed" to lyse. Marucci suggested that the lag phenomenon could be due to the progress of several sequential steps leading to lysis or to the necessity for the accumulation of a critical number of "hits" before the erythrocytes is destroyed. Lo and Fackrell (1979) defined 2 events immunologically: first, the binding of the toxin to erythrocytes and, second, destruction of the erythrocytes. Each of the events could be blocked by specific antibodies. Cassidy and Harshman
(1976) proposed 3 sequential events: firstly, binding of the toxin to erythrocytes; secondly, induction of foci of membrane injury that lead to the leakage of small, inorganic molecules; and thirdly, the eventual osmolysis of the cells.

Mode of Action

Alpha toxin was categorized under the group of "toxins for which the most significant event in toxin-membrane interaction is not known" (Bernheimer 1976). There are two schools of thought: the enzymatic versus the nonenzymatic mechanism.

Studies on the kinetics of hemolysis revealed 2 pieces of evidence that implicated an enzymatic mode of action for alpha toxin (Lominski and Arbuthnott 1962). First, all of the hemolytic activities were recovered in the supernatant after hemolysis. This observation suggested that the toxin, like a catalyst, was not "used up". Secondly, the plot of the rate of hemolysis versus the concentration of alpha toxin was compatible with an enzyme reaction. Although Marucci (1963a,b) confirmed the first piece of evidence, he argued that the phenomenon may merely reflect a situation of toxin in excess. In an attempt to ascertain whether or not a lytic agent is "used up", Bernheimer (1970) compared curves of "lysis versus the log of erythrocyte concentration" among lytic agents of enzymatic and nonenzymatic nature. Although alpha toxin conformed to the enzymatic curve, staphylococcal beta-lysin, which is an enzyme, did not. One of the arguments against the enzymatic implication was that some of the bound toxin was released from the membrane prior to the onset of hemolysis (Cassidy and Harshman 1973) and kinetic studies did
not taken into account the dynamics of binding and release (Bernheimer 1976). However, the finding of partial release of the toxin after binding was retracted (Cassidy and Harshman 1976). The enzymatic theory did not receive serious attention until the work of Wiseman and coworkers (Wiseman and Caird 1970, 1972; Wiseman et al. 1975), who found that proteinaceous materials were released from erythrocytes or erythrocyte membranes (ghosts) by the toxin. Furthermore, they showed that the native toxin, which did not hydrolyse the artificial substrate tosylarginine methylester (TAME), could be activated by insolubilized trypsin. The activated toxin not only hydrolysed TAME but exhibited a different Km from that of the trypsin. These investigators proposed that the native toxin is an inactive protease (pro-toxin) which can be activated by membrane protease(s) to become proteolytic itself (Wiseman et al. 1975, Wiseman 1975). Freer et al. (1973) were unable to confirm this proteolytic mechanism. The difference in the method of preparing the 'ghosts' (Dodge et al. 1963) could be an explanation for the conflicting findings.

The nonenzymatic view stresses the "surface activity" of the alpha toxin on membranes. Weissman et al. (1966) first showed the release of marker molecules such as chromate and glucose from liposomes by alpha toxin. The toxin showed no charge preference since liposomes containing lecithin, cholesterol, and either dicetylphosphate or stearylamine were equally sensitive. A hydrophobic interaction between alpha toxin and membrane lipids in general was confirmed
by further studies on mixed lipid monolayers (Buckelew and Colacicco 1971). Simply through the contact with deoxycholate detergent micelles, the toxin monomers self-assemble to form amphiphilic hexamers (Bhakdi et al. 1981).

Freeze etch studies revealed plaques having few membrane intercalating particles on the fractured plane of toxin treated ghosts, and suggested that the toxin may penetrate the hydrophobic interior of the membrane (Bernheimer et al. 1972; Freer et al. 1973). These changes resembled closely those described for erythrocyte membranes treated with phospholipase A or saponin (Speth et al. 1972). However, alpha toxin has been shown not to be a phospholipase (Coulter 1966; Wiseman and Caird 1970).

Freer et al. (1968) showed by electron microscopy that alpha toxin polymerizes to form regular arrays of ring-like structures on liposomes and on erythrocyte membranes. Each ring was believed to be hexamer of the toxin. These rings on the membrane were morphologically indistinguishable from those nonhemolytic polymeric 12S forms which occurred naturally in toxin preparations or arose from heat-inactivation of the toxin at 60 C. The ring-like structures, isolated from membranes solubilized by Triton X-100, were identified to be alpha toxin on the basis of immunological, biochemical and ultrastructural properties (Fussle et al. 1981). The ring-like structures harbored central pits 2-3 nm in diameter; this dimension coincided with the effective size of lesions measured by the release of markers from toxin-treated, resealed ghosts. Thus, they proposed that the native, hydrophilic, 2.8S toxin molecules oligomerized on membranes to
form an amphiphilic ring-like structure that embedded within the lipid bilayer and, consequently, generated a transmembrane channel. The ring-like structures on membranes have been observed by two independent group of investigators (Cassidy and Harshman 1976; Barei and Fackrell 1979). These two groups posed the argument that the ring structures, which were seen only at high concentrations of alpha toxin, were unrelated to the binding of alpha toxin to high affinity receptors at low, but lytic, concentrations of the toxin. Yet, one could pose the counter-argument that the failure to observe ring structures at low concentrations of the toxin simply means the rings were difficult to find, and therefore did not preclude their role in hemolysis. The sensitivity of erythrocytes from different animal species to alpha toxin varies widely (Bernheimer 1965; Cooper et al. 1966). This phenomenon is not explained by the nonspecific interaction of alpha toxin with lipids (Bernheimer 1974) but is related to the number of receptors on the surface of the different erythrocytes (Barei and Fackrell 1979).

The following quotation best describes the status of the surface activity studies: "... It must be emphasized that, although these findings strongly support the suggestion that, alpha toxin is capable of interacting with lipids of biological membranes, they do not in themselves provide an explanation for the mechanism of action of staphylococcal alpha toxin, nor do they explain the high specificity of alpha toxin for rabbit erythrocytes.... Although the bulk of recent work has been concentrated on the surface active properties, the enzyme
hypothesis cannot, and indeed should not, be excluded as a possible mechanism..." (Bernheimer 1976).

While the mode of action has been debated, progress has been made in studies designed to characterize the receptor for alpha toxin. Knowledge of the receptor may, in the future, help to elucidate the mode of action of the toxin.

Receptor for alpha toxin

A number of workers have postulated the existence of specific receptors on the membrane for alpha toxin (Marucci 1963a; Cooper et al. 1964a,b; Cassidy and Harshman 1973; Klainer et al. 1964, 1972; Wiseman and Caird 1972; Wiseman et al. 1975; Kato et al. 1975, Kato and Naiki 1976). As well, the binding of alpha toxin to erythrocytes always involves a specific region of the toxin (Lo and Fackrell 1979).

Cassidy and Harshman (1976) found approximately 5,000 receptors on the surface of rabbit erythrocytes and none on human erythrocytes. Barei and Fackrell (1979) estimated 125,000 receptors on rabbit erythrocytes and none on human erythrocytes. The disagreement in the number of receptors on rabbit erythrocytes may be due to the difference in methods; the former workers used toxin whereas the latter workers used toxoid. The latter workers also established a correlation between receptor number on erythrocytes from different animal species and hemolytic sensitivity to alpha toxin. Furthermore, they observed a threshold value of 37,000 receptors, below which hemolytic sensitivity was no longer a function of the receptor.

Kato et al. (1975) postulated that the receptor was a
glycoprotein, but later suggested a N-acetyl glucosaminyl-ganglioside was the receptor (Kato and Naiki 1976). Cassidy and Harshman (1976) suspected a proteinaceous nature of the receptor because pronase treatment of erythrocytes resulted in a decreased number of binding sites. Maharaj and Fackrell (1980) provided convincing evidence that Band 3, the transmembrane protein involved in anion transport, was the high affinity receptor in rabbit erythrocytes. They showed that only those enzymes and lectins that reacted with Band 3, affected the hemolytic sensitivity of the erythrocytes. Furthermore, purified Band 3 inhibited the hemolytic activity of alpha toxin, and erythrocytes preincubated with antibodies to Band 3 became resistant to the toxin. Although purified glycophorin, the major transmembrane glycoprotein, also inhibited the activity of alpha toxin (Bernheimer and Avigad 1980), there has been no evidence that native glycophorin on the erythrocyte membrane reacts with the toxin.
MATERIALS and METHODS
BIOLOGICAL METHODS

Cultures

The *Staphylococcus aureus* strain Wood 46 used in this study was passed on from Gow and Robinson (1969) at the University of Western Ontario to Wiseman, Caird and Packrell (1975) at the University of Manitoba and then to Lo and Packrell (1979) at this university. This strain fulfilled the criteria for *S. aureus* given by Baird-Parker (1974). This strain was preserved by lyophilization. Working stocks were cultured in Dolman-Wilson (DW) medium (Dolman and Wilson 1940) with or without agar supplement as desired. This strain was found to be "non-typeable" when the phage susceptibility pattern was tested by the Canadian Communicable Disease Centre in Ottawa.

After several transfers, the culture frequently produced less toxin. Streaking of such cultures on blood agar plates revealed nonhemolytic colonies which were about twice the size of the wildtype colonies. Spontaneous nonhemolytic mutants have been reported by Dalen (1976d). Cultures giving less than 500 hemolytic units (see Hemolytic Titration below) were discarded, and a new working culture was reconstituted from the lyophilized stock. When the lyophilized stock was depleted, the problem with nonhemolytic mutants was corrected by passage of the bacteria through mice. A 0.2 mL sample from a 12 h culture in T-soy broth was injected into a mouse intraperitoneally. Twelve to 18 h later, the sick mouse was sacrificed and dissected. The hemolytic strain was recovered by swabbing the chest cavity and then cultured on blood agar plates. Two passages were sufficient
to restore the original hemolytic titre to the culture.

Production of Crude alpha toxin

The method of Wiseman et al (1975) was used. Twenty four sterile 1-litre flasks, each containing 500 mL of DW liquid medium, were inoculated with 10 mL of an 18 h culture of S. aureus. The flasks were incubated at 37 C in an atmosphere containing 10 % (V/V) CO₂, and shaken at 150 rotations per minute. After 36 to 48 h, the bacteria were pelleted by the Sorvall® TZ-28 continuous-flow centrifugation system (DuPont Instruments, Biomedical Division, Newtown, CT 06470). The supernatant thus recovered contained alpha toxin.

It should be noted that CO₂ exerts different effects on the production of toxin by different isolates of Wood 46. For instance, the Wood 46 strain obtained from the American Type Culture Collection (ATCC 10832) was hemolytic when cultured aerobically, but was nonhemolytic when cultured under 10 % CO₂. In contrast, 10 % CO₂ increased the hemolytic titres of our strain of Wood 46 by 32-fold when compared to aerobic cultures.

Concentration of Crude Alpha Toxin

Two methods to concentrate the crude toxin were studied. The procedures for each method are outlined below.

The first method (Wiseman et al 1975) involves the overnight precipitation of the crude toxin at pH 4 by 35 % (V/V) final concentration of methanol at -20 C. The precipitate was obtained by continuous centrifugation as described above and redissolved in 500 mL of 0.01 M sodium phosphate buffered saline (PBS), pH
7.0. Saturated ammonium sulphate solution was added to a final concentration of 40\% (V/V). The mixture was left at 23 °C for 8 to 12 h and then centrifuged at 10,000 \times g for 30 minutes. The pellet was discarded. To the supernatant, saturated ammonium sulphate solution was added until the final concentration of the latter was 60\% (V/V). This mixture was allowed to sit at 23 °C for 8 to 12 h. The precipitate thus formed contained semi-purified alpha toxin in a concentrated form.

The other method of concentration (Dalen 1976c) involved heating the crude toxin at 60 °C, pH 5, for 30 min and collecting the precipitate by centrifugation. After two washes of the precipitate in 0.03 M borate buffer, pH 8.6, the precipitate was dissolved in the same buffer containing 8 M urea.

The two methods were compared. A batch of crude toxin was divided into two identical portions. Each portion was processed by one of the two methods, respectively. The second method (Dalen 1976c) recovered about 5 times more hemolytic units than the first method, and was therefore adopted.

**Purification of Alpha Toxin**

Purification of alpha toxin was achieved by adsorption chromatography on controlled pore glass (CPG-10-350 by Electro-Nucleonics) (Bock et al. 1976; Lo and Fackrell 1979). Unfortunately, the manufacturer could no longer supply this material. Controlled pore glass of similar specifications purchased from Sigma Chem. Co. (PG-350-120, lot 99C-0498) did not purify alpha toxin according to the method of Bock et al. (1976). Therefore, this method of purification was discontinued in our
laboratory.

Two alternative methods were available but only one of these methods preserved the hemolytic activity of the toxin. The method of choice depended on the experimental requirement for hemolytic activity.

When hemolytic toxin was required, the method of Wiseman et al (1975) was used. The concentrated crude toxin was dialysed against 20 volumes of Hallander's buffer (0.01 M sodium phosphate buffer, pH 7.0, supplemented with 0.5 % (W/V) NaCl) at 4°C for 2 days during which the buffer was changed twice daily. The dialysate was fractionated by gel filtration through a Sephadex G-75 column which was eluted with the same buffer. A sample (100 microlitres) of each fraction was added to 100 microlitres of a 2% suspension of rabbit erythrocytes in PBS. Fractions which showed complete hemolysis were pooled and concentrated in 80% (V/V) final concentration of ammonium sulphate solution. Samples of the purified toxin were dialysed against PBS before use. Purity of the toxin was established when a single precipitin line was obtained in Ouchterlony immunodiffusion in agar-gel test (Ouchterlony 1958) with antisera that were raised against concentrated crude toxin.

Whenever hemolytic activity was not an experimental requirement, alpha toxin or cross-reactive materials were purified in a single step by affinity chromatography using antibodies raised against purified toxin (Maharaj 1980, Machowski-Syrowik 1980).
Toxoid

For some experiments, purified alpha toxin was rendered nonhemolytic by heat at 60°C for 30 min. This turbid material was centrifuged at 8,000 x g for 1 h and the clear supernatant was used as toxoid. The usage of the term "toxoid" was in accordance with Burnett (1931) who restricted this term to physically denatured, nontoxic forms and applied the term "anatoxin" to formalin detoxified forms.

Erythrocytes

Human blood (type A or O, Rh+) was obtained from the Red Cross or donated by volunteers. Citrated sheep blood was supplied by Woodlyn Laboratory, Guelph, Ontario. Rabbits, guinea pigs, rats and mice were maintained and cared for in accordance with "The principle of care of experimental animals -- A guide for Canada". These animals were bled for erythrocytes or sera, as required. Coagulation was prevented either by the use of glass beads or heparinized tubes. The former method was particularly useful when both erythrocytes and serum were desired. Erythrocytes were packed by centrifugation (600 x g, 10 min) and the supernatant was removed. Erythrocytes that were not used immediately were stored in an equal volume of Alserver's solution (Carpenter 1975). Prior to use, the erythrocytes were washed three times in 6 to 10 volumes of PBS and the top layer of white cells (buffy coat) was removed. The concentration of the erythrocytes is expressed in % which indicates the mL of packed cells (600 x g, 10 min) per 100 mL of final suspension. The washed erythrocytes were resuspended to a 2% concentration (V/V) in PBS unless otherwise specified.
Hemolytic Titration

The 50% end point method (Wiseman and Caird 1972) was followed except the volumes of the reagents were scaled down proportionally. Fifty microlitres of alpha toxin were serially diluted in 50 microlitres of PBS in Microtitre plates (Cooke Lab. Products, Alexandria, Virginia, USA) with U-shaped wells. Fifty microlitres of PBS were added to each well followed by 100 microlitres of a 2% suspension of rabbit erythrocytes. The mixture was incubated at 37°C for 1 h and then centrifuged at 600 x g for 10 minutes. One hundred microlitres of the supernatant was diluted 1:3 with PBS and absorbance was read spectrophotometrically at 541 nm (1-cm light path). The hemoglobin contents of the supernatants thus measured were translated into % hemolysis by comparison to standards. The standards were prepared from a lysate of 2% erythrocytes in distilled water. This comparison and the subsequent determination of the 50% end point were done using a computer program for linear regression. One hemolytic unit (HU) is the reciprocal of that dilution of the toxin giving 50% hemolysis.

Antisera

Eight adult New Zealand white rabbits (Triangle Rabbitry, Paris, Ontario) were hyperimmunized with purified toxoid at a concentration of 2 mg/mL in PBS. On day zero, 0.1 mL of toxoid was injected intravenously while 0.5 mL was mixed with 0.5 mL of Freund's complete adjuvant (Difco Lab.) and injected subcutaneously in 4 sites on the rabbit's shaved back. On day 3 and 6, 0.25 and 0.5 mL of toxoid, respectively, was administered
intravenously. Thereafter, 1 mL of toxoid was injected intravenously every 3rd day up to the 21st day. The rabbits were bled for sera on day zero before injection. Bleedings were repeated every 7th day for 7 weeks.

Normal human sera were obtained from volunteers between the ages of 19 to 25. Sera from other animals have been described in the section for erythrocytes above. All sera were clarified by centrifugation ($6,000 \times g$, 30 min).

All sera were checked for antibodies that cross-reacted with the test erythrocytes, and were adsorbed when necessary, according to the procedures described under the Indirect Hemagglutination Test below.

Those sera to be assayed in the complement fixation test (see below), and those sera to be used as amboceptors (see below), were heated at 56°C for 30 min prior to use.

For some experiments, portions of the sera were purified to IgG fractions according to the method of Campbell et al. (1970). This method involves initially the repeated precipitation of gamma globulins by one-third final concentration ($V/V$) of saturated ammonium sulphate solution, and then the purification to IgG by ion exchange chromatography using DEAE cellulose.

Immunoadsorbent (L9CE)

Rabbit erythrocytes were coated with alpha toxoid, fixed with glutaraldehyde and washed with lysine as previously described (Lo and Packer 1979). The coating step involved adjusting washed erythrocytes to a final suspension of 10% ($V/V$) in alpha toxoid (1 mg/mL), and shaking this mixture periodically
over 30 min at 23 C. Unbound materials were removed by washing the coated erythrocytes three times in PBS. The bound toxoid was covalently bonded to its carrier erythrocyte via glutaraldehyde. The coated erythrocytes were adjusted to a 2% (V/V) suspension in 0.1% (W/V) glutaraldehyde solution prepared in PBS. The mixture was resuspended at 23 C for 1/2 h and then washed three times in PBS. To block excess aldehyde, the erythrocytes were resuspended to a final concentration of 20% (V/V) in 0.1 M lysine-HCl solution in PBS for 1 h at 23 C, and then washed thrice.

These lysine- and glutaraldehyde-treated, toxoid-coated erythrocytes (LGCE) were used as an immunoadsorbent to remove IHA-Ab from antisera. Subsequently, IHA-Ab were eluted from the LGCE by heat (56 C, 20 min) as reported previously (Lo and Fackrell 1979).

Indirect Hemagglutination (IHA) Test

A serial, twofold dilution of 50 microlitres of serum was prepared in PBS. To each well, 50 microlitres of a 1% suspension of LGCE were added. The hemagglutination patterns were graded from 4+ to 0 after 90 min of incubation at 4 C. The 4+ indicated a prozone reaction. The 3+ indicated a strongly positive reaction in which part of the layer of cells had rolled down from the edge of the well. A 2+ reaction showed an even layer of agglutinated cells covering the bottom of the well. Formation of a big ring was recorded as 1+ and a very small ring or a button as 0. The IHA titre of a given serum was the reciprocal value of the lowest serum concentration giving a 1+ reaction. Titres of less than 2 were not considered significant. A known pair of positive and negative sera were included to
ensure reproducibility.

A control for the LGCE consisted of 50 microlitres of LGCE in 50 microlitres of PBS. Tests were rejected if spontaneous hemagglutination occurred.

A control for the serum being tested consisted of 50 microlitres of serum plus 50 microlitres of a 1% suspension of uncoated erythrocytes. Hemagglutination indicated the presence of antibodies that cross-reacted with the erythrocytes and the IHA titre was invalid. Such a serum was adsorbed with 2 volumes of packed erythrocytes at 4°C for 5 min. The adsorption was repeated until no hemagglutination occurred when the adsorbed serum was titrated with the uncoated erythrocytes. Then, the IHA test was performed on the adsorbed serum.

Neutralization Test

Starting with 50 microlitres of serum, a twofold serial dilution was prepared in PBS. Fifty microlitres of toxin, which contained 2 HU as the challenging dose, was added to each well. Then, 100 microlitres of a 2% suspension of rabbit erythrocytes were added. After 1 h of incubation at 37°C, the supernatants of the mixtures were measured for hemoglobin content as described for hemolytic titration. The end point was the highest dilution of the serum exhibiting 50% hemolysis or 1 HU. One neutralization unit (N) was defined as the reciprocal of that dilution of serum inhibiting 1 HU (that is, at the end point).

Preparation of solid-phase antiglobulins

Goat antibodies to rabbit IgG, IgM or IgA respectively were purchased from Miles Laboratories. The specificity of each
antiglobulin was tested and guaranteed by the manufacturer. Then, each antiglobulin was semi-purified to IgG by precipitation with a final concentration of 33 % (V/V) saturated ammonium sulphate solution (Campbell et al. 1970). The protein content in each IgG fraction was determined spectrophotometrically using a 1 % extinction coefficient of 13 for goat globulins.

Solid-phase antiglobulins were prepared by coupling the IgG fraction of each of the antiglobulins to carboxymethyl-agarose gel beads. The beads and the coupling buffers were called "Immunobead reagent and coupling kit-G" (lot 16412) by the manufacturer, Bio Rad Laboratories. Coupling procedures were carried out according to the instructions of the manufacturer. The quantities of the beads described hereafter referred to the dry weight of the beads prior to swelling. The antiglobulins were coupled to the beads at a 6 % concentration (W/W) via amide bonds facilitated by 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride at 4 C, pH 6.3. Unbound materials were washed away with PBS. Materials noncovalently bound to the beads were washed away with 5 M guanidine hydrochloride which was removed by subsequent washings with PBS. Ten mg of the prepared beads were suspended in 1 mL of PBS containing 0.01 % (W/V) sodium azide as a preservative. The preparation was stored at 4 C until use.

Removal of a Specific Class of Immunoglobulin from Serum

One hundred microlitres of the prepared beads were packed (1,000 x g, 10 min) in a 1 mL conical centrifuge tube (Fisher Scientific). The supernatant was decanted leaving behind 1 mg of
beads. The tube was inverted so that the small drops of PBS that drained from the beads could be removed with a cotton swab without disturbing the beads. The beads were suspended in 300 microlitres of test serum and incubated at room temperature for 1 h, after which the mixture was resuspended and transferred to 4°C for 3 h. The beads were packed by centrifugation as before. Then, 50 microlitres of the adsorbed serum were removed for the IHA test and another 50 microlitres were removed for the neutralization test. The residual adsorbed serum was transferred to a second centrifuge tube which contained 1 mg of fresh beads and the adsorption procedure repeated. Each test serum was adsorbed twice and titrated after each adsorption.

Amboceptor

Rabbit anti-sheep erythrocytes (lot K7GAFW) were purchased from BBL, Division of Becton, Dickinson and Company, Cockeysville, MD 21030 USA, and used to sensitize sheep erythrocytes. The sensitised cells were used in the standard serological complement fixation tests (see below).

Rat anti-rabbit erythrocyte membranes were prepared as described by Maharaj and Packrell (1980) and were used to sensitize rabbit erythrocytes.

Complement

Sources of complement included sera from humans, rabbits and guinea pigs. Also, lyophilized guinea pig complement (lot 8111514 and lot E9HAVP) and diluent were purchased from BBL, Division of Becton, Dickinson and Company.
To ensure that the complement was free of antitoxin and antibodies that cross-react with the indicator erythrocytes, the following procedures were undertaken. Antitoxin in the complement was titrated by the neutralization test and the indirect hemagglutination (IHA) test. Those sera without AB-Ab were chosen. IHA-Ab were removed from such sera by adsorption with the immunoadsorbent LGCE. Then, these sera were checked for antibodies that cross-react with the test erythrocytes. If present, these cross-reactive antibodies were removed by adsorption as described in the IHA test. To ensure complete removal of the cross-reactive antibodies, adsorption was repeated three times even when hemagglutination was negative with the test erythrocytes.

Standard Serological Complement Fixation Test

The procedures employed were those of the Ontario Ministry of Health (anonymous 1974) except for two changes. Firstly, the buffer used was 5 mM barbitol buffered saline, pH 7.4 (BBS) containing 0.15 mM CaCl₂. The BBS was supplemented with 0.5 mM MgCl₂ when complement of either guinea pig or rabbit was used. The buffer contained 1 mM MgCl₂ when human complement was used (Hoffmann and Mayer 1967). Secondly, a 2.8 % (V/V) erythrocyte suspension was used in order to provide sufficient hemoglobin for the spectrophotometric determination of hemolysis.

The antigen box-titration and the CF test proper were performed in Microtitre® plates with U-shaped wells. The final reaction mixture consisted of 50 microlitres each of toxoid, antitoxin, complement (2 units) and 100 microlitres of sensitized
erythrocytes. When incubation was completed, the plates were centrifuged (600 x g, 5 min.) and supernatants were read at 541 nm (1-cm light path). The percent of hemolysis was plotted against serum dilution and the CF titre, which is the serum dilution that gave 50% hemolysis, was determined (Rapport and Graf 1957). Undiluted serum that produced more than 50% hemolysis in the test was considered negative (CF titre = neg). All tests were performed in duplicate.

Quantitative CF Test

The principle of the quantitative complement fixation test (Osler 1958, 1976; Mayer 1971) was adhered to except that the immune complexes were immobilized on the membrane of the red cell by use of LGCE. LGCE were incubated with an excess of antiserum (4°C, 4h) and then washed 3 times in PBS to remove unbound materials. The erythrocytes that were now coated with an insoluble toxoid-IHA complex were centrifuged at 600 x g for 10 min, resuspended in a known quantity of complement and incubated at 4°C overnight. After a second centrifugation the supernatant was titrated for residual complement. The recovery was compared to that of LGCE that was not exposed to antitoxin. The quantity of complement was defined in terms of the 50% hemolytic unit of complement (CH50), which is that amount required to produce 50% hemolysis (Mayer 1971).

Reverse Passive Arthus Reaction

This is a type of cutaneous reaction induced by intradermal inoculation of antibody in an animal with antigen in its circulatory system. This approach is suitable for investigating
hypersensitivity mediated by different types of antitoxins. The method used is detailed by Cochrane (1976) who considered that "Perhaps the most consistently and readily induced lesion is the Reverse Passive Arthus."

A New Zealand White rabbit, weighing 4.54 Kg, was injected intravenously with 3 mg of alpha toxoid in sterile PBS. Fifty min later, 50 mg of Evans Blue dye, dissolved in 0.5 mL of PBS, was administered intravenously. Then, 100 microlitres of antitoxins, containing approximately 50 micrograms of protein, were injected intradermally into the shaved back of the rabbit. The reactions were observed over a period of 36 h. The gross reaction is graded according to the following criteria (Cochrane 1976):

+- Mild edema and erythema lasting approximately 4 h
+
Mild edema with erythema, both lasting 24 h and measuring 1.5 cm in greatest diameter

2+ Mild to moderate edema, erythema measuring 1.5 to 3 cm in greatest diameter; brownish central discoloration measuring up to 0.5 cm at 4 to 6 h after injection.

3+ Moderate edema, erythema measuring 3 to 4 cm; hemorrhage and brownish discoloration measuring 0.5 to 1.5 cm in diameter

4+ Severe edema; marked erythema and hemorrhage measuring more than 1.5 cm; sloughing of central reaction site

Purification of Band 3

Band 3 was isolated from rabbit erythrocyte membranes according to Maharaj and Fackrell (1980) as follows. Erythrocyte membranes were prepared according to Dodge et al. (1963), and then solubilized in 0.5 % (V/V) Triton X-100 in PBS. The Triton X-100 was removed by adsorption onto Amberlite XAD—2 beads
(Sigma) as described by Cheetham (1979). The solution was clarified by centrifugation (20,000 x g, 30 min, 4 C) and applied to a concanavalin A-sepharose affinity column. Unbound material was eluted with 0.02 M sodium phosphate buffer, pH 7.4, supplemented with 0.1 M NaCl. Band 3 was desorbed with the same buffer containing 0.1 M methyl alpha-D-mannoside (Aspberg and Forath, 1970). This material was dialysed against 3 changes of PBS overnight and used immediately.

Enzyme-linked Immunosorbent Assay (ELISA)

An ELISA for alpha toxin was developed by Surujballi and Fackrell (1983). This method consisted of 3 steps: preparative, binding and detection.

In the preparative step, a portion of the IgG fraction containing antitoxin was coupled to the wells in Microtitre® plates. The IgG fraction was adjusted to a protein concentration of 10 µg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The plates were washed with 95 % ethanol and dried. Two hundred microlitres of the IgG solution were added to each well in a plate. The wells of the outermost two rows and columns of a plate were not used. After incubation at 37 C for 6 h, the plates were inverted to discard the fluids. Each well was washed 3 times with 250 microlitres of PBS supplemented with 0.05% (V/V) Tween 20 (PBS-Tween). At this point, the wells were coated with antitoxin.

In the binding step, 200 microlitres of test material such as alpha toxin were added to a well and incubated at 23 C for 12 h. Unbound materials were removed by three washings with PBS-
Tween, and the plate was inverted to dry for 10 min.

A portion of the IgG fraction of antitoxin was conjugated to alkaline phosphatase (EC.3.1.3.1, type 3, Sigma Chem. Co.) via glutaraldehyde (Clark and Adams 1977) ahead of time. This enzyme-linked antitoxin was referred to as the "ELISA-conjugate". In the detection step of the ELISA, 200 microlitres of a working dilution of the ELISA-conjugate was added to the wells. The plate was incubated and the wells were washed as before. Then, 200 microlitres of para-nitrophenylphosphate solution (0.67 mg/mL of 0.5 M Tris buffer, pH 9.8) were added to each well. The reaction was allowed to proceed for 2 h at 37°C and then stopped by 50 microlitres of 2N NaOH. The color developed was quantitated by reading the absorbance at 405 nm (1-cm light path).

Quantitative Precipitin Test

The principles of this test as outlined by Maurer (1971) were observed. Fresh preparations of alpha toxin were used on the same day the materials were purified. In the test series, various concentrations of alpha toxin in PBS were added to a constant volume of antitoxin. In the control series, PBS was substituted for the antitoxin. An additional control which consisted of antitoxin plus PBS was set up as the antitoxin control. The mixtures were incubated at 37°C for 7 days and mixed daily. The precipitates obtained by centrifugation (10,000 x g, 1 h, 4°C) were washed twice with 1 mL of cold PBS each time. The tubes were inverted to dry and the amount of protein assayed by the method of Bradford (1976).
The amount of non-immune precipitates at each concentration of the toxin was determined in the control series. The amount of non-immune precipitate due to the antitoxin alone was determined in the antitoxin control. Subtraction of these amounts from the amount of total precipitate in the test series provided an estimation for the quantity of immune precipitates at each concentration of alpha toxin added. The quantity of antibody precipitated at each concentration of alpha toxin was calculated by subtracting the quantity of toxin added from the quantity of immune precipitate. The mole ratio of antibody:antigen was calculated using M.W. of 150,000 for IgG and 40,000 for alpha toxin.

Preparation of Fab

Fab (fragment antigen-binding) was prepared by papain digestion of the IgG fraction of rabbit antitoxin according to Porter (1959) and to Putnam et al. (1961). Two mg of papain (Schwarz/Mann, Division of Becton, Dickinson and Co.) was added to 100 mg of IgG in 20 mL (final reaction volume) of 0.1 M sodium phosphate buffer, pH 6.0, supplemented with 0.01 M EDTA and 0.01 M cysteine. The mixture was shaken gently at 37 C for 18 h and then centrifuged at 1,000 x g for 15 min at 23 C. The supernatant was dialysed against two daily changes of distilled water at 4 C for 5 days with constant and vigorous stirring. The solution, which turned slightly milky due to the formation of Fc, was centrifuged (20,000 x g, 1 h, 4C). The supernatant was dialysed against 0.01 M sodium acetate buffer, pH 5.5 overnight and then applied to a carboxymethyl cellulose column (25 x 2.4 cm., flow
rate 20 mL/h, fraction size 4 mL) equilibrated with the same buffer at 37 C. The column was eluted further with 1 litre of the same buffer. Bound materials were desorbed by a continuous gradient of sodium acetate at pH 5.5. The molarities of the starting buffer and the final buffer were 0.01 and 0.9 respectively, and the volume of the mixing chamber was 1 litre. The first and second protein peaks were pooled, dialysed against distilled water and then concentrated by lyophilization. Upon reconstitution in PBS, a portion of the Fab was tested for purity by electrophoresis in 7.5 % polyacrylamide tube gels in the presence of sodium dodecyl sulphate (Laemmli 1969). This test revealed two bands with molecular weights of about 25,000 and 30,000 respectively, corresponding to the light and heavy chains of the Fab. No undigested IgG was detected.

Labelling the Fab with Fluorescein

Purified Fab were labelled with fluorescein isothiocyanate (FITC) (Sigma Chem Co.) according to Sternberger (1974). FITC was added to Fab in a mole ratio of 8 to 1. The conjugation was carried out in 0.1 M carbonate-bicarbonate buffer, pH 9.5, at 4 C for 1 h. The mixture was centrifuged (10,000 x g, 15 min, 4 C) and the supernatant was dialyzed against two daily changes of deionized distilled water over 4 days. The dialysate was desalted through a Sephadex® G25 column which was equilibrated with PBS. The absorbance of each fraction (0.5 mL) was taken at 280 nm and at 495 nm for the measurement of protein and fluorescein, respectively. A single fluorescent peak, which coincided with the protein peak, was obtained. Therefore, the preparation
did not contain any unbound FITC.

The fluorescein to protein ratio of the preparation was determined by the method of McKinney et al. (1964) as follows. The mole of fluorescein bound to the Fab was determined fluorometrically by comparison to a standard series of fluorescein diacetate. The protein concentration was determined by the method of Lowry et al. (1951) except the absorbance was read at 750 nm (this avoided the fluorescence at 516 nm). Moles Fab were calculated using a molecular weight of 50,000 for Fab.

The fluorescein label made possible the measurement of the quantity of Fab bound to, and free from, alpha toxin in solution (see below).

**Fluorescence Polarization**

The objective was to determine the number of antigenic determinants on alpha toxin. Scatchard analysis provides a measure for the number of binding sites. Since the valency of Fab is 1, the number of binding sites becomes an indication of the number of determinants. Since we wish to determine the maximum number of Fab bound per toxin, the Fab becomes the dependent variable. For this reason, Fab was labelled instead of the antigen. Aside from these crucial designs, the principles and methods used were that of Dandliker (1971) and Dandliker et al (1973, 1980) as outlined below.

The ratio of bound to free is solved by the equation:

\[ \frac{F_b}{F_f} = \frac{Q_f(P - Pf)}{Q_b(Pb - P)} \]

where \( F_b \) and \( F_f \) are the molar concentration of fluorescent Fab
in their bound and free forms, respectively. The 5 optical constants, P, Pf, Qf, Pb and Qb were defined in terms of \( \Delta v \) and \( \Delta h \); where \( \Delta v \) and \( \Delta h \) are the intensities (in arbitrary units) of the polarized components of the excess fluorescence (above that of the blank) in the vertical and horizontal directions, respectively.

The constant P, called polarization, is defined by the equation

\[
P = \frac{\Delta v - \Delta h}{\Delta v + \Delta h}
\]

The constant Pf, or polarization of free Fab, was determined simply in the absence of the toxin. Similarly, Qf or the molar fluorescence of free Fab, was determined as \( \frac{\Delta v + \Delta h}{M} \) in the absence of the toxin. M was the total concentration (in molarity) of Fab; that is \( M = Pb + Pf \).

The constants Pb and Qb are the polarization and molar fluorescence, respectively, of completely bound Fab. Since complete binding cannot be physically realized, these constants were extrapolated. First, equilibrium values of P were plotted against M, using at least 8 data points. When extrapolated to \( M = 0 \), P approached a limit, viz. \( P' \). Then, values of \( P' \) were obtained using at least three different concentrations of alpha toxin. Finally, \( P' \) values were plotted against

\[
\left( \frac{P' - Pf}{\text{relative toxin concentration}} \right)
\]

The intercept of a straight line on the \( P' \) axis yields the value
for Pb according to the mass law

\[ Q'_f(P' - P_f) \quad \text{where} \quad P_b^{\text{max}} \quad \text{is the maximum number of} \]
\[ P' = P_b - \frac{\text{Fab bound per toxin}}{Q_b \cdot K \cdot P_b^{\text{max}}} \quad \text{Fab bound per toxin} \quad (\text{= number of binding sites} \quad \text{= number of determinants}) \]

In the above plot, the values for K and P_b^{\text{max}} need not be known because \(1/(\text{relative concentrations of the toxin})\) was substituted for the quantity \(\frac{Q_f}{Q_b \cdot K \cdot P_b^{\text{max}}}\).

A similar extrapolation for the curves of \(Q\) versus \(M\) yielded the limiting values of \(Q\), viz. \(Q'\), which were used to determine \(Q_b\) by similar plots according to the mass law

\[ Q' = Q_b + \frac{Q_f - Q'}{K \cdot P_b^{\text{max}}} \]

All extrapolations were computed using a program. The program was written by H. Fackrell, according to the mathematical principles (Dandliker 1971) outlined above.

A spectrofluorometer (Model 430, G. K. Turner Associates, California, USA) was used with instructions developed in this laboratory (Fackrell and McConkey 1983). In order to minimize light scattering, peak wave lengths for the excitation and emission of fluorescein were not used. Instead the excitation and emission wave lengths were set at 483 nm and 523 nm respectively. At these wave lengths, the correction factor (called alpha) for instrument error (due to the polarization effect of the emission monochromator) was 0.6987.

The reaction mixture consisted of 2.5 mL of PBS, 50 to 500 microlitres of toxin and stepwise addition of 10 microlitres of labeled Fab. After each addition of Fab, 30 min was allowed for equilibration at 23°C. Then, v and h were taken by setting the
emission polarizer in the appropriate directions. PBS was the blank for measurements of Pf and Qf. Toxin in PBS was the blank for P values at that toxin concentration. Dilution factors due to the stepwise addition of Fab were included in computation of M. To maximize accuracy in the readings of v and h, the voltage output from the spectrofluorometer was interfaced with a computer (Apple II®)(Packrell and Glasgow 1983), and the average of 500 readings over approximately 30 seconds was determined.
PHYSICAL AND CHEMICAL METHODS

Chemicals

All chemicals used were reagent grade.

Buffers

All buffers were prepared according to Chase (1968). Sodium phosphate buffer, 0.01 M, pH 7.2, was supplemented with 0.85% NaCl, and this had been referred to earlier as phosphate buffered saline (PBS). Reference standard buffer solutions were purchased from Fisher Scientific Co. (New Jersey 07410, USA). An Accumet® model 320 expanded scale research pH meter, manufactured by Fisher Scientific Co., was employed. Preservatives were used only when specified. The volumes of preservatives added to a litre of buffer were 1 ml of a 1% (W/V) sodium azide in sterile water and 0.1 ml of a 0.5% (W/V) pentachlorophenol in 95% ethanol.

Protein Assays

The relative concentration of proteins in column eluates were expressed in terms of absorbance at 280 nm. Samples were read in a quartz cuvette with a 1 cm light-path. The quantity of protein was measured by the method of Lowry et al. (1951). The rapid, one-step method which utilized Coomassie Blue G-250 dye (Bradford 1976) was also used. When Triton X100 was present, the Lowry method of protein determination as modified by Wang and Smith (1974) was used. Rabbit IgG, purified as described earlier, was used as a standard.
Gel Filtration

Sephadex\textsuperscript{R} and polyacrylamide beads were purchased from Pharmacia of Canada Ltd., Montreal, and from Bio-Rad, Richmond, California, USA. The manufacturer's recommended procedures (Pharmacia 1970) for the preparation of gels and the packing of columns were followed. Filtration of toxin was performed at 4 C and filtration of antibodies was at 23 C.

Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis under denaturing conditions with sodium dodecyl sulphate (SDS-PAGE) was performed in tube gels according to Laemmli (1970). The system involved a separation gel of 7.5 % acrylamide, pH 8.8, and a stacking gel of 1 % acrylamide, pH 6.8. Electrophoresis was carried out at pH 8.3, 23 C. Samples of 25 microlitres were stacked at 2.5 milliamperes per gel and run at 4 milliamperes per gel. After electrophoresis, gels were stained with 0.25 % (W/V) Coomassie Brilliant Blue R in 7 % acetic acid for 1 to 4 h and then destained by continuous shaking in a solution containing 5 % methanol and 7 % acetic acid.
RESULTS
CHAPTER 1
ANTIGENIC DETERMINANTS

The knowledge of the number of antigenic determinants is essential to the understanding of the antigenicity of any protein. Three lines of evidence were considered below. Initially, the immunodiffusion test provided evidence that there exist at least 3 determinants on alpha toxin. Subsequently, this number was confirmed by two types of direct measurements: the quantitative precipitin test, which measures secondary immune complexes, and the fluorescence polarization, which measures primary immune complexes.

I. Immunodiffusion Tests

AB-Ab and whole antiserum consistently formed precipitin lines with alpha toxin in Ouchterlony double immunodiffusion tests (Fig. 1.1). In contrast, numerous attempts to precipitate various concentrations of purified IHA-Ab with alpha toxin were unsuccessful even though the preparation of IHA-Ab used had higher antibody titres. The IHA-Ab preparation that was used had 128 neutralization units (N) and an IHA titre of 64. In comparison, the AB-Ab and the whole serum had only 25 N and 55 N, and IHA titres of 0 and 64, respectively. Hence, the inability of the IHA-Ab to precipitate alpha toxin was not an artifact of insufficient antibodies in the preparation.

According to the lattice theory (Pauling et al. 1943), all antigens capable of giving a precipitin reaction have a valency of at least 2. Therefore, the simplest interpretation of the
Figure 1.1 Immunodiffusion test of alpha toxin versus antitoxins.

Each well was charged with 10 microlitres of the following reagents:
Central well contained alpha toxin (1mg/mL).
Well number 1 contained undiluted antiserum.
Well number 2 contained AB-Ab.
Well number 3 contained IHA-Ab.
Well number 4 contained preimmune serum.
above data is that there is only one IHA determinant but at least 2 anti-binding determinants on alpha toxin.

II. Quantitative Precipitin Test

The Quantitative Precipitin Test (QPT) is a standard method to measure the number of determinants (Maurer 1971). The result of a QPT using AB-Ab versus alpha toxin is depicted in Figure 1.2. There were 5 data points (numbered 1 to 5) in the zone of antibody in excess. Regression analysis was performed on these data points so as to extrapolate for the value on the ordinate whereby the regression line intersected. The result of such analysis indicated that the limiting value for the mole ratio of antibody:antigen was approximately 2. A molar ratio of 2 for antibody:antigen means that there were 2 molecules of AB-Ab per molecule of alpha toxin. Thus, there existed 2 anti-binding determinants on alpha toxin. The result was entered in Table 1.1.

The QPT was repeated using different types of antitoxin and the results were summarized in Table 1.1. If there were 2 anti-binding determinants and 1 IHA determinant on the toxin, a mole ratio of antibody:antigen of 3 should be observed when whole antitoxin was tested. However, the observed value was 2 when the IgG fraction of antitoxin from rabbit or pig was tested (Table 1.1). The value of 6 for rabbit antiserum may be artificially high due to precipitation of IgM and IgA as well as coprecipitation of non-immune serum proteins (Maurer 1971).

The discrepancy between the observed and the expected number of determinants may be explained by the following rationale.
Figure 1.2

Determination of the number of Antibinding Determinants by the Quantitative Precipitin Test.

The mole ratio of antibody (Ab) to antigen (Ag) in the precipitate declined with increasing amount of antigen until a limiting value was reached, indicative of the zone of antigen excess. Data points (numbered 1 to 5) before the zone of antigen excess were used to extrapolate for the value on the Y-intercept.

The regression line of data points number 1 through 3 is shown. Value of the Y-intercept of this line is 1.97 (S.E. = 0.22). The values of the Y-intercept of regression lines through data points number 1 through 4, and number 1 through 5, are 1.71 (S.E. = 0.26) and 1.51 (S.E. = 0.30). These Y-intercept values were corrected to the nearest integer because the number of antigenic determinants must be a whole number. In all 3 regressions, the nearest integer value for the Y-intercept is 2.
<table>
<thead>
<tr>
<th>TYPE OF ANTITOXIN</th>
<th>NUMBER OF DETERMINANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG fraction of rabbit AB-Ab</td>
<td>2</td>
</tr>
<tr>
<td>IgG fraction of rabbit antiserum</td>
<td>2</td>
</tr>
<tr>
<td>IgG fraction of pig antiserum</td>
<td>2</td>
</tr>
<tr>
<td>Rabbit antiserum</td>
<td>6</td>
</tr>
</tbody>
</table>
Alpha toxin spontaneously polymerizes to form an insoluble precipitate (Coulter 1966). In the above experiments, the weight of the precipitate of the toxin alone was subtracted from the total precipitate to give the weight of the immune precipitate. This calculation assumed that the amount of spontaneous precipitation of the toxin was the same in the presence or absence of antitoxins. This assumption could be wrong if the formation of immune complexes hinders the polymerization among the toxin molecules. Consequently, the value for polymerization of the toxin may be artificially high and the quantity of IgG precipitated may be underestimated. The net result would be an underestimation of the mole ratio of antibody:antigen. Hence, the value of 2 determinants, based on the QPT, could be an underestimation.

The QPT measures a secondary reaction between the antigen and the antibody, namely, the amount of immune precipitates. The formation of immune precipitates is a time-dependent event. To ensure that equilibrium was reached, an incubation period of 7 days was used (Maurer 1971). Due to this long incubation time, the formation of non-immune precipitates became a problem inherent to the test. Unlike the QPT, fluorescence polarization (see below) measures the primary union between the antigen and the antibody; equilibrium was reached in less than 1 h (Dandliker 1971). For this reason, fluorescence polarization was chosen to settle the discrepancy discussed above.
III. Fluorescence Polarization
A. Quality Controls
1) Properties of fluorescein-labelled Fab

Fluorescein-labelled Fab were prepared using the IgG fractions from the antisera of 2 rabbits (number F105 and F116). These antisera were obtained seven weeks after the initial immunization (see antisera in Materials and Methods). The fluorescein:protein ratio of one preparation (F105) was 1.96:1 and that of the other preparation (F116) was 1.55:1. The protein concentration of preparation F105 was 31.25 mg/mL or 625 μM of Fab, and that of preparation F116 was 19.375 mg/mL or 387.5 μM of Fab. Both preparations retained their ability to neutralize alpha toxin. The neutralization titre of preparation F105 was 181 N per 50 microlitres and that of preparation F116 was 315 N per 50 microlitres. If 'molar specific neutralization capacity' is defined as the neutralization units per molar concentration of Fab (that is, N/M), this quantity is approximately 3x higher for preparation F116 compared to preparation F105. Preparation F116 was chosen for further studies because of its higher 'molar specific neutralization capacity', and because its fluorescein:protein ratio was closer to the ideal ratio of 1:1 (Sternberger 1974).

ii) Signals from spectrofluorometer to computer

The spectrofluorometer has a linear scale from 0 to 100. A reading on this scale was transmitted in the form of voltage output. The output signal was first amplified (Packrell and Glasgow 1983) and then digitized to 8 bits by an analog-digital converter. The digitized signal, which has a range between 0 and 220, was received by the computer. To ascertain that the signal
transmitted to the computer corresponds to the reading on the spectrofluorometer scale, readings of the two machines were plotted (Fig. 1.3). Since a linear relationship existed between readings of the two machines, the computer readings were accepted as true representation of readings taken visually on the spectrofluorometer scale. Thus, readings may be taken by the computer automatically with confidence instead of manually. Subsequently, the computer readings were programmatically adjusted to a range from 0 to 100.

iii) Correction for settings of the sensitivity range

Although the markings on the spectrofluorometer scale only range from 0 to 100, the sensitivity of the machine can be magnified 3x, 10x, 30x, 100x, 300x or 1,000x by setting the range control correspondingly. However, the voltage output to the computer did not take into account the setting of the range control. To solve this problem, the input program was written in such a way that the operator was asked the range setting, and then the true reading was computed.

iv) Selection for the sensitivity range

Technically, the fluorometric readings of a given sample can be taken with the range control set at any one of the magnifications described above. The effect of the magnification on the accuracy of fluorometric reading was revealed by the following experiment. The readings of a PBS sample were taken repeatedly (in triplicates) at the different ranges and at the same time, the position of the needle on the spectrofluorometer scale was noted (Table 1.2). It appeared that the lower the range was set at,
Correlation between Readings of the Spectrofluorometer and the Computer:

Readings of the spectrofluorometer were taken visually. The corresponding reading by the computer was accomplished by an input program. The cuvette contained air. The different magnitudes of the readings were achieved by manipulating the sensitivity dial and the sensitivity range settings.

Instrument settings were as follows:
Excitation wavelength = 483 nm.
Emission wavelength = 523 nm.
Band width of both wavelengths = 15 nm.
The meter damp was set at 1.
The blank switch was set on high.

The correlation coefficient of the regression line was 0.9997. Variation of the X-variable accounted for 99.94% of the variation of the Y-variable.
<table>
<thead>
<tr>
<th>Sensitivity Range Set At</th>
<th>Computer Readings in Triplicate</th>
<th>% Coefficient of Variation</th>
<th>Position of Needle</th>
</tr>
</thead>
<tbody>
<tr>
<td>x30</td>
<td>7</td>
<td>18.3</td>
<td>downscale</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>x100</td>
<td>10</td>
<td>5.0</td>
<td>downscale</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>x300</td>
<td>10</td>
<td>2.9</td>
<td>midscale</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>x1000</td>
<td>6</td>
<td>2.8</td>
<td>upscale</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

The % coefficient of variation was corrected to 1 place of decimal.
the higher was the % coefficient of variation. Hence for the reading of any given sample, the range setting recommended is the one which deflects the needle of the spectrofluorometer upscale.

v) Precision of the readings

As mentioned earlier, the computer determined an average of 500 readings taken over approximately 30 seconds. In order to assess the accuracy of the readings, standard deviations were calculated (Table 1.3). First, the V and the H readings were taken using PBS as a blank. Then, readings were repeated after the stepwise addition of each 10 μl of labelled Fab. This example represents the procedure for the determination of Pf. The sensitivity dial was set at maximum (clockwise) and the sensitivity range was selected according to section iv above. The program was written to accommodate the maximum reading range that can be resolved by the spectrofluorometer (i.e. from 1 to 99,999). The results (Table 1.3) indicate that the readings are highly accurate since the standard deviations are very small. The same range of Fab concentration (10 to 80 μl) was used in later tests.

B. Fluorescence polarization of alpha toxin and labelled Fab

i) Confirmation of basic principle

The PP assay relies on the principle that the quantity P or Polarization is dependent on molecular motion as follows: P is minimal when the molecular motion of the free label is fast; when the label is bound, its molecular motion is retarded, and P increases (Dandliker 1971). This principle was validated for the alpha toxin and fluorescein-labelled Fab (Fig. 1.4). In the
TABLE 1.3. Precision of the computer readings.

<table>
<thead>
<tr>
<th>µl of Labelled Fab Added to 3 mL PBS</th>
<th>Average of 500 Readings V</th>
<th>Average of 500 Readings H</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>251</td>
<td>274</td>
<td>2.436</td>
</tr>
<tr>
<td>20</td>
<td>505</td>
<td>560</td>
<td>1.849</td>
</tr>
<tr>
<td>30</td>
<td>749</td>
<td>824</td>
<td>1.900</td>
</tr>
<tr>
<td>40</td>
<td>984</td>
<td>1087</td>
<td>1.191</td>
</tr>
<tr>
<td>50</td>
<td>1204</td>
<td>1323</td>
<td>1.497</td>
</tr>
<tr>
<td>60</td>
<td>1438</td>
<td>1602</td>
<td>1.443</td>
</tr>
<tr>
<td>70</td>
<td>1643</td>
<td>1822</td>
<td>1.665</td>
</tr>
<tr>
<td>80</td>
<td>1862</td>
<td>2057</td>
<td>1.628</td>
</tr>
</tbody>
</table>

ND = not determined
absence of toxin, the value for $P$ of free Fab were practically
constant over the concentration range tested (Fig. 1.4a). The
average of these $P$ values was taken as Pf. In this example, 3
concentrations of toxin were tested. The stepwise addition of
labelled Fab to each concentration of toxin generated $P$ values
which were used to extrapolate a value for $P'$ as described in
Materials and Methods. Hence, 3 $P'$ values were obtained; one
for each concentration of toxin used. The 3 values of $P'$ were
then used for the extrapolation of the value Pb (Fig. 1.4b).
These results demonstrated the basic relationship between $P$ and
binding. Pf assumed the lowest value. $P'$ increased as the
concentration of toxin was increased, presumably as a result of
more Fab being bound. Based on these observations, we proceeded
to use the PP assay for the alpha toxin-antitoxin system with
confidence. All the readings in this experiment had coefficients
of variation less than 2%.

ii) Number of determinants

The values of the various optical constants determined
above were recorded in Table 1.4. The number of binding sites
($Pb_{\text{max}}$) was computed as described in Materials and Methods.
Since Fab (valency = 1) was used, the number of binding sites
reflects the number of antigenic determinants. By this approach,
the total number of determinants on the surface of alpha toxin
was found to be 3. The intrinsic association constant $Ko$ was
within a reasonable magnitude.
Figure 1.4
Polarization of fluorescein-labelled Fab

P was related to the state (bound or free) of the labelled Fab.

a. The open circles represent the values of P at the various concentrations of labelled Fab in the absence of alpha toxin. The line across represents the average of these P values, and was taken to be Pf (Pf = 0.129).

b. The experiment was repeated in the presence of alpha toxin. Experimental conditions were as described in Materials and Methods. Instrument settings were as in Fig. 1.3.

As indicated in Table 1.4, the 3 concentrations of toxin tested were (1) 4 µM, (2) 9.34 µM and (3) 16.818 µM, yielding 3 respective values of P'. These P' values were plotted against 1/U in order to extrapolate the value Pb. U was the relative concentration of the toxin with the lowest concentration (4 µM) set as 1. The Y-intercept of the regression line yielded the value Pb (Pb = 0.149).

Note that the value of P' increased with increasing amounts of toxin, presumably because more label became bound, and that all P' values were higher than Pf.
TABLE 1.4. Results of fluorescence polarization of labelled-Fab versus alpha toxin.

<table>
<thead>
<tr>
<th>TOXIN CONCENTRATION μM</th>
<th>RELATIVE (U*)</th>
<th>VALUE OF THE CONSTANTS</th>
<th>STANDARD ERROR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pf = 0.129</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Qf = 48.94</td>
<td>0.328</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>P' = 0.132</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q' = 53.32</td>
<td>ND</td>
</tr>
<tr>
<td>9.343</td>
<td>2.333</td>
<td>P' = 0.140</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q' = 57.24</td>
<td>ND</td>
</tr>
<tr>
<td>16.818</td>
<td>4.2</td>
<td>P' = 0.146</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q' = 53.67</td>
<td>ND</td>
</tr>
</tbody>
</table>

| Pb = 0.149             | 0.002         |
| Qb = 55.814            | 3.286         |

NUMBER OF BINDING SITES  
$F_{D_{max}} = 2.898$  
1.673

intrinsic association constant  
$K_o = 2.5 \times 10^{-7}$ L/mole  
3.2

ratio of Qf/Qb  
= 0.877

* U = the relative concentration of alpha toxin tested.
ND = not determined
The correction factor (alpha) was 0.6987.

$F_{D_{max}}$ was determined by general regression in a Scatchard plot using all the data points that were obtained with the 3 concentrations of toxin versus the 8 concentrations of labelled-Fab.
CHAPTER 2
CHARACTERIZATION OF ANTITOXINS

The characteristics of the antitoxins were examined in three aspects: antibody response, neutralization, and immunoglobulin classes. The reasons for concentrating on these three aspects are as follows. The toxin-antitoxin system in question presents an unique opportunity to examine antibody responses to the different determinants on the same protein. Next, since IHA-Ab and AB-Ab neutralize alpha toxin by different mechanisms (Lo and Fackrell 1979), we wished to investigate their interaction in order to understand the events in whole serum. Finally, the study on immunoglobulin classes was prompted by claims that antibodies which neutralize alpha toxin were found only in the IgG class (Felsenfeld and Felsenfeld 1966; Smith 1968).

I. ANTIBODY RESPONSES
a. IHA-Ab Response

Sera from a group of 6 rabbits, hyperimmunized with alpha toxoid as described earlier, were titrated in the IHA test. The IHA-Ab response is depicted in Figure 2.1. The mean IHA-Ab titre increased drastically to a peak of 2048 by the second week. After the last injection of toxoid at week 3, the titre gradually declined. This pattern exhibited by the IHA-Ab response was typical of many serological responses. As illustrated by the small standard error about the means, the IHA-Ab response was uniform among the rabbits tested. This uniformity is remarkable considering that the IHA test is semi-quantitative.
Figure 2.1
IHA-Ab response in rabbits.

Each data point represents the mean of IHA titres of the weekly antisera among 6 rabbits. The limits represent the standard errors of the means.
b. Response of Total Antibodies

The same antisera were titrated in the neutralization test and the results are depicted in Figure 2.2. Although the mean titre increased and decreased in a manner similar to many serological responses, the standard errors were so high that a pattern was not obvious. Since the neutralization test measured the total antibodies, and since the IHA-Ab responses were uniform, we suspected that the variation may be due to the AB-Ab.

It was interesting to note that on week 4, the mean neutralization titre dropped to its lowest level of less than 100 units while the IHA titre was still quite high.

c. AB-Ab Responses

To trace the AB-Ab response, IHA-Ab were completely removed by adsorption of the antisera with the immunoabsorbent LGCE (Lo and Fackrell 1979). The neutralization capacity remaining in the adsorbed sera was considered to be due to AB-Ab. A complete set of AB-Ab was available in 4 out of the 6 rabbits. The neutralization titres due to AB-Ab in these 4 rabbits are shown in Figure 2.3. As a group, the 4 rabbits did not exhibit any consistent pattern of AB-Ab response. However, two of the rabbits with relatively high AB-Ab titres in their preimmune sera (Fig. 2.3b) displayed a decreased titre throughout the period of inoculation until the injections were stopped. In comparison, the AB-Ab titres in the other two rabbits with low AB-Ab titre in their preimmune sera remained low throughout the same period (Fig. 2.3a).

The above data indicates that the IHA-Ab response is uniform and conventional but the AB-Ab response is variable and unusual.
Figure 2.2
Total antitoxin response in rabbits.

Each bar represents the average neutralization capacity of whole antisera (Nw) obtained weekly from 6-7 rabbits.
Figure 2.3
AB-Ab response in 4 rabbits.

a. Rabbits with low preimmune AB-Ab titres
   △△△△△△ Rabbit number F116
   ○○○○○○ Rabbit number F105

b. Rabbits with high preimmune AB-Ab titres
   ●●●●●● Rabbit number F112
   △△△△△△△ Rabbit number F106

Note: Immunization was terminated after week 3. (See Materials and Methods for detail of immunization schedule).
Thus, antibody responses to alpha toxin were heterogeneous. This heterogeneity explained why a conventional antitoxin response was not readily demonstrable on the total antibodies (Fig. 2.2). 

d. Antitoxin Responses to LGCE

We wondered if the low titres of AB-Ab in response to immunization were related to the membrane-binding function of the antigen. We speculated that intravenous injection exposed the antigen to numerous erythrocytes with high affinity receptors. The binding of the antigen to erythrocytes masked the anti-binding determinants so that subsequent antigenic presentation was affected. To test this speculation, we immunized 3 rabbits intraveneously with 1 ml of a 2% LGCE once a week for 5 weeks, and the rabbits were bled every 7 days after each injection. Since the toxoid was covalently linked to the erythrocyte in LGCE, the antibinding determinants were masked (Lo and Packrell 1979). According to the above speculation, high IHA titres and low AB-Ab titres were expected of the immune sera. However, the opposite result was observed. The AB-Ab titres in all of the immune sera were very high (>128) but the IHA titres were very low (8 to 16). It seemed that the IHA determinant was affected whereas the antibinding determinants were enhanced. Hence, we discarded the above speculation and offered alternative explanations in the discussion.

II. NEUTRALIZATION STUDIES

The neutralization reaction of antiserum was analysed in terms of its constituents: AB-Ab and IHA-Ab. Some of the questions were: Which population of antibody predominates? How
many antibody molecules are required to neutralize a single toxin molecule? Is there an antibody that binds but does not neutralize the toxin?

a. The Ratio of AB-Ab in Whole Serum

The neutralization capacity due to AB-Ab ($N_{AB}$) and the total neutralization units in whole serum ($N_W$) were determined previously (section I.b and I.c). The ratio $N_{AB}/N_W$ describes the proportion of neutralization capacity contributed by AB-Ab to whole serum. When the 32 sera from 4 rabbits (corresponding to Fig 2.3) were examined, the value of this ratio was found to fluctuate not only among the different rabbits but also with time in any given animal. Examples of extremes were found in which sera were completely devoid of AB-Ab (ratio = 0) or of IHA-Ab (ratio = 1) (Fig 2.4). The mean value among the 32 ratios was 0.35 (standard error = 0.22); meaning that, on the average, AB-Ab accounted for about 35% of the total neutralization capacity. The median value of 0.27 was lower than the mean value of 0.35. This indicates that in the majority of cases, AB-Ab accounted for less than 35% of the total neutralization capacity. Therefore, IHA-Ab appeared to be responsible for the neutralization capacity of whole serum to a greater extent than AB-Ab.

b. Neutralization by Mixed Populations of Antibodies

We wished to check if all of the neutralization capacity in whole serum was accounted for by the sum of the neutralization units of the individual antibody populations. In other words, we wished to test the validity of the equation:

$$N_W = N_{AB} + N_{IHA}$$
Figure 2.4
Frequency distribution of $N_{AB}/N_W$ among 32 rabbit antisera.

arithmetic mean = 0.35 (standard error = 0.22)
median = 0.27

$N_{AB}$ = neutralization units of antibinding antibodies
$N_W$ = neutralization units of whole serum
For a given serum, we could measure experimentally, the quantities $N_W$ and $N_{AB}$, but not the portion of neutralization units due to IHA-Ab ($N_{IHA}$). On the one hand, $N_{IHA}$ in a serum could be estimated, based on the IHA titre of that serum (see 'indirect approach' below). On the other hand, $N_{IHA}$ could be determined directly for a purified IHA-Ab preparation. These two alternatives constituted the two approaches, direct and indirect, for checking the validity of the above equation.

In the direct approach, we compared the neutralization units of AB-Ab and IHA-Ab before and after they were mixed. When 50 microlitres of IHA-Ab with 128 N was mixed with 50 microlitres of AB-Ab with 32 N, the arithmetic sum of N in 100 microlitres of the mixture would be 128+32 N or 160 N. Thus, the expected N in 50 microlitres of the mixture was 80. However, the experimentally observed N in 50 microlitres of the mixture was only 48.5 N. Thus, the neutralization capacity of the two populations are not arithmetically additive. The mixture exhibited less N than the sum of its constituents.

Next, we used the indirect approach to see if the phenomenon holds true in whole serum. Since the quantity $N_{IHA}$ had to be estimated, we employed the arithmetic means of a number of observations to provide a reliable estimate. In the first step, the quantities $N_W$ and $N_{AB}$, and the IHA titres, of 28 serum samples from rabbits, were measured. The mean values for this group of sera were as follows:
The mean $N_W = 103.5$ (standard error = 9.8)
The mean $N_{AB} = 33.15$ (standard error = 4.14).
The mean IHA titre = log₂ 8.71 (standard error = 0.33).

These mean values were used to check the validity of the equation: mean \( N_W = \text{mean } N_{AB} + \text{mean } N_{IHA} \).

In the second step, the quantity 'mean \( N_{IHA} \)' was estimated based on a semi-quantitative relationship between \( N \) and IHA titre. A purified IHA-Ab preparation with an IHA titre of log₂ 6 was found to contain 48 N. The mean IHA titre of the group was 8.71 minus 6, or 2.71-fold higher than the purified IHA preparation. Since the IHA preparation contain 48 N, the mean \( N_{IHA} \) of the group could be expected to be 2.71-fold higher or (2.71 \times 48) approximately 130 N.

In the final analysis, the estimated mean \( N_{IHA} \) (130 N) plus the mean \( N_{AB} \) (33.15 N) was equal to 163.15 N which is higher than the observed value of 103.5 for \( N_W \). Therefore, the observed neutralization capacity in whole serum was less than the theoretical sum of the \( N \) of the constituents. We conclude that neutralization capacities between IHA-Ab and AB-Ab are not additive arithmetically, and that non-additivity holds true in both artificial mixture and whole serum.

To compare the data from the two approaches we used the ratio of observed \( N \) : expected \( N \). In the artificial mixture this ratio was \( \frac{48.5}{80} \) or approximately 0.61. In sera this ratio was \( \frac{103.5}{163.15} \) or approximately 0.63. Thus, the ratios represent a 97% agreement between the direct and the indirect approaches. This close agreement reinforced the validity of the above conclusions that \( N_W < N_{AB} + N_{IHA} \).
c. The Minimal Number of Determinants Required for Neutralization

Antitoxins were composed of 2 fractions: IHA-Ab and AB-Ab (Lo and Packrell 1979). We have shown earlier that IHA-Ab neutralized toxin, and were directed to a single determinant. However, in the case of AB-Ab, which were directed to at least 2 anti-binding determinants on alpha toxin, the possibility of non-neutralizing antibodies must be considered. One of the possibilities was that one of the subpopulations of AB-Ab may not neutralize. The other possibility was that more than one AB-Ab molecules were required for the neutralization of a single toxin molecule. In any case, non-neutralizing AB-Ab were defined as antibodies that bound alpha toxin but did not prevent the toxin from destroying the erythrocytes. The existence of non-neutralizing AB-Ab was investigated according to the working hypothesis described in Figure 2.5. According to the rationale of this model, if one of the subpopulations of AB-Ab cannot neutralize, immune complexes would be present on the erythrocytes in the zone of antigen in excess (Figure 2.5b). Conversely, if the binding of the toxin to erythrocytes were prevented when any one of the determinants is occupied by a specific AB-Ab, all of the immune complexes would remain in the supernatant (Figure 2.5a). The possibility existed that the hypothetical subpopulation of AB-Ab may be so minor that detection might be difficult. Hence, we chose the enzyme-labelled AB-Ab for maximum sensitivity in our detection method (Surujballi and Packrell 1983). Since the pH of this detection method (ELISA) is deleterious to erythrocytes, we measured the consumption of
Figure 2.5
Models to test the existence of non-neutralizing AB-Ab.

The circles represent alpha toxin (constant amount).
The blocks represent antibodies (varying amount).
The arcs represent erythrocyte membranes (constant amount).

Possibility a: One molecule of AB-Ab is sufficient to prevent binding of alpha toxin to membranes.

Consequence: All labelled AB-Ab remain in the supernatant at all concentrations of antibodies.

Possibility b: More than one molecule of AB-Ab is required to prevent the binding of alpha toxin to membranes, or
One subpopulation of antibodies does not prevent binding.

Consequence: The non-neutralizing antibodies are removed from the aqueous phase.
labelled AB-Ab in the supernatant. Toxoid instead of toxin was employed as the antigen so as to circumvent hemolysis due to the toxin.

The experimental procedures were as follows. Enzyme-labelled antitoxin (IgG fraction) was excessively adsorbed with LGCE to remove all the IHA-Ab. Adsorption was repeated using a portion of the test erythrocyte. This adsorbed antitoxin represented labelled AB-Ab. A working dilution of the labelled AB-Ab was determined by titration so that a 1/4 dilution of this working dilution gave an absorbance of just below 2.0 in the ELISA. In the test, 50 microlitres of various concentrations of the working dilution of labelled AB-Ab was preincubated with 50 microlitres of toxoid (which had 256 HU before heat inactivation) at ambient temperature for 15 min. Then, 100 microlitres of a 2% suspension of rabbit erythrocytes was added to each mixture. The mixtures were incubated for 30 min. The temperature of incubation was 4 C in one experiment and 23 C in another, and the final results were similar. After incubation, the mixtures were centrifuged at 600 x g for 10 min. at 23 C. Fifty microlitres of supernatant was removed from each test dilution and the enzyme activity measured by ELISA. Parallel control series included varying concentrations of: labelled AB-Ab alone; labelled AB-Ab plus toxoid; and labelled AB-Ab plus erythrocytes. Three other independent controls were set up for backgrounds. They were: toxoid alone, toxoid plus erythrocytes, and erythrocytes alone.

The results are depicted in figure 2.6. No difference was detected between the test series and the control series since the
Figure 2.6

Recovery of enzyme activities from the supernatants of:

- labelled AB-Ab + toxoid + erythrocytes (test)
- labelled AB-Ab + toxoid + PBS (toxoid control)
- labelled AB-Ab + PBS (AB-Ab control)
test values fell in between the control values. The results indicated that all of the label remained in the supernatant, suggesting that no AB-Ab-toxoid complex was able to bind to the erythrocytes. We concluded that the binding of any one of the antibinding determinants to its specific AB-Ab leads to neutralization, and that no non-neutralizing antibodies were detected.

III. CLASSES OF IMMUNOGLOBULINS

Previously we found neutralizing antibodies in purified IgG preparations. Despite several attempts, we were unable to purify antitoxin to the IgM or IgA classes by the classical methods of gel filtration combined with ion exchange chromatography using diethylaminoethyl cellulose (Fahey 1967; Fahey and Terry 1967). These IgM and IgA enriched preparations of antitoxin contained traces of contaminating IgG, which were detected by immunodiffusion against specific anti-IgG. This problem was resolved by adsorption with insolubilized antiglobulins (see Materials and Methods). To illustrate the general findings, the removal of IgG, IgM or IgA (Fig. 7a, b and c respectively) from immune serum decreased the IHA titre at least 4-fold, demonstrating that IHA-Ab were present in all three classes of immunoglobulins. Since the removal of IgG, IgM and IgA from immune serum also decreased the total neutralization capacity by 30%, 19% and 30% respectively, neutralizing antibodies must occur in all three classes of immunoglobulins. The decrease in neutralization reflects only the minimal amounts removed because of the complex, nonadditive nature of antibodies in the
Figure 2.7
Immunoabsorption of antitoxin with solid-phase antiglobulins.

Decrease in $N_w$ and IHA titre were observed in antisera
adsorbed with solid phase anti-IgG (a), anti-IgM (b), or anti-IgA (c). Open bars represent $N_w$. Shaded bars represent IHA titre.
neutralization test. For this reason, while antitoxin was demonstrated in the various classes by this approach, the quantity of antitoxin in each class was not determined.
CHAPTER 3

COMPLEMENT FIXATION

Alpha toxin is the most lethal among the toxic products of *Staphylococcus aureus* (Arbuthnott 1970). While attention has focussed on the alpha toxin, the possible involvement of complement in the toxic manifestations has been neglected. In the preceding chapter, the complexity of antitoxin was illustrated and antitoxins were found in the IgG, IgM and IgA classes. Since both IgG and IgM can fix complement, it is conceivable that the two different antitoxins and their respective reactions with complement, could influence the outcome of many *in vivo* experiments. This concern is substantiated by the following studies on complement fixation (*CF*) *in vitro* and *in vivo*.

I.

**Complement Fixation *in vitro***

a. **Optimal conditions for the Standard Serologic Complement Fixation Test**

To avoid any hemolysis other than that initiated by the amboceptor, toxoid was used as the CF antigen instead of toxin. Since the CF test requires an optimal rather than a maximal amount of antigen, the concentration of toxoid required for the CF test was determined by an antigen box titration with a representative rabbit antiserum which had an IHA titre of 4096 and an antitoxin titre of 73.5 neutralization units of which 48.5 neutralization units were due to AB-Ab. Sheep erythrocytes were the indicator. One unit of the amboceptor was contained in a
1:8000 dilution; and the guinea pig complement contained 5 CH50 in a 1:19 dilution. Under these conditions, the optimal concentration of toxoid was found to be approximately 4 ng/mL and no anticomplementary effect was observed.

It should be noted that the standard serological CF test measures CF reactions of immune complexes in solution.

b. Immune Response leading to production of CF Antibodies in Rabbit

When the amount of CF antitoxin in the antisera of immunized rabbits was measured under these optimal conditions, an intense response was demonstrable by the standard serologic CF test (Fig. 3.1). In this typical example, the preimmune serum already had a CF titre of 1:97. The CF titre reached 1:588 by the second week, and then decreased despite continued immunization. After the last injection on the third week, the CF titre declined to about twice the preimmune level by week 6.

To determine if AB-Ab fix complement the sera were adsorbed with LGCE to remove the IHA-Ab. Any residual CF antitoxin measured was considered to be due to AB-Ab. The result showed that AB-Ab fixed complement (Fig 3.1). Not unexpectedly, the response of the complement-fixing AB-Ab exhibited a similar general pattern but with a preimmune titre of 1:55.7 and a peak titre of 1:97.

Since removal of the IHA-Ab decreased the CF titres by two- to six-fold (Fig. 3.1), the IHA-Ab must contribute to the complement fixation of whole sera. To establish directly if IHA-Ab fix complement, IHA-Ab were eluted from the immunoadsorbent (LGCE) and titrated. Although the recovery was low (CF titre =
Figure 3.1
Immune responses as depicted by complement-fixing antibodies.

CF titres in rabbit number P112 hyperimmunized with alpha toxoid.

- o--o whole antisera
- - - AB-Ab.
1:2, IHA titre = 16) the IHA-Ab preparation did fix complement. c. Comparison of AB-Ab responses measured by CF and by Neutralization

The AB-Ab titres in the weekly antisera that had been LGCE adsorbed were obtained by CF test and by neutralization test. The antibody response curves, as depicted by these two types of tests for a single rabbit, were remarkably different (Fig. 3.2). During the period of immunization (up to week 3), the pattern of complement-fixing antibodies was similar to that of the IHA-Ab response, whereas $N_{AB}$ continued to drop. Hence, the description of antibody responses is dependent on the assay used.

d. CF Antitoxin in Humans

The sera of 39 nonimmunized humans were subjected to the Standard Serologic CF Test. Three sera were anticomplementary and were discarded. Of the remaining 36 sera 72% revealed the presence of CF antibodies (Fig. 3.3). Considerable variability of the CF titre was seen among the 36 individuals.

e. Comparison between CF Titre and IHA Titre

The same 39 sera from humans were subjected to the IHA test using LGCE prepared with human type O, Rh- erythrocytes. The result (Fig. 3.4) showed that 95% of the sera were positive for IHA-Ab.

When the 36 CF titres (from section d.) were regressed on their corresponding IHA titres, the correlation coefficient was found to be -0.243, and the variation in IHA titre accounted for less than 6% of the variation in CF titre. Therefore, the CF titre is, to a large extent, independent of the IHA titre in any given serum. This statement is consistent with the finding in
Figure 3.2
Comparison of immune response curves by different tests.

AB-Ab were prepared from the weekly serum of rabbit number F112.

○○○○○○○○○ AB-Ab response curve obtained by CF test.

△△△△△△△△△ AB-Ab response curve obtained by neutralization test.
Figure 3.3
Distribution of CF titre among 36 normal human sera.

Sera which were anticomplementary were not included in this survey.
Figure 3.4
Distribution of IHA titre among 39 normal human sera.
section b that IHA-Ab fix complement poorly, and with the contention in section c that different tests measure different parameters.

f. Complement fixation by membrane-bound immune complexes

Since IHA-Ab fixed complement, and since the immune complexes formed by IHA-Ab and toxin/toxoid were demonstrated on erythrocyte membranes (Lo and Packrell 1979), we sought to discover if these membrane-bound complexes fixed complement. IHA-Ab-coated LGCE (packed cell volume = 1.1 mL) were resuspended with 830 CH₅₀ of complement overnight at 4 C. After centrifugation, only 231 CH₅₀ were recovered from the supernatant. When the parallel experiment was done with LGCE that was not coated with IHA-Ab, 759 CH₅₀ were recovered. The drastic difference in recovery clearly demonstrated that the IHA immune complex fixed complement under conditions when the antigen was covalently bound to the membrane.

g. Lack of Passive Immune Hemolysis

In the above experiment, LGCE was resistant to the lytic effect of complement. This is not unexpected because glutaraldehyde-treated erythrocytes are known to be resistant to complement (Dalen 1976a). In order to determine whether or not complement fixation, by membrane-bound complexes, was lytic for the carrier erythrocyte, the following experiments were performed. Toxoid was allowed to bind onto erythrocytes noncovalently (Barei and Packrell 1979) and then IHA-Ab was added to the toxoid-coated erythrocytes, followed by complement. Hemolysis was determined after incubation at 37 C for 30 min or 4 C for 18 h. The protocol of this approach is given in Table 3.1.
TABLE 3.1
Cytotoxicity of the IHA-Ab Immune Complexes

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>ERYTHROCYTES (μL)</th>
<th>TOXOID (μL)</th>
<th>ANTIBODY (μL)</th>
<th>COMPLEMENT (μL)</th>
<th>BUFFER (μL)</th>
<th>PERCENT HEMOLYSIS</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td></td>
<td></td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>Erythrocytes intact</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>50</td>
<td></td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>Toxoid nonhemolytic</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>50</td>
<td>50 (A*)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>Amboceptor nonhemolytic</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>50</td>
<td></td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>Complement nonhemolytic</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td></td>
<td>50 (A*)</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>Complement fixed, cytotoxic</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td></td>
<td>50 (IHA-Ab)</td>
<td></td>
<td>100</td>
<td>0</td>
<td>IHA-Ab nonhemolytic</td>
</tr>
<tr>
<td>Test</td>
<td>50</td>
<td>50</td>
<td>50 (IHA-Ab)</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>IHA-Ab noncytotoxic</td>
</tr>
</tbody>
</table>

An rat anti-rabbit erythrocyte membrane

The concentration of each reagent and the test conditions were as described for CF test. Erythrocytes and complement were drawn from the same rabbit. The rabbit complement had 800 CH50 and was devoid of antitoxin. The rabbit IHA-Ab had an IHA titre of 16 and a CF titre of 2. The toxoid-erythrocyte mixture was preincubated at 23°C for 15 min. IHA-Ab was added 15 min prior to the addition of complement.
Although complement was fixed by membrane-bound immune complexes, the carrier erythrocytes did not lyse (Table 3.1). Similar results were obtained when erythrocytes from sheep or human were substituted for those of rabbit. Neither the source of IHA-Ab, whether rabbit or human, nor the source of complement, be it rabbit, human or guinea pig, altered this observation. Rabbit erythrocytes activate complement of human or guinea pig by the alternate pathway (Platts-Mills and Ishizaka 1974). Nevertheless, rabbit erythrocytes plus guinea pig complement were used as an additional positive control for the tests in Table 3.1.

The protocol in Table 3.1 was repeated with 1,2 or 3 HU of toxin instead of toxoid. As expected, hemolysis was observed in controls 2, 3, and 4 with rabbit erythrocytes but not with human erythrocytes which are resistant to lysis by alpha toxin (Bernheimer 1965; Cooper et al. 1966). In the test (bottom row of Table 3.1) the toxin was neutralized by preincubation with the antitoxin (15 min, 23 C) prior to the addition of erythrocytes. After 15 min, complement was added. The test showed no lysis of erythrocytes of rabbit, sheep or human indicating that fixation of complement by membrane-bound toxin-IHA-Ab complex did not lyse the carrier erythrocytes.

II. Complement fixation in vivo

The above data established that immune complexes fix complement in vitro. The following experiment demonstrates that these immune complexes could induce a hypersensitivity reaction in vivo.

Positive reactions of the Reverse Passive Arthus type were produced by IHA-Ab, AB-Ab and whole antiserum (Table 3.2). The
reactions may be described as immediate since they appeared within 4 h. Relative to the other antitoxins, whole antitoxin produced the most prominent erythema but the least edema. The IgG fraction of antitoxin produced only a weak reaction. The reaction induced by $\lambda B$-Ab seemed slightly more prominent than that induced by IHA-Ab. The reason could be that the IHA determinant is single, and the fixation of complement by IgG requires the cooperation of 2 antigen-bound IgG molecules in close proximity. It should be pointed out that the above speculations, based on the intensity of the gross reaction, may be invalid because the exact quantities of specific antibodies were not defined. The use of 50 micrograms of protein for each antitoxin did not necessarily mean an equivalent amount of antitoxin in each case.
TABLE 3.2. Reverse Passive Arthus Reaction induced by Antitoxins

<table>
<thead>
<tr>
<th>TIME AFTER INJECTION (hours)</th>
<th>DIAMETER (cm) OF REACTIONS AT THE SITE INJECTED WITH</th>
<th>antiserum</th>
<th>IgG of antiserum</th>
<th>IHA-Ab</th>
<th>AB-Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ed*</td>
<td>Er**</td>
<td>Ed</td>
<td>Er</td>
</tr>
<tr>
<td>zero</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.2</td>
<td>1.2</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>0.2</td>
<td>1.2</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td>0.2</td>
<td>1.2</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.15</td>
<td>1.5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>11.5</td>
<td></td>
<td>0.2</td>
<td>1.4</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>0.2</td>
<td>1.5</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>26.5</td>
<td></td>
<td>0.2</td>
<td>1.7</td>
<td>0.8</td>
<td>0</td>
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<tr>
<td>35</td>
<td></td>
<td>0</td>
<td>1.5</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Grade

* Ed = Edema as indicated by the Evans Blue dye.
** Er = Erythema as indicated by the area of reddening.
DISCUSSION
The measurement of the number of antigenic determinants by conventional approaches such as the quantitative precipitin test is complicated by the self-association of alpha toxin molecules. This complication was overcome by the use of an fluorescence polarization (FP) immunoassay with a special design. The design involved the use of Fab instead of IgG. Fab can only form primary immune complexes with alpha toxin. Therefore, complications with secondary immune complexes are abolished. Furthermore, the use of Fab instead of IgG has the following theoretical advantage. Antibodies directed to determinants that were physically close together, may fail to bind the antigen simultaneously (Atassi 1967, 1975). The probability of this kind of hindrance is reduced when Fab, which is smaller than IgG, is used instead of IgG. To my knowledge, this is the first time the FP assay has been designed to measure the number of antigenic determinants.

Three determinants are present on alpha toxin because three Fab were detected on the antigen by the FP assay. It should be noted that the number 3 remains as the minimal estimate due to the following reasons. First, only epitopes (determinants on the surface of the antigen) were detectable by this method. Cryptotopes (determinants hidden inside the interior portion of the protein) would escape detection. Secondly, different antisera may recognise different determinants (Atassi and Saplin 1968, Atassi 1975). This possibility remains to be tested for the alpha toxin-antitoxin system. Thirdly, the possibility
remains that determinants may be so close together that even the use of Fab could not resolve the determinants. In the mean time, the number 3 may be taken as a minimal number of determinants.

IHA-Ab did not precipitate with alpha toxin. According to the lattice theory, antibodies directed to a single determinant cannot form precipitates with the antigen. Also, the immune complexes, formed between IHA-Ab and toxoid, fixed complement poorly in solution. These observations are consistent with the contention that the IHA determinant is single. For instance, monoclonal antibodies in other systems also fix complement poorly (Milstein 1980).

The antibinding determinants must be multiple because AB-Ab precipitated, and fixed complement, readily with alpha toxin in solution. Furthermore, quantitative precipitin tests revealed 2 determinants when AB-Ab were used. These findings, taken together with the result of the FF assay, suggested there are 3 determinants on the surface of alpha toxin: one IHA determinant and two antibinding determinants. A model that accommodates the present data on the antigenic structure of alpha toxin is presented (Fig. 4.1). This model proposes a specific orientation of the toxin molecule with respect to the erythrocyte surface.

The complete antigenic anatomy of alpha toxin awaits the isolation and amino acid sequencing of the various determinants. Preliminary work had begun along this line. Twenty four hours digestion of alpha toxin by trypsin resulted in no detectable antigenic fragments (data not shown). However, in subsequent experiments (Suruja, personal communication), a 25000 dalton antigenic fragment was obtained after 10 minutes of tryptic digestion.
Figure 4.1. The antigenic structure of alpha toxin.

Three determinants are shown on alpha toxin (circle). The 2 antibinding determinants are labelled as $AB_1$ and $AB_2$. They are thought to be located near the membrane-binding region (M) of the toxin. The arc represents the erythrocyte surface bearing the receptor (Band 3) for alpha toxin. The IHA determinant, but not the antibinding determinants, remains accessible to antibodies when the toxin is bound to the erythrocyte surface. Neutralization is effected by any one of the three antibodies. The membrane-bound toxin is neutralized by IHA-Ab whereas the binding of the toxin to the erythrocyte is prevented by any one of the two AB-Ab.
Other workers (Watanabe and Kato 1978; Kato 1979) isolated a biologically active fragment by limited tryptic digestion. The two fragments by the two laboratories remain to be compared.

The ratio $N_{AB}/N_w$ revealed that the proportion of antibodies directed to different determinants of alpha toxin varied from animal to animal. The phenomenon, that the proportion of different antibodies varied from individual to individual, is not unique to the antisera under study. The same phenomenon has been observed in the studies of human serum albumin (Oliveira and Lapresle 1966), myoglobin (Atassi and Saplin 1968), thyroglobulin (Dussault and Guay 1974), and bovine collagen (Nowack et al. 1975).

Several tests were used to examine the course of antibody responses among a group of rabbits immunized with alpha toxoid. The IHA test and the CF test depicted conventional curves of antibody responses. Yet, unusual patterns were observed when the neutralization test was used. The different descriptions of antibody responses was clearly illustrated when the immune response curve of AB-Ab obtained by CF and by neutralization were superimposed (Fig. 3.2). The different patterns resulted from the fact that different tests measured different parameters. On the one hand, the IHA titre and the CF titre are dependent primarily on the reaction between the antigen and the antibodies. On the other hand, the neutralization titre (especially $N_{AB}$) primarily reflects the competition between the receptor and the antibodies for alpha toxin. This competition is affected by the amount as well as the affinity of the antibodies.

The ratio $N_{AB}/N_w$ varied with time in any single
individual. This observation implied that antibody responses to the different determinants of the same antigen follow independent courses. Similar observations were reported in other studies. For example, variation in the proportion of antibodies in an individual over time was observed in rabbits immunized with human serum albumin (Kabat 1976). In anamnestic responses, the production of antibodies to one particular determinant may be selectively favoured while the production of antibodies to some other determinants on the same antigen ceased. This peculiar phenomenon was observed in 1 out of 6 rabbits immunized with bovine serum albumin (Porter 1957). Numerous other studies on antibody maturation confirmed the fact that the affinity of antibodies rise and fall during immune responses (Urbain et al. 1971, Doria et al. 1972, Macario and Conway de Macario 1973, Goidl et al. 1975, Haimorich and Du Pasquier 1976, Tasiaux et al. 1978). According to the network theory (Jerne 1974; Urbain et al. 1981) which deals with the control of antibody synthesis, fluctuation of affinity is expected during the immune response. However, the network theory did not explain the peculiar phenomenon of antibody responses that selectively favor one determinant over another. Since we are working with antibodies to different determinants and affinities can be measured by fluorescence polarization, the alpha toxin-antitoxin system may be a useful model for future studies on the course of antibody responses to different determinants of a protein antigen.

The phenomenon of low AB-Ab titre in response to immunization remains unexplained. It should be stressed that the
sample size is small (4 rabbits) and more studies are needed before generalizations can be made. Assuming this phenomenon is reproducible, one might take advantage of those rabbits with high preimmune titres to selectively elicit antisera enriched for IHA-Ab. The availability of IHA-Ab-enriched sera that are deprived of AB-Ab, would facilitate the immunologic research of alpha toxin because the existing method for the isolation of IHA-Ab produces a poor yield of IHA-Ab. Another possible method of enrichment for IHA-Ab is to see if immunization with toxoid-AB-Ab complex or toxoid-IHA-Ab complex would selectively alter the immune responses. This idea is based on the example in which passive immunization of Rh- mothers with anti-Rh antibodies prevents antigenic stimulation by erythrocytes from Rh+ newborns (Friedman and Friedman 1969).

There are striking similarities between antibodies to alpha toxin and antistreptolysin O. Streptolysin O has 2 topologically distinct sites: a fixation site (f) which binds to the receptor (cholesterol) on the cell surface and a lytic site (l) which triggers cell damage after fixation (Prigent et al. 1974). When antistreptolysin O was elicited in horses (Alouf et al. 1965), the ratio of neutralization titre/hemagglutination titre varied from serum to serum and even in the same animal with time. In 2 out of the 3 horses, the neutralization titres dropped despite an increase in hemagglutination titres (comparable to the antisera of week 4 in our system). The neutralization titres, and their corresponding hemagglutination titres, of 12 sera were taken from this report and regression analysis was performed. About 70% of the variation in neutralization was accounted for by the
variation in hemagglutination (correlation coefficient = 0.84, p = 0.01). It appears that the hemagglutinating antibodies accounted for the majority of the neutralization capacity in whole sera. Sera that contained only anti-f or only anti-l were found (Seligmann et al. 1968; Prigent et al. 1974). The similarities between antistreptolysin O and antibodies to staphylococcal alpha toxin are summarized in table 4.1.

The earlier report of Felsenfeld and Felsenfeld (1966) that all of the neutralizing antibody in humans occurred in the IgG fraction does not conflict with our observations that the IgG, IgA and IgM classes contained neutralizing and IHA antibodies to alpha toxin. Their sera were from individuals with chronic staphylococcal skin lesions and thus would be expected to be in secondary responses, whereas our animals were immunized over a short period of time.

Jozefczyk (1976) found, in patients with active staphylococcal infections, that there was often an elevated level of IgG, sometimes accompanied by an elevation in IgA but rarely an increase in IgM levels. The present study demonstrated directly the presence of antitoxin in the three classes of immunoglobulins in rabbits.

The CF test for antibodies to alpha toxin was highly sensitive in comparison to CF tests for other antibodies important to the serodiagnosis of other diseases. For example, syphilitic sera from humans fixed less than 30 CH₅₀ with Wassermann antigens (Osler and Knipp 1957) and the titre of complement-fixing antibodies, following Rubella infection in
TABLE 4.1. Similarities between antistreptolysin O and antibodies to staphylococcal alpha toxin.

<table>
<thead>
<tr>
<th>ANTIBODIES TO ALPHA TOXIN</th>
<th>ANTISTREPTOLYSIN O</th>
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<tbody>
<tr>
<td>2 populations</td>
<td>anti-binding</td>
</tr>
<tr>
<td></td>
<td>IHA-Ab</td>
</tr>
<tr>
<td></td>
<td>anti-fixation</td>
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<tr>
<td></td>
<td>anti-lytic</td>
</tr>
<tr>
<td>Ratio that varied from</td>
<td>N_AB</td>
</tr>
<tr>
<td>serum to serum and with</td>
<td>N_W</td>
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<tr>
<td>time in an individual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neutralization titre</td>
</tr>
<tr>
<td></td>
<td>hemagglutination titre</td>
</tr>
<tr>
<td>% neutralization capacity</td>
<td>65</td>
</tr>
<tr>
<td>in whole sera accounted</td>
<td>70</td>
</tr>
<tr>
<td>for by hemagglutinating</td>
<td></td>
</tr>
<tr>
<td>antibodies</td>
<td></td>
</tr>
<tr>
<td>Sera that contained only</td>
<td>found</td>
</tr>
<tr>
<td>one or the other</td>
<td>found</td>
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<tr>
<td>population of antibodies</td>
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humans, did not exceed 64 (Sever et al. 1965). We found CF titres of 1:16 in normal humans and titres from 1:100 to 1:500 in immune sera from rabbits. Yet, the use of CF as a routine assay for antitoxin is restricted by two considerations. Firstly, the ubiquitous presence of antitoxins in the amboceptor and complement reagents poses a practical problem. Sera must be screened for antitoxin and only those sera devoid of both AB-Ab and IHA-Ab are suitable for use as amboceptor or as a source of complement. While IHA-Ab can be removed from these reagents by adsorption with LGCE, we lack a method to eliminate endogeneous AB-Ab. Secondly, although the use of toxoid as the antigen in the CF test, circumvented the problem of hemolysis due to the toxin, the binding of toxoid to erythrocytes renders the cells fragile (Barei and Fackrell 1980). Fragile erythrocytes are not ideal indicators in the CF test. In view of these considerations, the neutralization test and the IHA test are superior as routine assays for antitoxin.

The Standard Serologic CF Test demonstrates that both AB-Ab and IHA-Ab fix complement in solution. Furthermore, immunologic injuries were induced by both types of antibodies as illustrated by the Reverse Passive Arthus reactions. These findings fulfilled the necessary prerequisite for type III or complex-mediated hypersensitivity (Osler 1976). This type of hypersensitivity may be clinically manifested as glomerulonephritis (Cochrane and Koffler 1973). Glomerulonephritis has been associated with both coagulase-positive (Tu et al. 1969) and coagulase-negative (Black et al. 1965, Dobrin et al. 1976) staphylococci. In many of these cases,
the demonstration of IgM, IgG and complement deposited in the glomeruli strongly implies complex-mediated hypersensitivity. The one attempt to demonstrate the presence of toxin-antitoxin complexes in the glomeruli (Black et al. 1965) was with coagulase negative staphylococci and no such complexes were found. However, the role of toxin-antitoxin complexes in infections of coagulase positive staphylococci warrants investigation.

Antigens or haptens that are not normally part of the cell membrane can be chemically coupled to the membrane and serve as a target for the action of the specific antibody and complement (Borsos and Langone 1981). This phenomenon, called passive immune hemolysis, did not occur in our system. Although the IHA-Ab immune complexes consumed complement, the carrier erythrocytes did not lyse. Based on modern concepts of the sequence of activation of complement, we think that there is an interference with one of the early events. Antibody in the immune complex binds the first component of complement, C1, which hydrolyses C4 to yield C4a and C4b. C4b binds to a membrane receptor and positions C2 so that it can be hydrolysed by C1. Subsequent activation steps are independent of the antigen-antibody complex (Rapp and Borsos 1970; Muller-Eberhard 1975; Osler 1976). Unlike the nonspecific distribution of antigen that predominates in passive immune hemolysis, alpha toxin specifically binds to Band 3. Band 3 is a transmembrane protein that is anchored in the cytoskeleton and thus is relatively immobile (Peters et al. 1974; Bennet and Stenbuck 1979). If band 3 is not in the vicinity of the C4b receptor, C2 may not be split even though C1 and C4 may
be consumed. The above rationale could explain the lack of passive immune hemolysis in our system. The lack of passive immune hemolysis was also observed in human anti-Rh antibodies and "naturally occurring" anti-A antibodies of the A, B, O blood typing system (Zmijewski 1968, Friedman and Friedman 1969).

The erythrocyte is merely a model for the study of the mode of action of alpha toxin, and many other cell types are targets for the toxin. The lack of passive immune hemolysis does not preclude immunologic injuries to cells other than erythrocytes in vivo. This point is substantiated by the positive Reverse Passive Arthus Reactions, and should be considered since receptors for the toxin have been found in cultured cells (M. Thelestan, personal communication). This consideration is crucial in any attempts to establish the specific target cells in vivo.

Alpha toxin is thought to induce a number of pharmacological effects including the liberation of 5-hydroxytryptamine (serotonin) and histamine (Jeljaszewicz 1972, Jeljaszewicz et al. 1978). However, these vasoactive amines are also released by anaphylatoxins which are products of complement fixation (Johnson et al. 1975, Osler 1976). Before any pharmacological effects can be attributed solely to the toxin, the involvement of complement must be ruled out. This concern is all the more significant because of the prevalence of complement-fixing antitoxin in nonimmunized rabbits and humans. Since toxoid which is not hemolytic fixes complement, we propose the use of toxoid in control animals in experiments in which in vivo effects of toxin are of concern. In this manner, the toxic effect of the toxin
may be discerned from its immunologic effect.

We propose that the protective value of IHA-Ab is superior to that of AB-Ab. Since this proposal is valuable in clarifying the protective value of antitoxins, or in the design of a vaccine, further testing of this proposal is warranted. This proposal is based on the following five considerations. First, IHA-Ab account for the majority (approximately 65%) of the neutralization capacity in antiserum. Second, IHA-Ab confer more protection than AB-Ab to cells already exposed to alpha toxin; a phenomenon explained by the IHA-Ab's ability to neutralize the toxin on the membrane (Lo and Packrell 1979, Lo 1979). Third, IHA-Ab fix complement poorly. Therefore, the chance of immunologic injury due to complex-mediated hypersensitivity should be less in comparison with AB-Ab. Fourth, it appears that most humans are immunocompetent to the challenge by the IHA determinant because IHA-Ab prevailed in 95% of normal human sera. The fifth point is merely conjectural. Erythrocytes are now being recognized to be important in the clearance of antigens from the body (Siegel and Liu 1981; Medof and Oger 1982). It is possible that erythrocytes may "dilute out" the toxin in vivo, especially in the case of human erythrocytes which are resistant to lysis by alpha toxin (Bernheimer 1965, Cooper et al. 1966) or lysis by complement (Osler 1976). If this mode of antigenic clearance does apply to alpha toxin, IHA-Ab would not hinder such actions, but AB-Ab would.
SUMMARY

This investigation achieved the 3 objectives; namely, the measurement of the number of antigenic determinants on alpha toxin, the characterization of the antitoxins, and the examination of complement fixation by the various immune complexes. These findings constitute a basic knowledge of the immunology of alpha toxin. This knowledge is essential to the assessment of alpha toxin as a virulence factor. Thus, the conflicting reports on the protective role of antitoxin in vivo may be enlightened by the complexity of the antitoxins, in conjunction with the complement fixation by the different immune complexes. In the future, all experiments concerning the effects of alpha toxin in vivo must fulfill 2 prerequisites: the knowledge of the prereactive status of the host, and the pharmacological effects of toxoid.

Alpha toxin contains a minimum of 3 antigenic determinants: 1 IHA determinant and 2 antibinding determinants. The toxin is neutralized by any one of the three antibodies, although more than one antibody can bind the toxin at the same time. There is no evidence for the existence of antibodies that bind but do not neutralize the toxin. Since the protective value of IHA-Ab appears to be superior to that of AB-Ab, the IHA determinant is the logical candidate for the design of vaccines.


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