1979

Antibodies to staphylococcal alpha toxoid.

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ANTIBODIES TO STAPHYLOCOCCAL ALPHA TOXOID

by

CHING YEE LO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

WINDSOR, ONTARIO

February, 1979

This thesis is dedicated to my parents.

ABSTRACT

Erythrocyte, a target cell for staphylococcal alpha toxin, was used as a template to orient alpha toxoid. The toxoid erythrocyte complex was covalently bonded with glutaraldehyde and used as an immunoadsorbent. By immunoadsorption and desorption, antitoxin was separated into two distinct populations. One population prevented binding of alpha toxin onto erythrocyte membranes. The other population neutralized after the toxin was bound to erythrocytes and thereby brought about an indirect hemagglutination reaction. Competition between the two populations of antibodies was observed in neutralization tests.

Elucidation of the modes of neutralization of these two antibodies led to the conclusion that bound toxin is immunologically oriented on erythrocyte membranes. Subsequent testing by the same methods indicated that alpha toxin is oriented similarly on erythrocytes from different animal species.

ACKNOWLEDGEMENTS

Special thanks is due to my advisor, Dr. H.B. Fackrell, for his guidance and enthusiasm. I would like to thank Dr. A. H. Warner of this Department and Dr. K.E. Taylor of the Department of Chemistry for serving on my committee. The valuable discussions with Mr. Indar Maharaj and Mr. Gary Barei are much appreciated.

I am most grateful to my wife, Teresita, for her patience and moral support during this study, and for her time and endurance in typing this thesis.

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INTRODUCTION

According to the International Committee on Systematic Bacteriology (Subcommittee, 1965), alpha hemolysin is one of the six crucial criteria to distinguish the pathogen Staphylococcus aureus from the saprophyte Staphylococcus epidermidis (9). A renowned investigator considered that " of the several toxic and potentially toxic products of pathogenic staphylococci, the most potent is the alpha toxin" (3). Despite the close association of alpha hemolysin with pathogenicity, the role of this toxin in disease processes is obscure. Also, numerous attempts to determine the value of antitoxin in the protection against the onset of infection or in the alteration of the course of the disease, have met with conflicting results. These latter studies were critically reviewed by Elek in 1959 (48) and extensively discussed by Ekstedt in 1972 (49). The differences in the methods of immunization and methods of challenge, as well as host differences, may account for the conflicts. However, it seems unlikely that the pathogenic role of the toxin and the protective value of antitoxin could be elucidated unless the toxin is chemically and antigenically characterized. While considerable data on the physicochemical characteristics of the toxin are available (103),information regarding antigenic characteristics is so crude that even the class of immunoglobulins to which antitoxin belong (52) is questionable. It has been stated that " A further detailed study of the nature of the immunoglobulins produced in experimental immunization with alpha toxoid would seem to be required."(3)

Moreover, staphylococcal toxoid (69), which is defined in terms of alpha hemolysin, is used in everyday medical practice without an understanding of the basic immunologic parameters of this vaccine.

In addition to all of the above medical implications, the study of the antigenic characteristics of alpha hemolysin fulfills an inadequate area in the knowledge of protein immunology. Several proteins such as sperm whale myoglobin (7), human hemoglobin (90), and egg white lysozyme (8) have been well characterized antigenically. However, these proteins, unlike alpha hemolysin, do not interact with biomembranes. It has been suggested that alpha toxin, along with cytolytic toxins of bacterial origin, may prove useful in probing the molecular organization of biomembranes (12, 1). Hence, immunologic study of alpha toxin should provide some basic knowledge on the immunology of membrane-associated protein leading to its use as a molecular probe for biomembranes.

The objective of this investigation is to establish some basic immunologic parameters upon which the antigenic anatomy of alpha toxin could be built.

LITERATURE REVIEW

Before the turn of the century, medical investigators (43,68,21,92,99,82,67) 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 (23,25). He put forth the "unitarian hypothesis" that "... the one toxin is responsible for the three characteristic activities of staphylococcal filtrates..." (25). His view was substantiated when purified toxin preparations were shown to exhibit the various toxicities described under Biological Effects on page 7 (65,66). Similar findings were repeated independently (14,59,61,72) and summarized (11). 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 (77).

The recognition of the four types of staphylococcal hemolysin was accelerated by the resolving power of specific antisera in researches during the 30's and 40's.

In the 20's, a "hot-cold" hemolysin was described (17) that was subsequently found to be relatively active on sheep erythrocytes (18). 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 (56) distinguished immunologically the sheep hemolysin from the one which rabbit erythrocytes are most

sensitive. They designated the rabbit hemolysin alpha- and the sheep hemolysin beta-, a terminology adopted ever since. In the following year, Morgan and Graydon (81) reported two antigenically different hemolysins which they termed alpha$_1$ and alpha$_2$. Two years later, Smith and Price (96) proposed the existence of gamma lysin which was subsequently confirmed by Marks (74). In 1947, Williams and Harper (102) discovered delta hemolysin which was not neutralized by anti-alpha and anti-beta and which had a hemolytic spectrum unlike that of gamma lysin. Existence of the four hemolysins was confirmed by Marks (74) 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 (103).

Production and Purification

Numerous methods for production and purification of alpha toxin have been vigorously compared and reviewed (103) and will not be reiterated here. Recently, two relatively simple methods of purification were reported: one takes advantage of the aggregation of the toxin by heat (41) and the other uses adsorption chromatography on controlled pore glass (19).

Physicochemical Characteristics

Purified alpha hemolysin is a protein. Several reports (14,34,50,95) on the amino acid composition are in reasonable agreement, and revealed the presence of more acidic than basic

amino acids. As expected, the protein migrates anodically. Aspartic acid contributes to more than 10% of the total amino acid content.

Various reports on molecular weights of alpha hemolysin, ranging from 10^4 to 4.5×10^4 daltons, have been summarized (103). Although the difference in methods used may be important, the apparent discrepancy in molecular weight (M.W.) determination may be attributed, to a greater extent, to the proteolytic degradation of the hemolysin during production. Dalen (40) found that in culture, alpha hemolysin (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 (34), histidine (104, 50) and alanine (94, 95) have been ascribed to be the N-terminal amino acid.

Alpha hemolysin occurs in multiple forms. In 1963, Bernheimer and Schwartz (14) obtained 4 biologically similar, but electrophoretically distinct, peaks from pure hemolysin by sucrose density gradient centrifugation. In isoelectric focusing, although 80% of the hemolytic activity was recovered in a major component with pI 8.5 ± 1 (100, 79, 58, 51), 3 minor components, which are biologically equally active, were located at other pH's (100, 79). When each of the 4 components were refocused, the same pattern of the 4 bands appeared (100). In 1907, Arrhenius (6) reported that brief heating at 60°C converted alpha hemolysin to nonhemolytic aggregates which became

hemolytic after heating to 100°C. In 1966, Coulter (34) found that aggregates, which precipitated upon storage of the hemolysin at 4°C in physiological buffer, was nonhemolytic but immunogenic. Multiple forms of alpha hemolysin are observed in sedimentation studies. Fackrell and Wiseman (51) discovered that the sedimentation coefficient of the hemolysin changed from 1.4S, when freshly prepared, to 2.8S after 3 days of standing at 4°C in physiological buffer. Other studies revealed that purified hemolysin preparations contain 70 to 90% of 3S molecules and 10 to 30% of 12S forms (5,12). This nonhemolytic 12S form is indistinguishable by electron microscopy from the nonhemolytic 12S form which arose from heating at 60°C; both 12S structures were similar on membranes (5). Therefore, the inactivation of alpha hemolysin at 60°C and the subsequent reactivation at 100°C, or by 8 M urea (4), may be assumed to be the result of interconversion between the 3S and the 12S forms, and that the soluble 12S form aggregates further to produce insoluble precipitates.

Biosynthesis

Little is known about the biosynthesis of alpha hemolysin. Arginine, glycine and proline (38,55) were found to be essential in hemolysin production. Parker et al (87) increased production by incubating cultures under 10% CO_2 in air. Burnet (24) found that 20-40% CO_2 and 0.3% agar

enhanced yield in his "Wood 46" strain but not in the "Wood Albus" variants. The use of CO_2 is followed by others(65,72, 91,106). Dalen suggested that the stimulating effect of CO_2, serine and glycine was related to their role as precursors of histidine (36,37). However, he found that , unlike tetanus toxin (80), alpha toxin production was not directly correlated with the intracellular level of free histidine (37).

Alpha hemolysin is formed during the logarithmic phase of growth. It is an exotoxin because it is released by intact cells (45). It constitutes 2% of the dry weight of the cell (45,14) and is localized solely on the inner surface of the cytoplasmic membrane (35) prior to release.

The genetic control of alpha toxin biosynthesis was examined by McClatchy and Rosenblum (77,78). They obtained 2 groups of alpha toxin mutants. One group produced immunologic cross-reacting proteins with varying degree of dermonecrotic and lethal activities. The other group produced no cross-reacting materials and is usually fibrinolysin negative. Based on these data, they postulated that two genetic loci, a regulator and a structural gene, are involved in alpha toxin biosynthesis. Their data did not resolve whether the multiple biological effects (see below) are attributed to one or multiple sites on the toxin molecule.

Biological Effects

In addition to hemolysis, the property that conveys the name "hemolysin" to this toxin, alpha toxin is lethal,

dermonecrotic, platelet damaging, and cytotoxic. Although these pharmacological effects are well described (60), the underlying mechanisms are poorly understood. Any one of these toxicities qualifies alpha hemolysin to be a toxin. Therefore, the term alpha hemolysin and alpha toxin will be used interchangeably in this thesis.

The diversity of toxicity on various cell types forces the search of a common denominator among different target cells. Since all susceptible cells have, in common, a membrane which is the primary contact with the toxin, studies were focused on the interaction between alpha hemolysin and membranes. Various methods used in these studies included fluorescein labeled antitoxin (63), fluorescein labeled anti-immunoglobulin (64), ^{125}I-labeled toxin (28), adsorption of the toxin with ghosts (106), and electron microscopy (53,54). The conclusion, that alpha toxin is bound to membranes from erythrocytes of rabbit, is the same. Hence, most investigators today considered the cell membrane to be the primary site of action of the hemolysin.

Mode of Action

As of 1976, alpha hemolysin was categorized under the group of "toxins for which the most significant event in toxin-membrane interaction is not known" (13). Two schools of thought remain : the nonenzymatic versus the enzymatic mechanism.

The nonenzymatic view stresses the 'surface activity' of the hemolysin on membranes. Weissman (101) first showed the release of marker molecules such as chromate and glucose from liposomes by alpha toxin. Since liposomes containing lecithin, cholesterol and either dicetylphosphate or stearylamine were equally sensitive, alpha toxin was not interacting specifically with any individual phospholipid. A nonspecific hydrophobic interaction between the toxin and membrane lipids in general was confirmed by further studies on mixed lipid monolayers (22). Freer et al showed by electron microscopy that alpha toxin polymerizes to form regular arrays of ringlike structures on liposomes and erythrocyte ghosts (53). These rings are identical with the naturally occuring 12S nonhemolytic polymeric form described earlier (P. 6). Recently, these ring structures have been reproduced but unpublished by two independent groups of investigators (29, 70). More important is the argument proposed by both of these two groups that the ring structures, which are found at high concentration of alpha toxin, reflect merely bindings unrelated to the actual high affinity binding at low but hemolytic toxin concentration at which these rings are not found.

Freeze etch studies revealed plaques having few membrane intercalated particles on the fracture plane of toxin treated ghosts, and suggested that the toxin may penetrate the hydrophobic interior of membranes (54, 16). The surface activity theory was further advocated since these changes

resembled closely, those described for erythrocyte ghost treated with phospholipase A and saponin (97). However, alpha toxin has been shown not to be a phospholipase (34,104).

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..." (13).

During the 60's the enzymatic mode of action of alpha toxin was implicated by kinetic studies which indicated that the rate of hemolysis is directly proportional to the concentration of the toxin (71,75,76,31). 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 (28) and kinetic studies did not take into account the dynamics of binding and release (13). However, the finding of partial release of toxin after binding was retracted recently (29). After a series of experiments on the enzymatic mode of action of alpha toxin, Wiseman et al (103,104,105) proposed that the toxin is secreted as a protease zymogen, which is activated by membrane bound protease and itself becomes a protease

capable of hydrolysing membrane polypeptides. Freer et al (54) were unable to confirm this proteolytic mechanism. The difference in the method of obtaining "ghosts" could affect the results (46).

It should be pointed out that most investigators do not uphold either of the two views exclusively, and many have worked on both hypotheses.

In addition to providing an enzymatic implication for the mode of action of alpha toxin, the kinetic studies in the 60's also reveal a sigmoid curve that indicates three stages in the time course of hemolysis and a tailing off in the rate of lysis (71,75,76,31,32,73,3). In the prelytic phase, loss of selective permeability of the membrane is indicated by the leakage of potassium ions. The lytic phase reflects a period of rapid, linear release of hemoglobin (31,32,71,3). Marucci (76) identified two steps in the hemolytic process. The first step involves the reaction of alpha toxin with the red cell. The second step, leading to the release of hemoglobin, is an intrinsic reaction of the damaged cell and takes place without further participation of the toxin. The first step is reversible by antitoxin but not the second step at which point the cells are "committed" to lysis. Marucci suggested that the lag can 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 erythrocyte is destroyed. Cassidy and Harshman (29) recognized three sequential events: firstly,

binding of free toxin; secondly, induction of foci of membrane injury that leads to the leakage of small inorganic molecules; and thirdly, the eventual osmolysis of the cells.

While the enzymatic versus nonenzymatic mechanism of the destructive event is debated, the initial binding event is now better understood. The existence of receptor sites on membranes have been postulated by several workers since the 60's (64,28,106). Recently, the number of receptors on erythrocytes and the affinity of binding with the toxin have been independently measured by Cassidy and Harshman (29) and by Barei and Fackrell (report in preparation). The receptor is assumed to be proteinaceous in nature because the treatment of erythrocytes with pronase reduces the number of receptors (29) (Maharaj and Fackrell, unpublished results). Based on this assumption, attempts to isolate the receptor for alpha toxin are in progress (29).

MATERIALS AND METHODS

BIOLOGICAL METHODS

Cultures

Staphylococcus aureus strain Wood 46 (23) used in this study fulfilled the criteria for S. aureus given by Baird - Parker (10). This strain was preserved by lyophilization and by subculturing once a month on 1.5% agar (Difco) slants with Dolman Wilson media base (44). The subcultured organisms were incubated at 37°C for 24h and stored at 4°C. The strains were examined periodically for purity, hemolysis on rabbit or sheep blood agar plates, and for positive reactions in coagulase, mannitol and deoxyribonuclease tests. The phage susceptibility patterns were confirmed recently by the Canadian Communicable Disease Centre in Ottawa.

Production of Crude Hemolysin

The method of Wiseman et al (106) was used. The supernatant obtained by centrifugation of cultures will be referred to as crude hemolysin.

Purification of Alpha Hemolysin

The method of Wiseman et al (106) was used. Briefly, this involves precipitation of the crude hemolysin at pH 4 by methanol at -20°C. The precipitate was obtained by centrifugation at 10,000 g for 10 min. The precipitate was partially purified and concentrated by stepwise fractionation with 40%

saturated and then 60% saturated ammonium sulphate solution. The hemolysin at this stage will be referred to as semi-purified.

Final purification was achieved by gel filtration through a Sephadex G 75 column eluted with Hallander's buffer — 0.01 M phosphate buffer, pH 7.0, supplemented with 0.5% (W/V) NaCl. One hundred fractions (15 ml/tube) were collected. A volume of 50 µl of each fraction were added to 50 µl of a 2% (V/V) rabbit erythrocyte suspension in Microtitre® plates and incubated for 1 h at 37°C. Fractions which showed complete hemolysis were pooled and concentrated in $\geqslant$ 80% saturated ammonium sulphate solution. The precipitate obtained was stored in saturated ammonium sulphate solution. Samples of the purified toxin were dialysed against phosphate buffered saline (PBS) before use. Purity of the toxin was established when a single precipitin line was observed in immunodiffusion tests against antisera raised to semi-purified toxin.

Purification was also achieved by adsorption chromatography on controlled pore glass (19). However, this method is limited.

Toxoid

For some experiments, the hemolysin was rendered non-hemolytic by heat at 60°C for 1/2 h (5). This product was referred to as toxoid in accordance with Burnett who restricted the term "toxoid" to nontoxic physical forms and applied the term "anatoxin" to formalin detoxified forms (25).

Erythrocytes

Human type A, Rh^+ blood was obtained from the Red Cross through the blood bank at Hotel Dieu Hospital in Windsor. Type O, Rh^+ blood was donated by a volunteer in this laboratory. Citrated sheep blood was supplied by Woodlyn Laboratories, Guelph. Rabbit, guinea pig, rat, and mouse erythrocytes were obtained by bleeding animals maintained in this department. Coagulation was prevented by either using heparinized tubes or defibrination by shaking with glass beads. Blood obtained from departmental animals, if not used immediately, were stored in an equal volume of Alserver's solution (27).

Prior to use, the erythrocytes were thrice washed in PBS and resuspended to a 2% concentration (V/V) in the same buffer, unless otherwise specified.

Hemolytic Titration

The 50% end point method (105) was followed except the volumes of the reagents were reduced proportionally. Starting with 50 µl of alpha toxin, a 2-fold serial dilution was made in PBS in Microtitre® plates (Cooke Lab. Products) with U-shaped wells. Then, 50 µl of a 2% (V/V) suspension of rabbit erythrocytes were added. After the mixture was incubated at 37°C for 1 h, 50 µl of PBS was added and the plates were shaken gently. After centrifugation at 600 x g for 5 min., 100 µl of the supernatant was diluted 1:3 with PBS and the absorbance was read spectrophotometrically at 541 nm (1 cm light path). The hemoglobin content of the supernatant thus measured was translated

into % hemolysis by comparison to standards prepared from a 2% erythrocyte (V/V) lysate in distilled water. One hemolytic unit (HU) is the reciprocal of that dilution of toxin giving 50% hemolysis.

Kinetic Hemolysis (KH) Test

When the turbidity of rabbit erythrocyte suspensions is monitored at 650 nm (Coleman 124 double beam spectrophotometer), a proportionality is observed between absorbance and concentration (Fig. 1). Based on this relationship, the extent of hemolysis is visualized by a corresponding decrease in the absorbance of the reaction mixture with time. The final reaction volume in a cuvette of 1 cm light path was 0.3 ml. This volume contained washed erythrocytes in PBS and hemolysin so that the initial absorbance of the mixture was about 1.6 at RT. The course of hemolysis was graphically traced by a Sargent Recorder. The slope of the plot of absorbance decrease versus time, expressed as absorbance change per min. (Δ_{OD}), reflected the rate of hemolysis.

Antisera

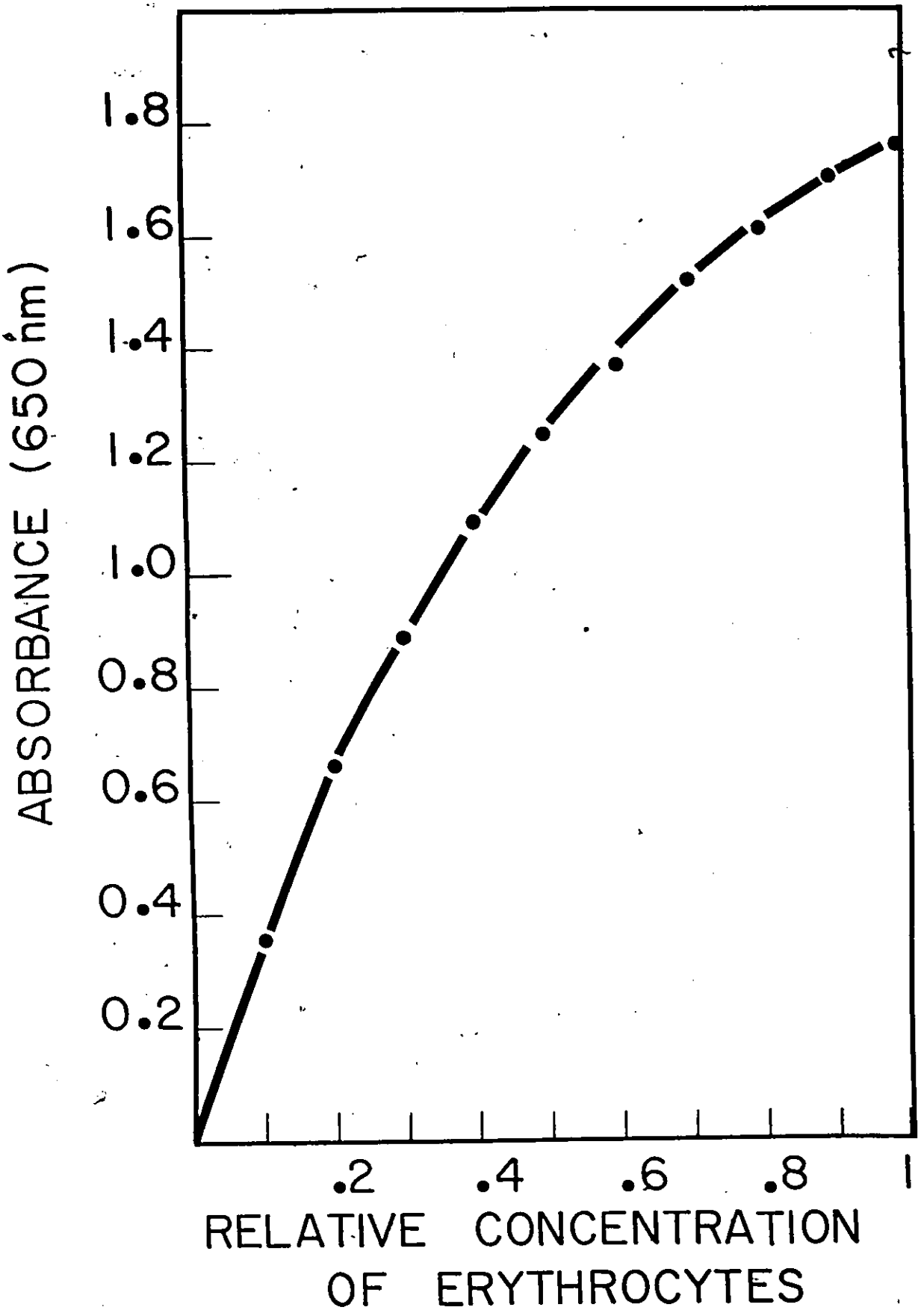
Antitoxoid was raised in rabbits and pigs.

Twelve 8 month old New Zealand White rabbits were hyper-immunized 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. ON day 3 and 6, 0.25 ml and 0.5 ml of toxoid, respectively were administered intravenously.

FIGURE 1

Proportionality between absorbance and concentration of erythrocytes.

The concentration of rabbit erythocytes which had an absorbance of 1.7 at 650 nm was normalized to a value of 1. This suspension contained 1.5×10^6 cells per ml.


ABSORBANCE (650 nm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
.2
.4
.6
.8
1
RELATIVE CONCENTRATION
OF ERYTHROCYTES

Then 1 ml of toxoid was intravenously injected every 3rd day up to the 21st day. The rabbits were bled for sera before injection and every 7th day thereafter for 7 weeks.

Two young pigs were injected intravenously with 2 ml of toxoid at 1 mg/ml concentration, once a week for 6 weeks. An equal volume of Freund's incomplete adjuvant was mixed with the toxoid given in the first injection. On the 7th week, the pigs were bled from the inferior vena cava. After the erythrocytes were removed by centrifugation, the pig antisera were pooled.

All sera were titrated for heterophile antibodies to the test erythrocytes by hemagglutination reaction as described for the Indirect Hemagglutination Test (see below). Heterophile antibodies, when found, were removed by adsorption at 4^{o}C for 1h with the corresponding erythrocytes. Heterophile -free sera were stored at -20^{o}C until use.

Portions of pig's and rabbit's sera were purified to IgG fractions by ion exchange chromatography on DEAE-cellulose according to the method of Campbell et al (26).

Immunological Tests

Immunodiffusion in agar gel was performed by the method of Ouchterlony (85).

Complement fixation procedures were those employed by the Laboratory Branch of the Ontario Ministry of Health, Virology Section, Toronto (83). The 100% end point method was used.

Toxin neutralization tests were performed as follows. The concentration of toxin which produced 90% hemolysis was determined by hemolytic titration. Then, this concentration of toxin was doubled because 25 µl instead of 50 µl was used in neutralization tests. A serial 2-fold dilution of 50 µl of antiserum was done in PBS. Then, 25 µl of toxin at a concentration determined above, and 25 µl of a 4% suspension of rabbit erythrocytes (V/V) were added. After incubation at 37°C for 1 h, the supernatants of the mixtures were measured for hemoglobin content as described for hemolytic titrations. Serum dilutions were plotted against hemoglobin contents. From this plot, the dilution which produced 40% hemolysis was determined. The reciprocal of this dilution was designated as 1 neutralizing unit (N) because 50% of the hemolysis or 1 HU was inhibited. Controls for background hemolysis included erythrocytes alone, and erythrocytes plus serum without toxin, both made up to 100 µl with PBS.

Two types of antiglobulin tests were conducted to determine whether the antibody-toxin complex is in solution or on erythrocyte surfaces. When rabbit antitoxin was used for neutralization, goat anti-rabbit globulins were employed as anti-antibodies. The goat antiglobulin preparations were heterophile adsorbed (P. 41) prior to use.

The first type of test is based on the principle of the direct Coomb's reaction (107). Certain antibodies do not

agglutinate erythrocytes even when bound to erythrocyte surfaces (30). However, their presence on erythrocytes is detectable by indirect hemagglutination brought about by the addition of antiglobulin. In the neutralization tests, erythrocytes were protected from lysis when sufficient antitoxin was present. To these protected erythrocytes, 50 µl of goat anti-rabbit whole serum (Pantex®, Miles Lab.) was added directly. Goat antiserum with final dilutions of 2:3, 1:6, and 1:24 were tested. The mixture was incubated for 1 h at RT and the hemagglutination pattern read. The test was transferred to 4°C for overnight incubation and the patterns were rechecked next morning. The use of anti-rabbit serum allowed the specificity of the test to extend to all antigenic serum globulins including all classes of immunoglobulins.

The second type of antiglobulin test is indirect immunofluorescence. Fluorescein conjugated anti-rabbit IgG was purchased from Cappel Laboratory. This labelled goat antiglobulin preparation had been fractionated to IgG and was specific for the heavy chain of rabbit IgG. Erythrocytes that were protected by rabbit antitoxin in the neutralization tests were transferred into small tubes and centrifuged at 600 g for 10 min. The supernatants were removed. Then 100 µl of undiluted, fluorescein labelled anti-rabbit IgG was added so that the erythrocyte suspension was about 1%. Unreacted erythrocytes were included as controls for nonspecific fluorescence. After 1/2 h incubation at RT, the

erythrocytes were gently washed twice in PBS and mounted under PBS or FA mounting fluid (Bacto, Difco Lab.) Examination was done with a UV microscope with vertical fluorescence (Model 1070, American Optical Corp.)

PHYSICAL AND CHEMICAL METHODS

Buffers

All buffers were prepared according to Gomori (57). Sodium phosphate buffer, 0.01 M, pH 7.2, was supplemented with 0.85% NaCl, and this will be referred to as phosphate buffered saline (PBS).

Protein Assays

The amount of protein was measured by the technique of Lowry et al (108) with bovine serum albumin as a standard. The rapid, one step method which utilized Coomassie Blue G-250 dye (20) was also used.

Gel Filtration

Sephadex gel G-75 was supplied by Pharmacia of Canada Ltd., Montreal. The procedure for preparation of the gel and packing of columns were those recommended by the manufacturer (88). Glass columns 40 x 100 cm. were used and all filtrations were performed at 4°C.

RESULTS

CHAPTER 1

COMPLEMENT FIXATION TEST

Reichlin considered that "... for adequate sensitivity and convenience, coupled with the capacity for reproducible quantitation, the complement fixation techniques are very difficult to surpass" (90) for the study of antigenic determinants.

Unlike many antigens previously studied, alpha toxin can bind to erythrocyte membranes. Therefore, the complement fixation (CF) test may or may not be applicable. The applicability of CF to the study of alpha toxin, and some of the problems encountered, are described below.

In the CF test, the amount of complement fixed is indicated by the degree of hemolysis. Yet alpha toxin can hemolyse erythrocytes. To circumvent this problem, one must either use erythrocytes that were resistant to the toxin or render the toxin nonhemolytic.

Human erythrocytes are most resistant to alpha toxin (11, 33). However, human erythrocytes are also relatively resistant to lysis by CF (84). The feasibility of using human erythrocytes as the indicator was investigated by a series of titrations with various combinations of reagents. The results (Table I) allow several conclusions to be made. Firstly, sheep erythrocytes are the best indicator (most sensitive to complement lysis). Secondly, fresh guinea pig

TABLE I

COMPLEMENT FIXATION BY VARIOUS COMBINATIONS OF REAGENTS

COMBINATION	INDICATOR ERYTHROCYTE	SOURCE OF AMBOCEPTOR	SOURCE OF COMPLEMENT	TITRATION RESULTS UNITS OF AMBOCEPTOR	TITRATION RESULTS UNITS OF COMPLEMENT
1	sheep	rabbit	guinea pig	8,000	72
2	sheep	rabbit	guinea pig	8,000	32
3	sheep	rat	guinea pig	4,000	40
4	human	rat	guinea pig	0	0

* Commercial complement (Bacto, Lot. 3256 56, Difco Lab.)

All guinea pig sera, before their use as a source of complement, were heterophile antibodies adsorbed with the corresponding indicator erythrocytes.

complement is more potent than commercially lyophilized preparations. Thirdly, the rat antibodies are capable of fixing guinea pig complement. Finally, human erythrocytes are resistant to lysis by rat antibodies and guinea pig complement although the presence of anti-human erythrocyte in the rat amboceptor preparation was confirmed by positive slide agglutination tests.

Since the use of resistant erythrocytes failed to measure complement fixation, the alternative to use nonhemolytic toxoid was attempted. Reagent combination 1 was used because this combination proved to be the most sensitive indicator (Table I). In order to determine the CF titre of the toxoid, a "chess-board" titration of 10-fold dilutions of the toxoid versus 2-fold dilutions of antitoxin was done. The antitoxin used was purified to IgG. The results are presented in Table II. Toxoid at 1:4 and 1:8 dilutions exhibited anticomplementary effects in the absence of antitoxin. That is, toxoid alone interfered with normal hemolysis of the indicator erythrocytes. In addition, non-antibody induced hemagglutination by toxoid alone at 1:1 and 1:2 dilutions was observed. Therefore, this toxoid must be diluted $\geq$ 1:16 for use in CF tests. However, hemagglutination at all toxoid dilutions was also observed in the presence of undiluted antitoxin. Thus, the value of the CF test was depreciated by two undesirable side effects, namely, anticomplementarity and hemagglutination.

The above titrations were repeated substituting toxin for toxoid and the results are presented in Table III. As expected,

TABLE II

COMPLEMENT BOX TITRATION WITH TOXOID AS ANTIGEN

		Rabbit Antitoxin Dilution 1:					
		1	10	100	1,000	10,000	antitoxin absent
toxoid dilution 1:	1	A	A	A	A	A	A
	2	A	A	A	A	A	A
	4	A	●	●	●	●	●
	8	A	●	●	·	·	·
	16	A	●	·	·	H	H
	32	A	●	·	H	H	H
	64	A	●	·	H	H	H
	128	A	·	·	H	H	H
	256	A	·	·	H	H	H
toxoid absent		H	H	H	H	H	H

H = hemolysis
A = hemagglutination
● = erythrocyte button without hemolysis or hemagglutination
· = small button with partial hemolysis

TABLE III

COMPLEMENT BOX TITRATION WITH TOXIN AS ANTIGEN

		Pig Antitoxin Dilution 1:					
		1	10	100	1,000	10,000	antitoxin absent
toxin dilution 1:	1	H	H	H	H	H	H
	10	A	H	H	H	H	H
	100	A	•	H	H	H	H
	1,000	A	•	•	H	H	H
	10,000	A	•	•	•	•	•
	toxin absent	H	H	H	H	H	H

H = hemolysis
A = hemagglutination
• = erythrocytic button without hemolysis or hemagglutination

this system did not measure complement fixation because sheep erythrocytes are sensitive to lysis by the toxin. Hence, the result reflected merely a neutralization phenomenon. However, hemagglutination was observed when erythrocytes were protected by undiluted antitoxin. Thus, it was suspected that antitoxin could bring about an indirect hemagglutination of erythrocytes through the bound toxin and toxoid. This reaction offered a possible alternative to the CF test.

Although the CF test may be theoretically possible with other combinations of reagents, attention was diverted to the hemagglutination reaction because the latter is a simpler system, involving only the toxin, the antitoxin and the erythrocytes. Furthermore, the hemagglutination test is considered more sensitive than the CF test (47). In addition, assuming both tests are standardized, the hemagglutination test is much less cumbersome to perform on a routine basis than the CF test. Standardization of the hemagglutination reaction is described in the next chapter.

CHAPTER 2

STANDARDIZATION OF THE INDIRECT HEMAGGLUTINATION (IHA) TEST

I. Description

The IHA test for anti-alpha toxin involves coating erythrocytes with toxoid followed by quantitating the agglutination of the coated erythrocytes (CE) in the presence of antibodies.

The tests were performed in Microtitre® plates with U-shaped wells. Starting with 50µl of antiserum, 2-fold serial dilutions were made in appropriate diluents and an equal volume of CE was added to each well. After incubation, the hemagglutination patterns were graded from 4+ to 0 (Fig. 2). The titre of a given antibody or serum was the reciprocal of the highest serum dilution giving + reaction. A titre of < 2 was considered insignificant.

The optimal conditions for coating of the erythrocytes and for testing for antibody were experimentally determined and are described below.

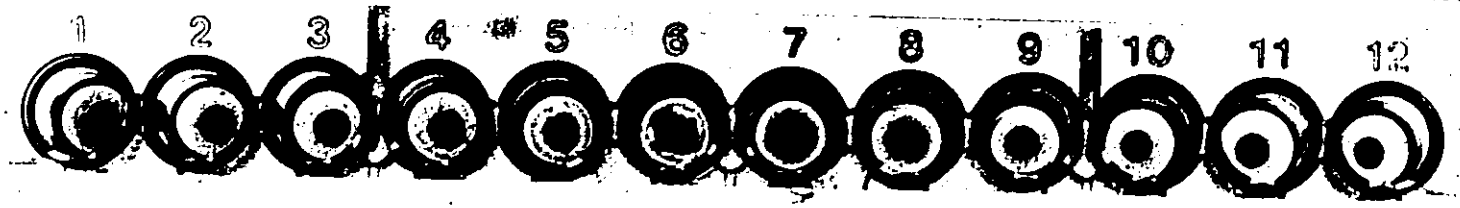
Since alpha toxin is hemolytic, toxoid was used as an antigen.

FIGURE 2

Grading of hemagglutination patterns

The agglutination pattern was graded from 4+ to 0. The 4+ indicated prozone reactions. The 3+ indicated strongly positive reactions in which part of the layer of CE had rolled down from the edges of the well. A 2+ reaction showed an even layer of CE covering the bottom of the well. Formation of a big ring of agglutinated cells was recorded as + and a very small pellet of cells or a button was recorded as 0. The titre of a given serum was recorded as the reciprocal value of the lowest serum concentration giving + reaction. Titres of < 2 were considered insignificant.

CELL CONTROL



GRADING 4 4 4 3 3 2 1 0 0 0 0 0

II. Optimal Conditions for Coating Erythrocytes with Toxoid

Parameters examined for their effect on coating included toxoid concentration, time, temperature, washings, coating media, and purity of antigen.

A. Toxoid Concentration

Rabbit erythrocytes were added to different concentrations of purified toxoid in PBS (Table IV). The final concentration of the erythrocyte suspensions in toxoid was 10% (V/V) in each case. The mixtures were incubated overnight at $4^{o}C$. After three washings, the coated cells were adjusted to a 1% final concentration (V/V) and titrated against the same antiserum A proportionality was observed between IHA titre and the concentration of toxoid used up to 1 mg/ml. To produce a sensitive IHA test, the minimum concentration of toxoid required for coating 10% (V/V) rabbit erythrocytes was found to be 1 mg/ml.

To determine if the concentration of 1 mg/ml represents antigen excess, the supernatants of the above toxoid erythrocyte mixtures were tested for their ability to coat additional erythrocytes. Supernatant with initial toxoid concentrations of 2.0, 1.5, 1.0 and 0.5 mg/ml respectively, were found to be capable of sensitizing more erythrocytes with which IHA titres 64 were obtained with rabbit antiserum. Hence, for maximum sensitivity in IHA test, the erythrocytes should be coated with the antigen in excess.

TABLE IV

EFFECT OF COATING CONCENTRATION OF TOXOID ON THE SENSITIVITY OF THE IHA TEST

Toxoid Concentration used for coating	IHA Titre
2.0 mg/ml	32
1.5 mg/ml	32
1.0 mg/ml	32
0.5 mg/ml	8
0.1 mg/ml	8
50 µg/ml	4
10 µg/ml	2
5 µg/ml	1
1 µg/ml	1
0.5 µg/ml	1

B. Time and Temperature of Coating.

Rabbit erythrocytes were adjusted to 10% (V/V) suspensions in toxoid (1 mg/ml). The mixtures were incubated at 4°, 23°, 30°, and 37°C. Samples at each temperatures were withdrawn after 10, 30, 60, and 120 min. and were thrice washed at their respective incubation temperatures. The washed cells were adjusted to 1% (V/V) final concentration and added to dilution series of antitoxin. After 90 min. incubation at 4°C, the titres were read and recorded (Table Va). The intensity of the reaction at each end point is presented in Table Vb.

Titres of erythrocytes coated up to 30 min. at 4°C were 2-fold lower than the other titres.

While the titre of the others were constant at 128, the intensity of the IHA reaction at the end point seemed higher for erythrocytes coated at RT.

Therefore, coating was performed at 23°C for 15-30 min.

C. Washing Coated Erythocytes

Excess unbound toxoid was removed by washing CE twice in PBS. Washing at 4°C resulted in CE that produced a 4-fold drop in IHA titre from 64 to 16 as compared to those CE washed at RT. This may indicate that the toxoid erythrocyte interaction is susceptible to fluctuation in temperature.

D. Coating Media

Erythrocytes coated with 1 mg/ml toxoid in PBS were compared to those coated in unbuffered saline. More hemolysis was observed of erythrocytes coated in saline but when

TABLE V

EFFECTS OF COATING TIME AND TEMPERATURE ON IHA TITRES

a. IHA titres

COATING TIME (min)	COATING TEMPERATURE (°C)			
	4	23	30	37
10	64	128	128	128
30	64	128	128	128
60	128	128	128	128
120	128	128	128	128

b. Intensity of IHA reaction at the respective end points

COATING TIME (min)	COATING TEMPERATURE (°C)			
	4	23	30	37
10	1+	4+	2+	3+
30	1+	3+	3+	3+
60	1+	4+	3+	4+
120	1+	4+	2+	3+

adjusted to 1% suspensions, both erythrocyte samples gave titres of 64.

E. Purity of the Antigen

Although erythrocytes coated with purified toxoid consistently produced a titre lower by a single tube compared to cells coated with crude or semi-purified material, the difference was considered insignificant since such variation occurs frequently in many serological tests.

III. Optimal Conditions for the Indirect Hemagglutination Test

A. Concentration of CE

A 2% (V/V) suspension of CE produced undesirably large patterns and obscure end points. However, 0.5% and 0.2% CE suspensions were too dilute to produce clear patterns in negative controls within 3 h. In compromise, a 1% suspension was found to give the most distinct patterns.

B. Temperature of Incubation

Hemagglutination occurred equally at all temperatures tested (4°, 23°, 37°C) but 4°C was chosen because least prozone reaction occurred.

C. Time of Incubation

Generally, 4+, 3+ and 2+ hemagglutination patterns were quite stable for 48 h. Occasionally, a 2 fold drop in titre was observed after overnight incubation because a 1+ reaction withdrew into the form of a button. Therefore, the titres were recorded after 90 min. when the negative controls showed distinct buttons.

D. pH

Saline buffered with sodium phosphate (0.01 M) was adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Since the IHA titres were constant at 64 when tested at all these pH as well as in unbuffered saline (pH 5.6), a neutral pH was used in routine tests.

E. Proteinaceous Additives.

One of the inherent problems of any hemagglutination test is the occasional spontaneous agglutination of the erythrocytes in the absence of antibodies. The addition of certain substances such as albumin and gelatin to overcome spontaneous hemagglutination is a common serological practice. However, in our test, spontaneous hemagglutination reactions were intensified in the presence of unpurified Bovine Serum Albumin (BSA) or pure gelatin at 2% (W/V) final concentrations.

Purified BSA (Sigma Chem.) at a final concentration $\geq$ 1.5% (W/V) was found to prevent spontaneous hemagglutination of coated rabbit erythrocytes but not of uncoated erythrocytes. When an antiserum titrated in PBS supplement with 2% pure BSA was compared with a similar test in PBS alone, a 4-fold drop in titre (128 to 32) was observed. Therefore, albumin and gelatin are found not applicable.

IV. Stabilization of Coated Erythrocytes

We wished to modify the coated erythrocytes so that the adsorbed antibodies could be eluted from their surfaces. Modification was necessary because of two reasons. Firstly, the drop in IHA titre produced by erythrocytes washed at 4°C (p.32) suggested that the binding of toxoid is reversible. Likewise, the binding of toxin is thought to be reversible (75, 28). Secondly, the method of elution (56°C, 10 min.) resulted in lysis of CE.

A. Method of Stabilization

Dalen (39) suggested that "glutaraldehyde has many advantages as a coupling reagent of infectious material to erythrocytes for serological use. The antigens remain intact to a large extent, and the cells can be stored for very long periods of time". Hence, CE were treated with glutaraldehyde to covalently link the bound toxoid onto the erythrocytes.

To determine the optimal reaction conditions, a 1% (V/V) CE was incubated at 23°C in concentrations of glutaraldehyde solution in PBS (Aldrich Chemical Co.) ranging from 2.5% to 0.1% (W/V) in PBS. Samples were removed at 15, 30, 60, 90 and 120 min. After three washings in PBS, these samples of treated CE were adjusted to 1% suspensions and titrated by IHA with the same antibody. Nonspecific hemagglutination occurred only with CE treated in 2.5% glutaraldehyde for 15 and 30 min.

No significant difference in titre was observed among the rest of the samples. The minimal concentration of glutaraldehyde investigated (0.1%) was chosen so as not to offset the osmolarity of the medium. The time of exposure to glutaraldehyde was chosen to be 30 min.

Under these conditions, CE suspensions ranging from 0.1% up to 10% (V/V) in glutaraldehyde solutions were stabilized. However, at 10% concentration, CE began to clump, presumably due to linkage of erythrocytes in proximities. Clumping was not seen with 2% CE, a concentration thereby adopted.

Clumping was thought to be due to excess free aldehyde groups on CE. To block excess aldehyde groups, CE was incubated in 0.1 M lysine-HCl solution in PBS (1h, RT).

Lysine and glutaraldehyde treated CE were abbreviated as LGCE.

B. Antigenicity of LGCE

The antigenicity of CE appeared unaffected by these treatments. The IHA titres of antitoxin were identical among CE before and after glutaraldehyde or glutaraldehyde-lysine treatment.

Similarly, the antigenicity of the carrier erythrocytes appeared unaffected. Uncoated erythrocytes at each step of treatments produced constant heterophile titres (64) with a heterophile positive serum indicating that the heterophile determinant(s) is unmodified.

Also, uncoated erythrocytes at each step remained unreactive with a heterophile negative serum indicating that no new determinants arose.

C. Physical Characteristics of LGCE

LGCE assume the color of deoxygenated blood. The cells are intensely cohesive such that resuspension requires stirring. Microscopically, LGCE were indistinguishable from uncoated red cells (fig. 3a) whereas CE tends to crenate (fig. 3b).

D. Advantages of LGCE

Antigenicity was unaffected. Spontaneous hemagglutination was abolished. Preparation and standardization became possible. LGCE was reactive and hemolysis-free when stored at $4^{o}C$ up to a month under PBS without antibiotics, making lyophilization unnecessary. They resisted heating at $56^{o}C$ for 1/2 h with constant agitation making elution of antibodies by heat possible.

FIGURE 3

Morphology of CE and LGCE

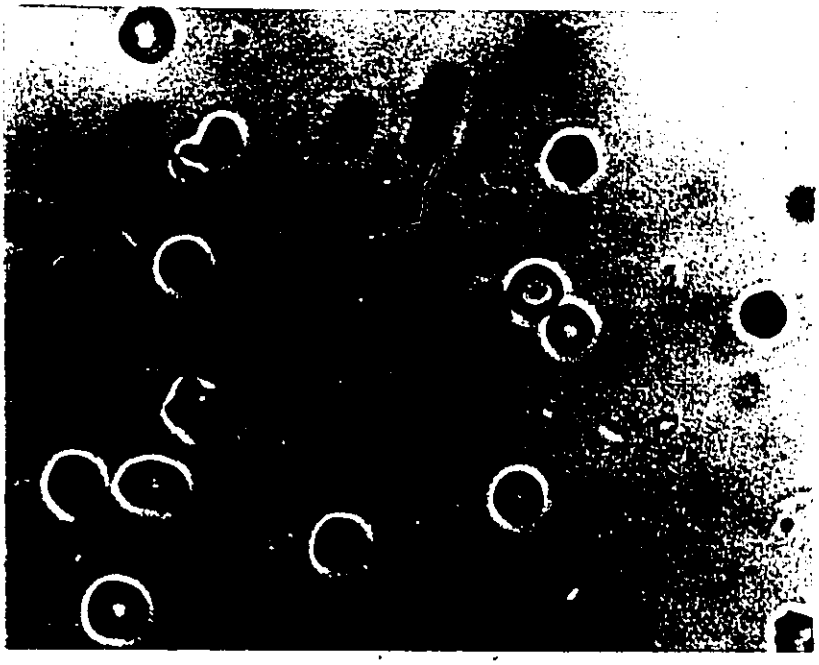
A. Two months old LGCE (x400).

Note that the morphology of erythrocytes is well preserved.

B. Fresh rabbit erythrocytes coated with toxoid (x400).

Note the intensive crenations.

A



B

V. Controls for IHA Test

A. Cell Control

Tests were rejected if spontaneous hemagglutination occurred when 50 µl of CE or LGCE were added to 50 µl of PBS.

B. Heterophile Control

A heterophile antibody is one that can react with an antigen that is not instrumental in producing the antibody response. Heterophile antibodies, which frequently create problems in serological tests, can be removed by adsorption.

The antisera used in this study contained heterophile antibodies which agglutinated not only erythrocytes from a different species (Table VIa) but also erythrocytes from a different individual of the same species (Table VIb). The titre of the heterophile antibody varied from serum to serum. Hemagglutination activity remained in antitoxin that was semi-purified to IgG. Therefore, the heterophile antibodies were directed probably to the native antigenic determinants on the red cells.

For the above reason, antitoxins to be tested for IHA were also titrated with uncoated erythrocytes. Heterophile antibodies, when found, were removed by adsorption with uncoated erythrocytes —— the same batch from which the CE or LGCE were prepared. Adsorption was performed at $4^{o}C$ for 2 h with packed cells so that the dilution factor introduced to the antitoxin was minimal. The volume of packed cells used was calculated according to the heterophile titre of

TABLE VIa

HETEROPHILE ANTIBODIES TO ERYTHROCYTES FROM DIFFERENT ANIMAL SPECIES

SERA from	ERYTHROCYTES from	NUMBER of SERA with	HETEROPHILE TITRE of
1 rabbit	rabbit	1	0
	rat	1	4
	sheep	1	2
	mouse	1	16
	human	1	64
4 guinea pig	sheep	1	16
		2	32
		1	64
4 guinea pig	human (B, Rh^+)	pooled	16
1 guinea pig	human (A, Rh^+)	1	32
33 human	rabbit (pooled)	4	8
		11	16
		9	32
		5	64
		4	64

TABLE VIb

HETEROPHILE ANTIBODIES IN RABBITS TO RABBIT ERYTHROCYTES

SERA from	ERYTHROCYTES from	NUMBER of SERA with HETEROPHILE TITRE of	
		4	0
		19	1
		8	2
		2	4
40 rabbits	rabbits (pooled)	0	8
		2	16
		2	32
		0	64
		0	128
		1	256
		1	512

the antitoxin, and an excess of erythrocytes were used. After adsorption, the heterophile titre was rechecked. Heterophile antibodies were titrated the same way as described for antitoxin except for the use of uncoated erythrocytes. A typical set of results is presented in Table VII.

C. Known Positive and Negative Controls

A pair of previously titrated sera, one with a high and the other a low IHA titre, were included to ensure reproducibility.

VI. Summary

The following procedures were adopted as routine in the laboratory.

A. Coating of Erythrocytes

Erythrocytes are washed three times. The buffy coat is removed. The red cells are adjusted to a 10% suspension in alpha toxoid (1 mg/ml). The mixture is periodically shaken at RT for 30 min. The unbound toxoid is removed by two washings in PBS at RT. If desired, the CE is adjusted to a 1% suspension for IHA test.

B. Stabilization

CE are adjusted to a 2% suspension in 0.1% (W/V) glutaraldehyde solution in PBS. The mixture is agitated at RT for 30 min. After three washings in PBS, the cells are adjusted

TABLE VII

REMOVAL OF HETEROPHILES IN IMMUNE PIG ANTIBODIES

ADSORPTION	HEMAGGLUTINATION TITRE with UNCOATED ERYTHROCYTES*	COATED ERYTHROCYTES*	INTERPRETATION
before	64	128	heterophile titre = 64
after	0	128	IHA titre = 128

* Rabbit erythrocytes were used for adsorption and testing.

to a 20% (V/V) suspension in 0.1 M lysine-HCl solution in PBS. The mixture was agitated at RT for 1 h. The LGCE is then washed three times with PBS and stored at 4°C as a 10% (V/V) suspension in PBS.

C. IHA Test

Starting with 50 µl of antibody, a 2-fold serial dilution was made in PBS. Then, 50 µl of a 1% suspension of LGCE was added to each well. A cell control, a heterophile control, a positive control and a negative control were included. After incubation for 90 min. at 4°C, the tests were read as described.

Having standardized the IHA test, the next question was did the test measure all the anti-alpha antibodies in serum? The following chapter described investigations into this question which led to the discovery of two distinct populations of anti-alpha antibodies.

CHAPTER 3

TWO POPULATIONS OF ANTIBODIES

I. Evidence for Two Anti-Alpha Antibodies

The antibodies reactive in the IHA test were designated as indirect hemagglutinating antibodies (IHA-Ab). They could be removed from immune sera by adsorption with coated erythrocytes or LGCE. When 28 immune sera from rabbits were adsorbed, they became negative in the IHA test (Table VIII). However, the adsorbed sera still neutralized alpha toxin. The residual neutralization capacities which could not be removed by repeated adsorption were later shown to be anti-alpha antibodies that neutralized by prevention of binding of the toxin onto erythrocytes. The residual anti-alpha antibodies are referred to as anti-binding antibodies (AB-Ab).

TABLE VIII

EVIDENCE FOR TWO POPULATIONS OF ANTI-ALPHA ANTIBODIES

IMMUNOADSORPTION OF 28 IMMUNE RABBIT SERA	LOG_2 IHA TITRE (MEAN ± S.E.)	NEUTRALIZING UNITS (MEAN ± S.E.)
Before	8.7 ± 3.4	103.5 ± 9.8
After	0	33.2 ± 4.1

II. Separation of the Two Populations of Antibodies

The development of heat resistant LGCE earlier made the following separation method possible. The method is presented schematically in Fig. 4 . LGCE was used as an immunoadsorbent from which IHA-Ab were subsequently eluted by heat.

The antitoxin or antisera to be adsorbed were freed of heterophile antibodies. The amount of LGCE required to adsorb completely all of the IHA-Ab depends on the IHA titre of the antiserum. The minimal amount of LGCE sufficient for complete adsorption was calculated by the following empirical formula:

ml LGCE used = 0.04 x IHA titre of the serum

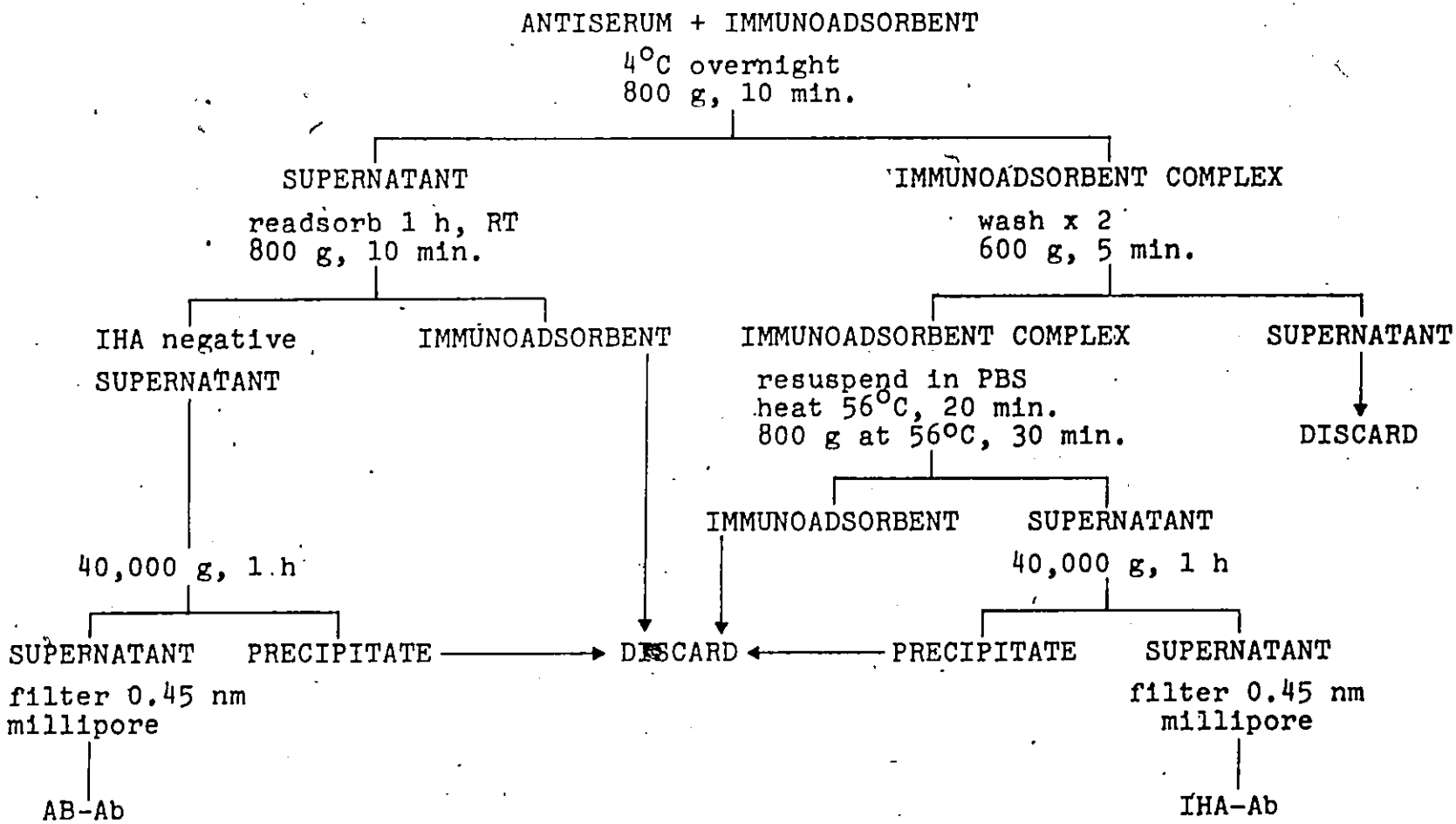
For instance, the formula calls for 0.25 ml of LGCE for adsorption of 0.1 ml of antiserum with an IHA titre of 64. Most, but not all of the IHA-Ab would be removed by this amount of LGCE (see supporting data on page 53 Table IX). Nevertheless, from experience, it is more effective to readsorb than to use too much LGCE initially. For practical convenience, packed LGCE and the antiserum were both diluted 1:2 and then the empirical formula was applied.

Adsorption was performed either at RT for 1 h with periodic resuspension or overnight at 4°C. The mixture was centrifuged at RT and the supernatant containing AB-Ab was centrifuged and filtered as outlined in Fig. 4.

Meanwhile, the LGCE complex was washed twice gently in PBS in order to remove unbound material. Each time, the wash suspension was incubated at RT for 1/2 h before centrifugation. To counteract loss, the volume of PBS added for

FIGURE 4

Separation of anti-alpha antibodies


ANTISERUM + IMMUNOADSORBENT
4°C overnight
800 g, 10 min.
SUPERNATANT
readsorb 1 h, RT
800 g, 10 min.
IMMUNOADSORBENT COMPLEX
wash x 2
600 g, 5 min.
IHA negative SUPERNATANT
IMMUNOADSORBENT
IMMUNOADSORBENT COMPLEX
resuspend in PBS
heat 56°C, 20 min.
800 g at 56°C, 30 min.
SUPERNATANT
DISCARD
IMMUNOADSORBENT
SUPERNATANT
40,000 g, 1 h
40,000 g, 1 h
SUPERNATANT
PRECIPITATE
DISCARD
PRECIPITATE
SUPERNATANT
filter 0.45 nm millipore
filter 0.45 nm millipore
AB-Ab
IHA-Ab

heat elution was half the initial volume of the antiserum. During heating in a 56°C water bath, the suspension was constantly agitated with an applicator stick. After 20 min., the tube of warm suspension was placed in a beaker of water at the same temperature and quickly transferred into a pre heated centrifuge and centrifuged. The supernatant was collected using prewarmed pasteur pipettes and tubes. Despite care in the above method, a 4-fold loss of IHA-Ab was not uncommon.

Did the above method elute all of the IHA-Ab from the immunoadsorbent? Repeated heating of used LGCE did not elute any more IHA-Ab, as detected by the IHA test. Therefore, either the first heating eluted most of the IHA-Ab, or there might be another residual subpopulation which was so tightly bound that could not be heat eluted. If the latter were true, the subpopulation might be expected to accumulate on the erythrocyte by reuse of the same LGCE in repeated adsorption and elution. As a result, subsequent adsorptions and elutions should produce less and less IHA-Ab. When this hypothesis was tested, the yield of IHA-Ab from LGCE reused several times was found to be comparable to that from freshly prepared LGCE; the IHA titre of the heat eluents were constant at 32 in each instance. Hence, all detectable amounts of IHA-Ab were eluted by the heat method.

Control Experiments

These experiments were designed to determine whether the separation methods described produced any artifacts.

A. Test for Coelution of Toxoid

One of the possible artifacts is that toxoid might be released from heated LGCE with the IHA-Ab. The previous experiment, which measured the amount of IHA-Ab adsorbed from reused LGCE, suggested that coelution did not occur. This supposition was confirmed in the following experiment.

LGCE were heated in PBS, centrifuged, and the supernatant removed at 56°C as previously described. Samples of 0.1 ml of pig antibodies were adsorbed with various amounts of heated versus unheated LGCE. After overnight incubation at 4°C, the supernatants were obtained by centrifugation at RT, and were tested for their residual IHA activities (Table IX). No significant difference was observed. It was therefore concluded that no significant amount of toxoid was eluted by heat.

B. Negative Controls

Preimmune serum when adsorbed with LGCE did not neutralize alpha toxin, indicating the absence of AB-Ab (Fig.7).

In view of the presence of a low level of IHA-Ab in preimmune sera (Table X, P. 68), PBS was used in equivalent amounts in the place of antiserum and the heat eluent from this control was negative in neutralization and IHA tests.

TABLE IX

HEAT STABILITY OF LGCE

ML of LGCE used to adsorb 0.1 ml antibodies	IHA TITRES of SUPERNATANT after adsorption by unheated LGCE	heated LGCE
1.4	0	0
1.2	0	0
1.0	0	0
0.8	0	0
0.6	0	0
0.4	2	2
0.2	4	4
0.05	8	16
0.01	32	32
0.0	64	64

III. The Antibinding Antibodies

A. Characteristics

AB-Ab exhibited the characteristics of antibodies in that they were found in purified IgG fractions of immune sera and that they were precipitated by anti-IgG in immunodiffusion in agar-gel test (Fig. 5). Also, they form a precipitin line of identity with whole antitoxin in immunodiffusion test when alpha toxin was used as the antigen (Fig. 6). The AB-Ab response to antigenic stimulation was demonstrated in a nonimmune rabbit (Fig. 7). Further, Maharaj and Fackrell demonstrated that purified AB-Ab could be coupled to Sepharose 4B (Pharmacia) via CNBr for use as an affinity immunoadsorbent from which alpha toxin was purified (report in preparation).

Toxin neutralization was dependent on AB-Ab concentration. Various amounts of AB-Ab were added to a constant amount of rabbit erythrocyte suspension and challenged with the same amount of toxin in KH tests (Fig. 8). Protection increased with increasing amounts of AB-Ab. This establishes a cause-effect relationship between AB-Ab and neutralization.

The degree of neutralization was found to be dependent also on the time when the AB-Ab were added to the erythrocyte-toxin mixture. In a series of KH tests, a constant amount of AB-Ab was added at different times to a constant amount of erythocyte-toxin mixture. The results indicates that the longer the population of erythrocytes was exposed to the toxin,

FIGURE 5

Immunodiffusion of AB-Ab against antiglobulins

Centre well contained undiluted rabbit AB-Ab.

Well 1 contained goat anti-rabbit whole serum.

Well 2 contained goat anti-rabbit IgG.

Well 3 and 4 contained PBS.

FIGURE 6

Immunodiffusion of alpha toxin against anti-alpha antibodies

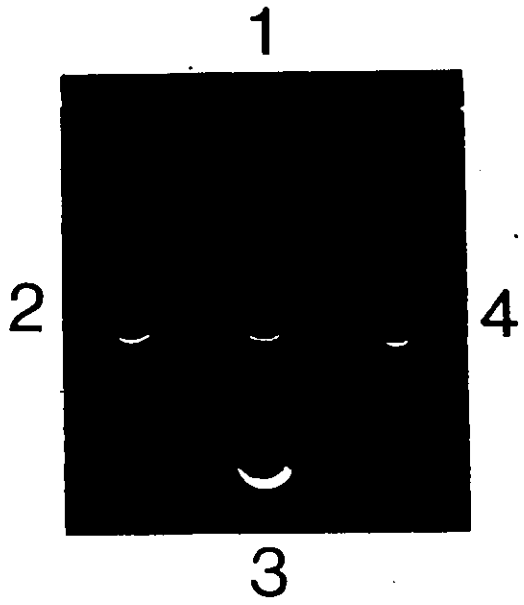
Centre well contained alpha toxin (1 mg/ml).

Well 1 contained unadsorbed, undiluted antiserum.

Well 2 contained undiluted AB-Ab.

Well 3 contained IHA-Ab.

Well 4 contained heat eluant control for IHA-Ab.


1
2
4
3

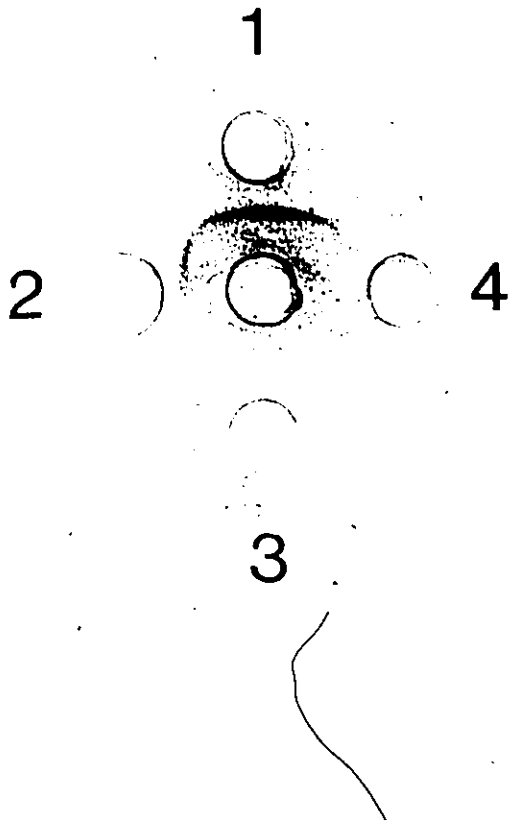

1
2
4
3

FIGURE 7

AB-Ab response in a rabbit

Rabbit F105 was immunized with alpha toxoid as described in Materials and Methods. The weekly antisera were IHA-Ab adsorbed. The residual neutralization capacities were due to AB-Ab.

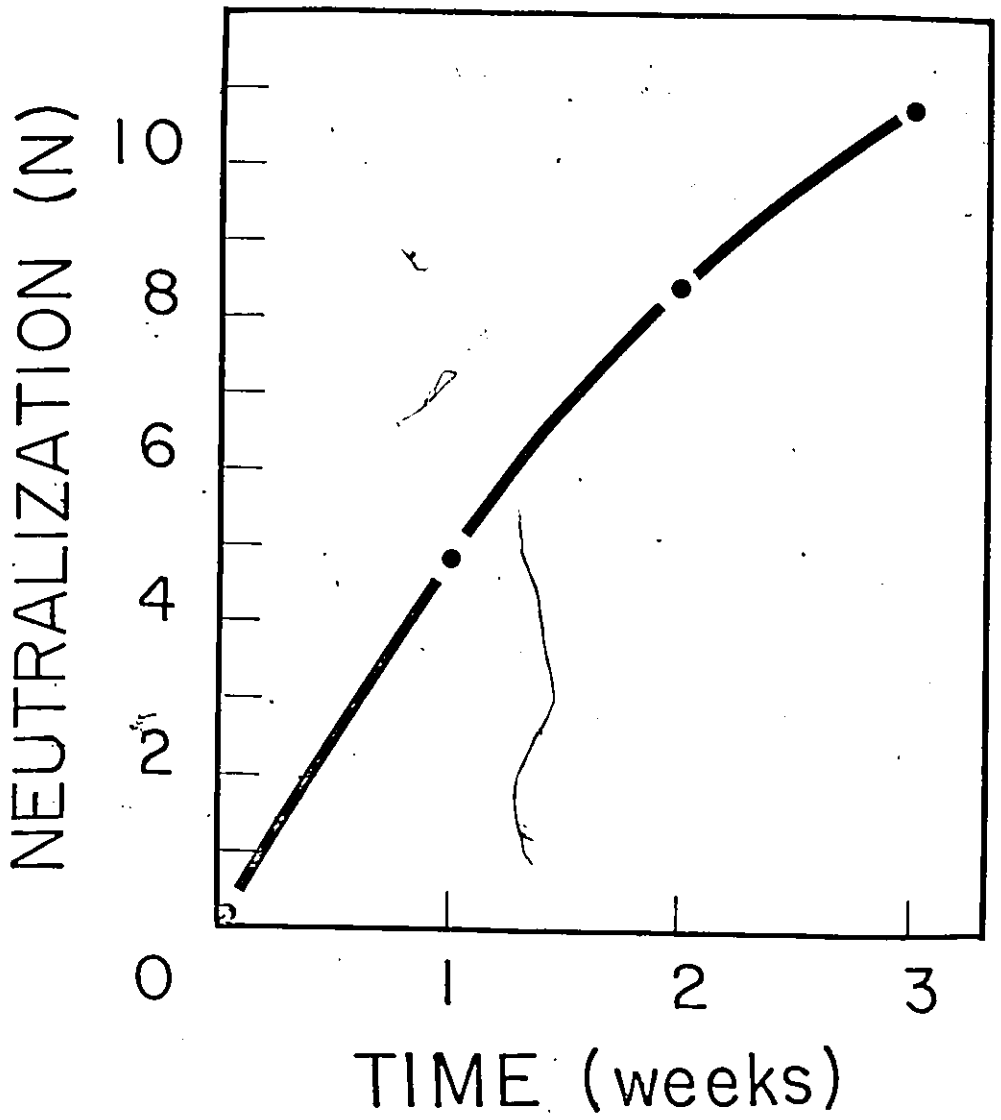

NEUTRALIZATION (N)
0
2
4
6
8
10
0
1
2
3
TIME (weeks)

FIGURE 8

The effect of AB-Ab concentration on neutralization.

The reaction mixture (final volume 350 µl) contained 100 µl of erythrocytes, 50 µl of toxin and various amounts of AB-Ab made up to 200 µl in PBS. B = baseline hemolysis in the absence of AB-Ab. The volume of AB-Ab in the tests were: 1 = 2.5 µl, 2 = 10 µl, 3 = 20 µl, 4 = 30 µl, 5 = 50 µl, 6 = 70 µl and 7 = 100 µl.

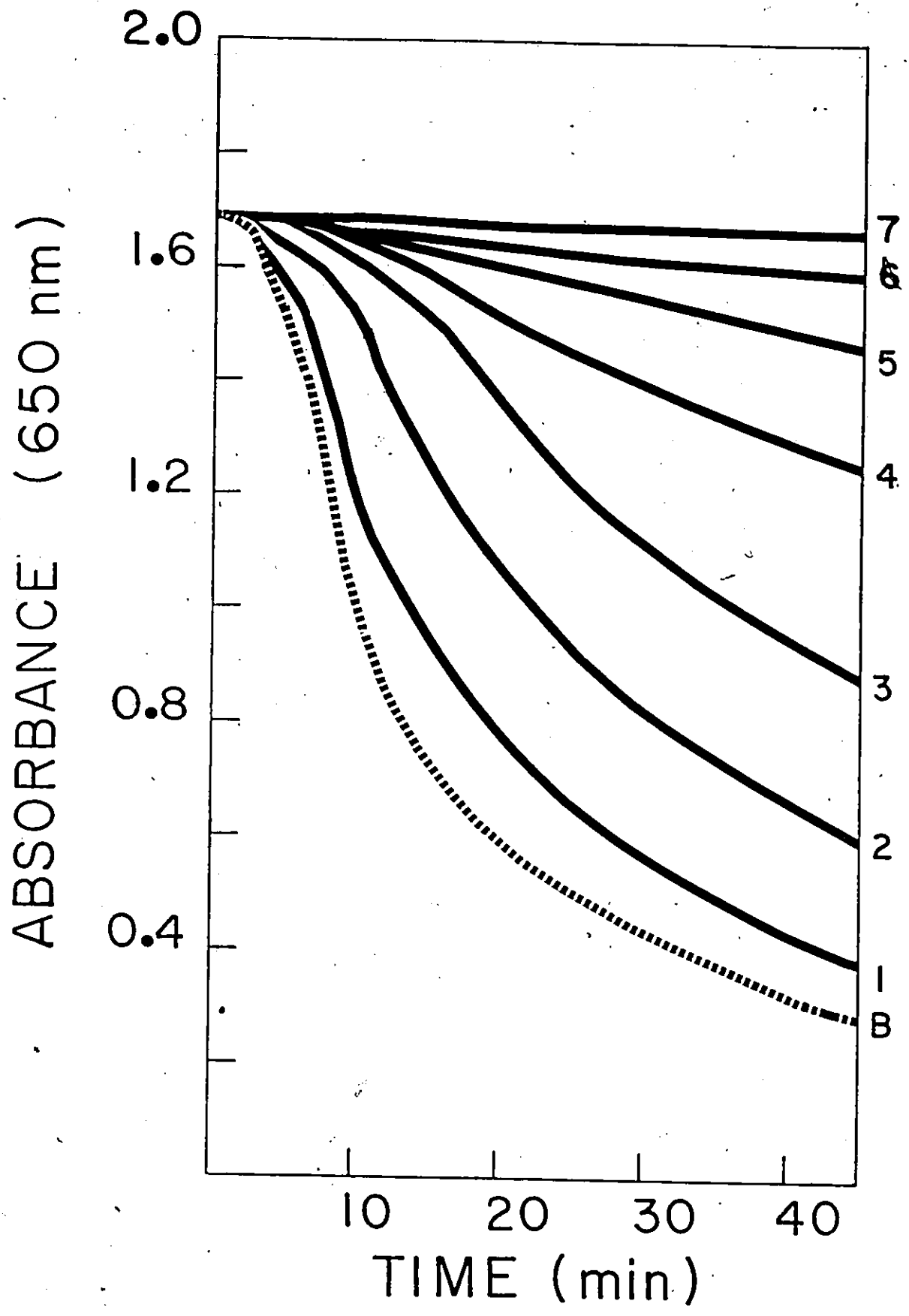

2.0
1.6
1.2
0.8
0.4
ABSORBANCE (650 nm)
7
6
5
4
3
2
1
B
10
20
30
40
TIME (min)

FIGURE 9

The effect of time of introduction of AB-Ab on neutralization.

The reaction mixture (final volume 310 µl) contained 100 µl of erythrocytes, 10 µl of toxin, 170 µl of PBS initially. Then, 30 µl of AB-Ab was added to different aliquots of the mixture at different times as indicated by the arrows. The solid black line represents the hemolytic baseline.

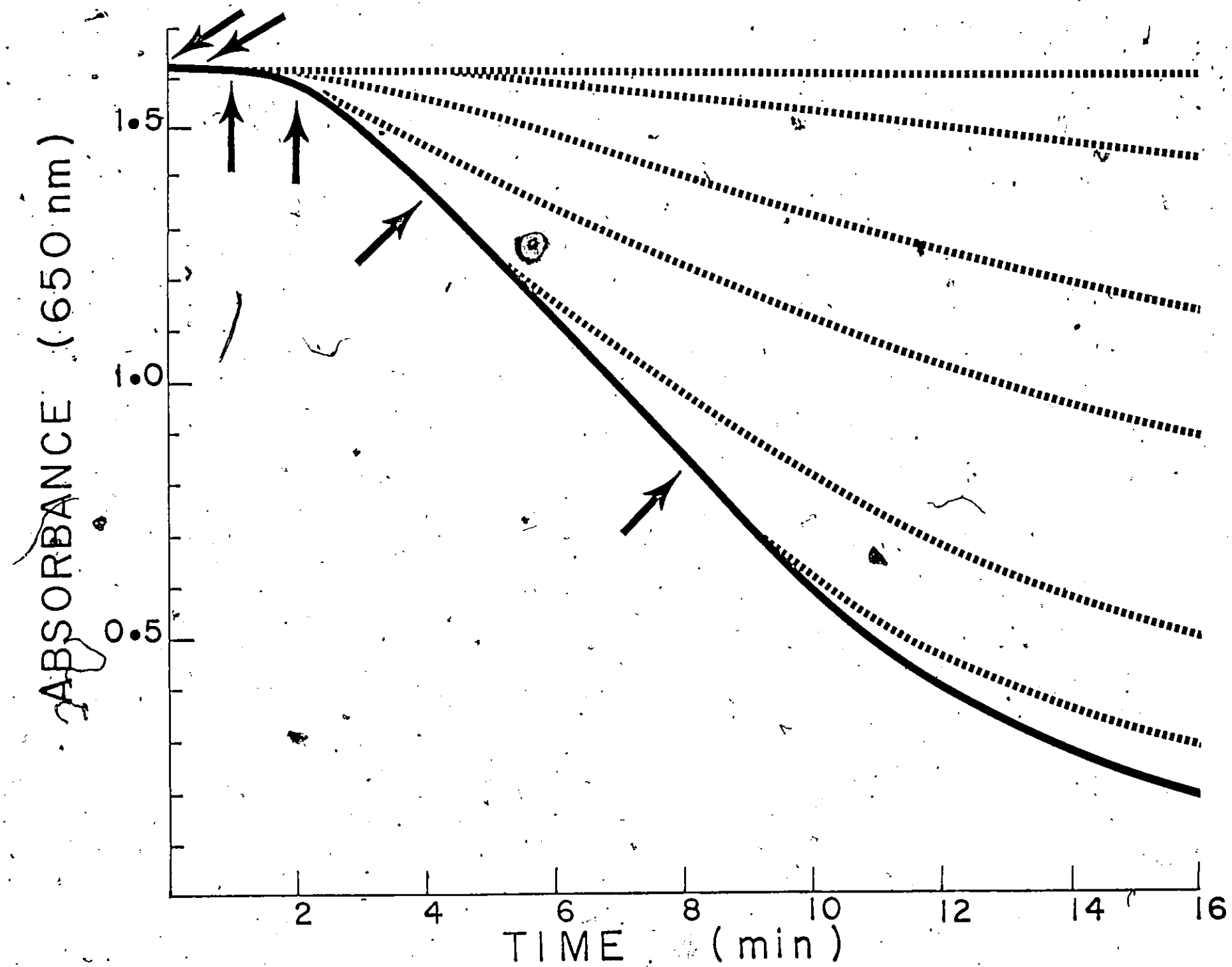
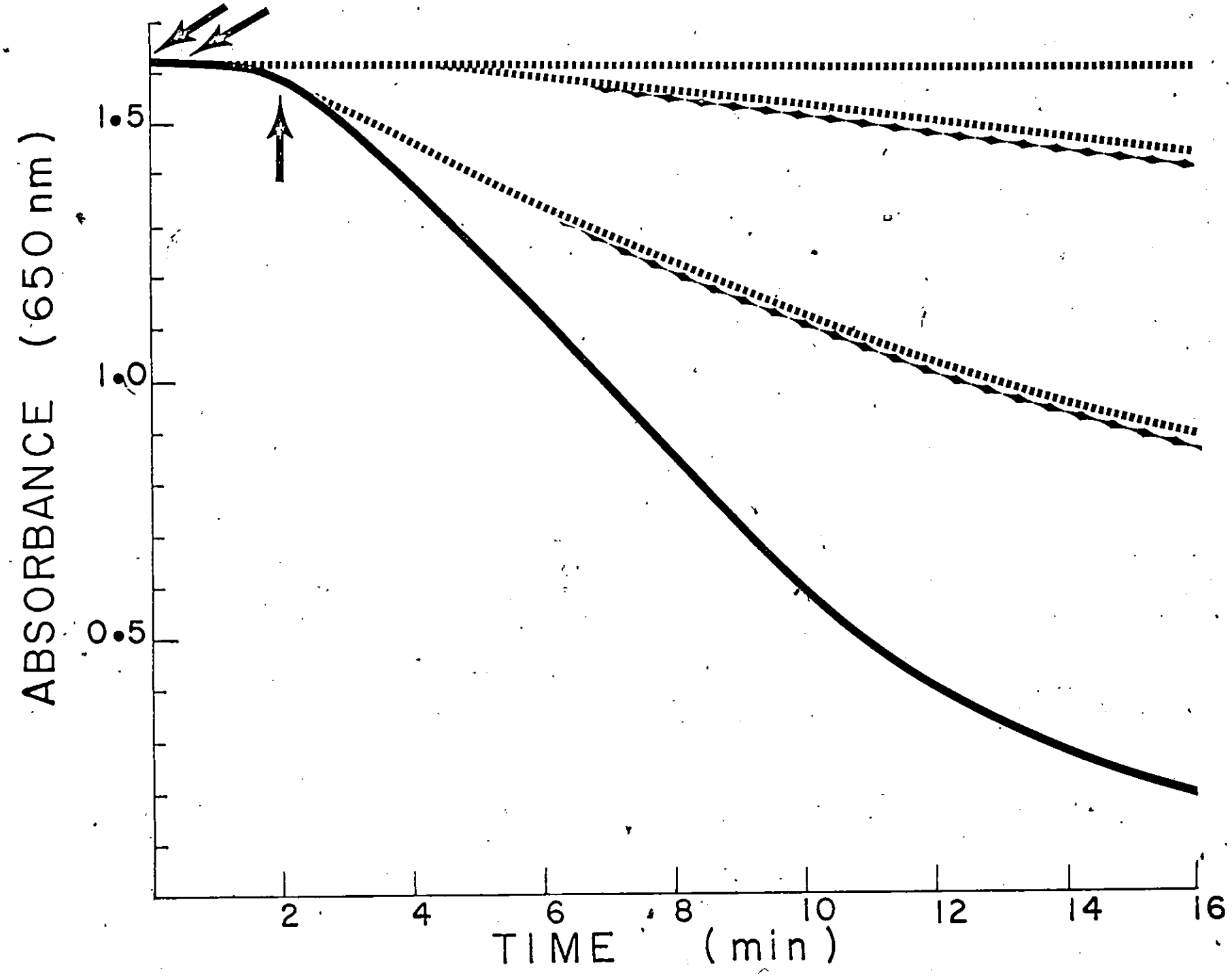

ABSORBANCE (650 nm)
1.5
1.0
0.5
2
4
6
8
10
12
14
16
TIME (min)

FIGURE 10

Rate of hemolysis unaltered by excess AB-Ab after time zero

▪▪▪▪▪▪▪▪▪▪▪▪▪▪ represents tests as described for FIGURE 9.

◆—◆—◆—◆ represents tests when double amount of AB-Ab was added at half a minute and at two minutes, respectively, after time zero.


ABSORBANCE (650 nm)
1.5
1.0
0.5
2
4
6
8
10
12
14
16
TIME (min)

the less protection the AB-Ab were able to confer on the remaining unlysed erythrocytes (Fig. 9). Furthermore, the addition of twice the amount of AB-Ab used in Fig. 9 did not give any additional protection (Fig. 10).

B. Mode of Neutralization

The antibinding action of AB-Ab was initially indicated by experiments with toxoid, subsequently elucidated by neutralization tests and finally confirmed by indirect antibody methods.

1. Hemagglutination

(i) with bound toxoid

Although AB-Ab react with toxin (Fig. 6, 8) and with toxoid (Fig. 12), AB-Ab did not hemagglutinate CE or LGCE. These observations together indicated that the AB-Ab determinant(s) of the toxoid became inaccessable to AP-Ab when the toxoid was bound to erythrocytes.

(ii) with unbound toxoid

When 25µl of soluble toxoid (0.1 mg/ml) were added to a mixture containing 25µl of a 1% (V/V) rabbit erythrocyte and 25µl of undiluted AB-Ab (which had 30 N), no hemagglutination occurred even after 48 h at either $4^{o}C$ or RT. This result indicated the absence of the immune complex on the erythrocytes.

2. Inhibition of Toxoid Protection Phenomenon

Rabbit erythrocytes were adjusted to a 2% suspension

FIGURE 11

Resistance of coated erythrocytes

coated erythrocytes plus toxin

uncoated erythrocytes plus toxin

control erythrocytes (coated or uncoated erythrocytes in PBS)

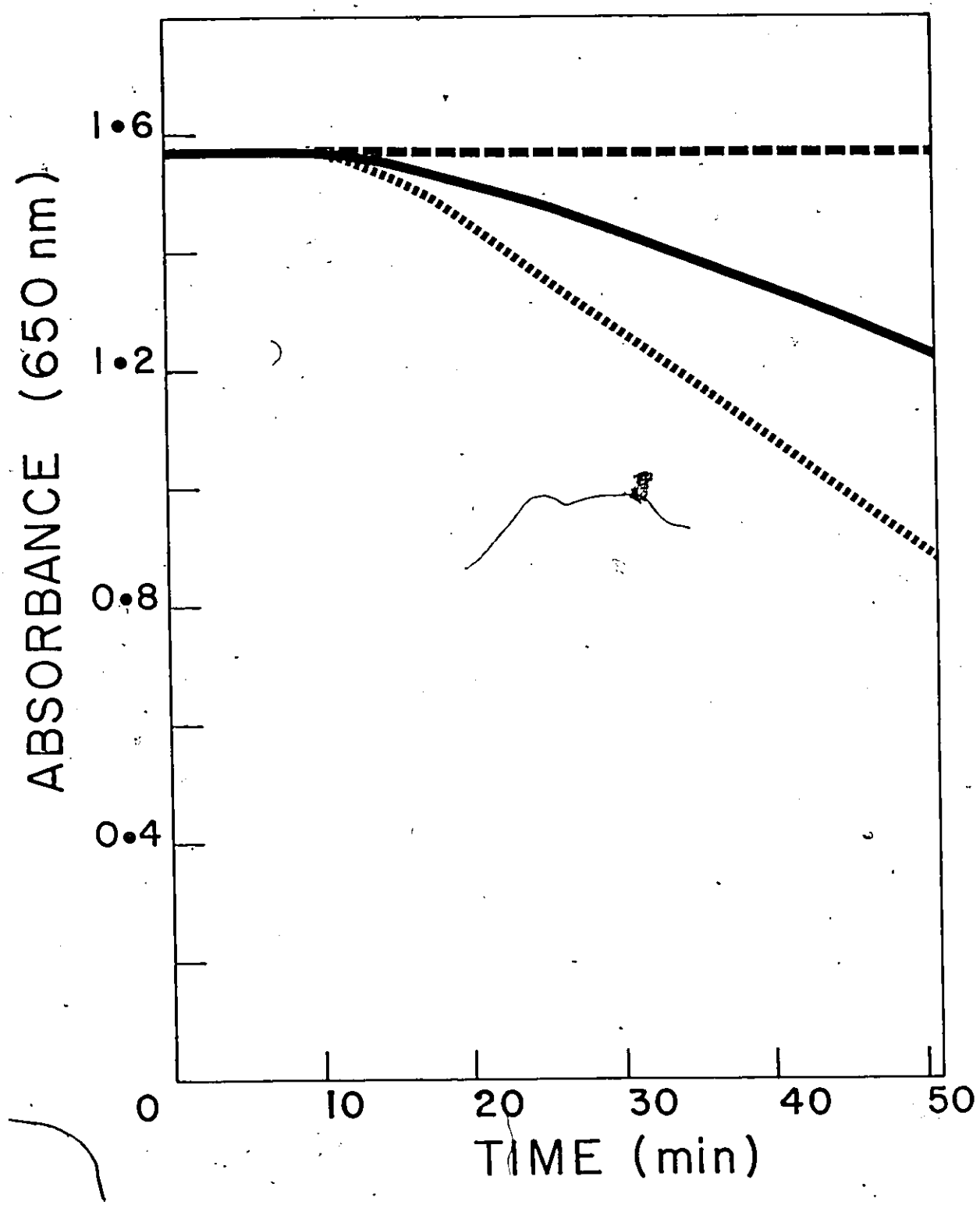

ABSORBANCE (650 nm)
1•6
1•2
0•8
0•4
0
10
20
30
40
50
TIME (min)

FIGURE 12

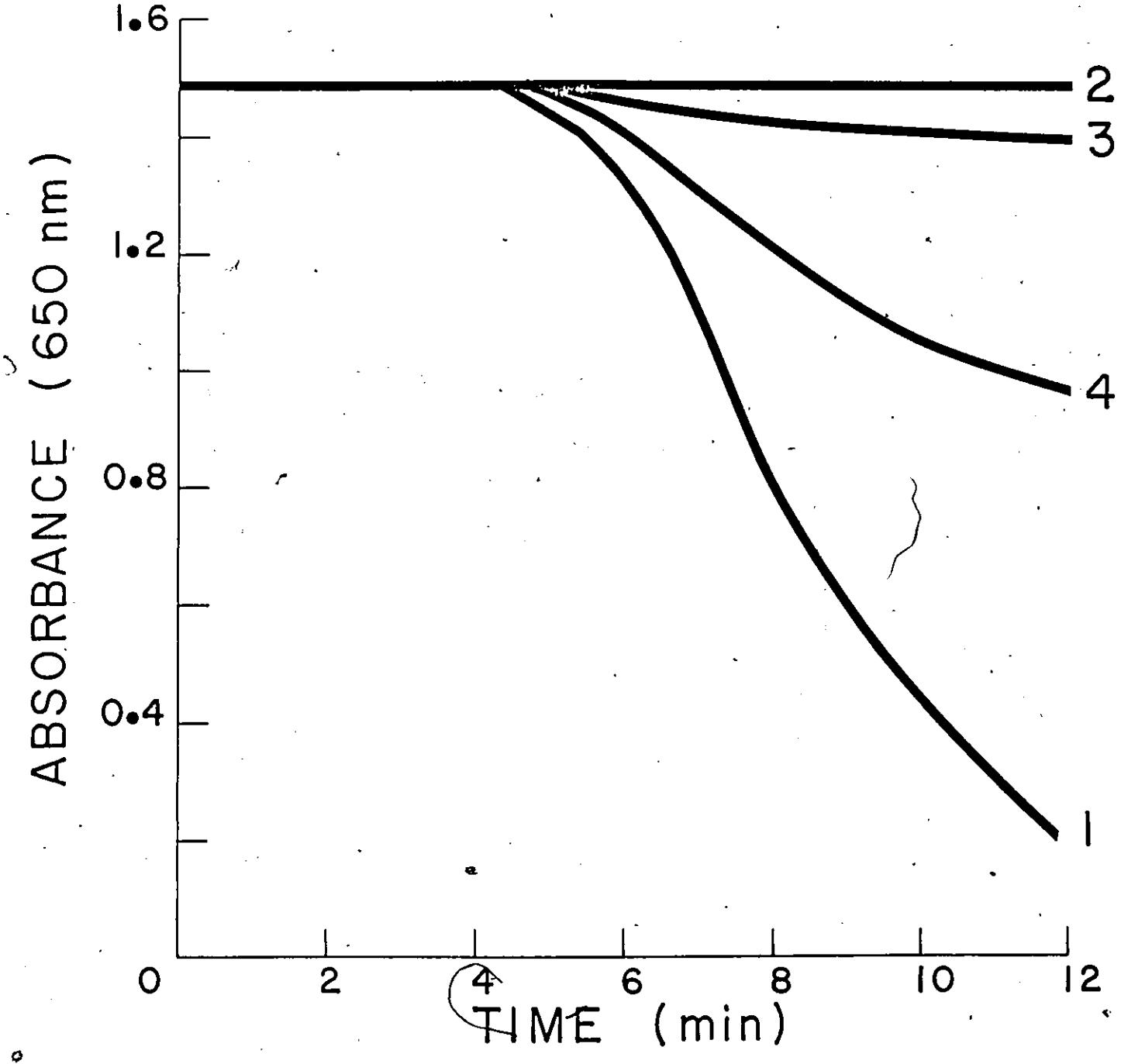
Inhibition of toxoid protection by antibodies.

Line 1 represents the hemolytic baseline when 3 ml of thrice washed rabbit erythrocytes at an absorbance of 1.5 was exposed to a small amount of alpha toxin.

Line 2 represents contents of line 1 including 0.1 ml of antibodies. Neutralization was evident.

Line 3 represents erythrocytes preincubated with toxoid, twice washed, adjusted to the same absorbance and challenged with the same amount of toxin. Protection was observed.

Line 4 represents erythrocytes which were preincubated in a toxoid-antibody mixture, twice washed and adjusted to the same absorbance, then challenged with the same amount of toxin. The degree of protection was reduced.


1.6
1.2
0.8
0.4
0
ABSORBANCE (650 nm)
2
4
6
8
10
12
TIME (min)
1
2
3
4

in toxoid of 1 mg/ml concentration. After incubation at RT for 1 h the coated cells were washed twice and challenged with toxin. They were found to be more resistant than uncoated erythrocytes (Fig. 11). The tentative explanation for this resistance was that toxoid protected coated erythrocytes from toxin by occupying the receptor sites.

If antibodies could prevent the toxoid from binding onto erythrocytes, protection of erythrocytes would be reduced. Fig. 12 depicts results which indicates that toxoid protection was reduced when erythrocytes were coated with toxoid preincubated with antitoxin (line 4) as compared to those coated with toxoid without antitoxin (line 3). Presumably, antitoxin prevented binding of toxoid onto erythrocytes thereby reducing protection.

3. Neutralization in Solution

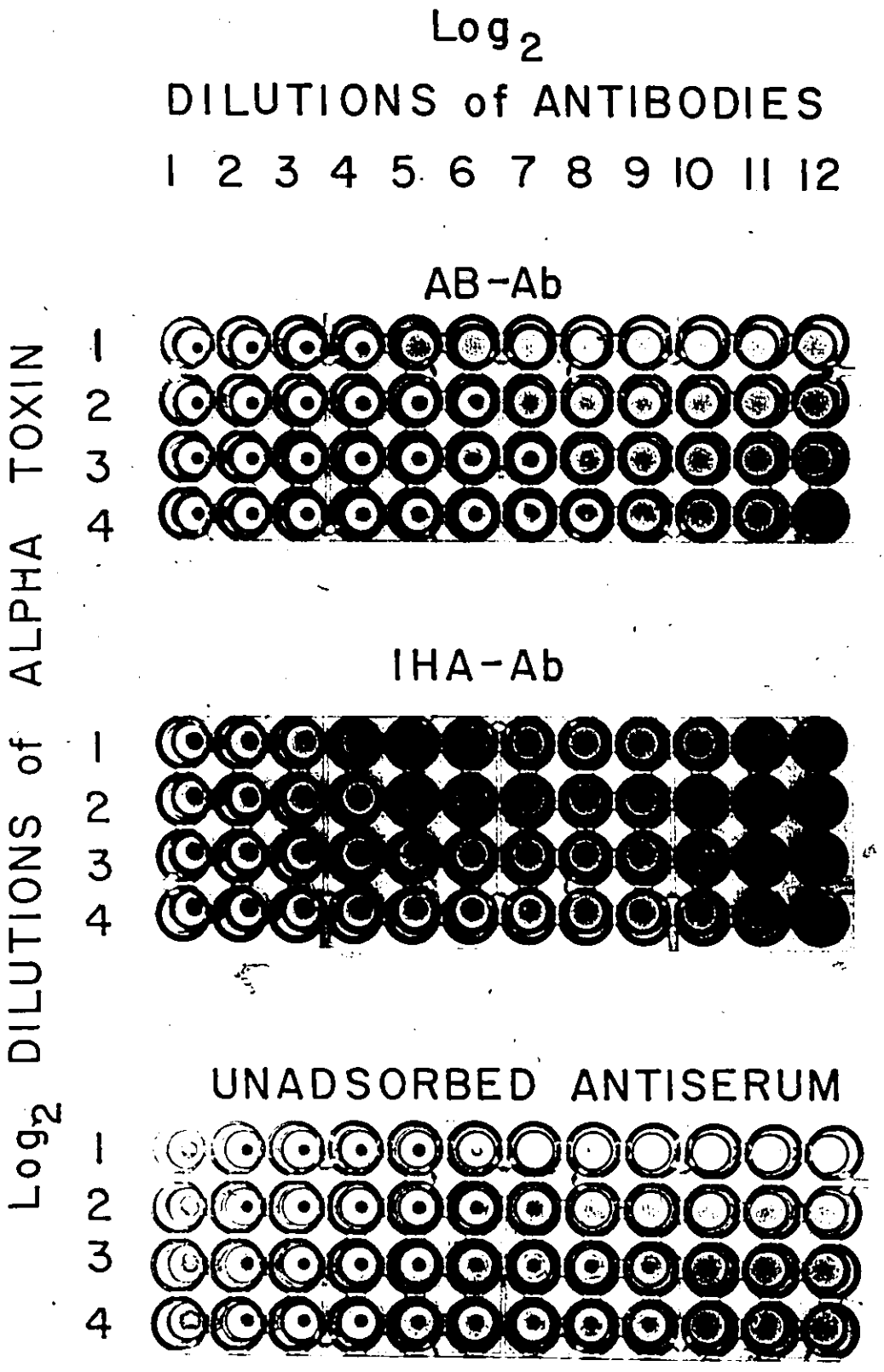
Neutralization tests were set up in the manner of a "chess board" titration with different dilutions of toxin versus different dilutions of rabbit antibodies (Fig. 13). When neutralization by AB-Ab and by unadsorbed, whole antiserum were compared, the following were observed.

(i) Neutralization was dependent on the concentrations of both toxin and antibodies.

(ii) Hemagglutination occurred only at high concentrations of the whole serum (1:2), but not at higher dilutions. This was unusual considering that the IHA titre of the whole serum was 128.

FIGURE 13

Toxin neutralization by AB-Ab, IHA-Ab, and unadsorbed antiserum.


Log2
DILUTIONS of ANTIBODIES
1 2 3 4 5 6 7 8 9 10 11 12
AB-Ab
IHA-Ab
UNADSORBED ANTISERUM
Log2 DILUTIONS of ALPHA TOXIN
1
2
3
4

(iii) No hemagglutination occurred whenever erythrocytes were protected by AB-Ab indicating the absence of immune complexes on the erythrocytes.

4. Indirect Antibody Methods

Following neutralization tests, two different anti-immunoglobulin tests were performed to establish that AB-Ab were absent from the protected erythrocyte surfaces. Firstly, addition of goat anti-rabbit whole serum to AB-Ab protected erythrocytes did not cause hemagglutination. Secondly, indirect immunofluorescence was negative when fluorescein labeled anti-rabbit IgG was added as described in the method section. Anti-IgG was used because AB-Ab was found in IgG fractions (Fig. 5).

From this series of experiments on the mode of neutralization, it was concluded that AB-Ab neutralize predominately in solution and that they neutralize primarily by prevention of binding of the toxin onto erythrocytes.

IV. The Indirect Hemagglutinating Antibodies

A. Characteristics

IHA-Ab were found in purified IgG fractions of immune sera and were precipitated by anti-IgG in immunodiffusion tests (Fig. 14).

The IHA titres of sera from four rabbits increased in response to antigenic stimulation as shown in Table X. The course of the response was depicted in figure 15.

These characteristics, plus the ability to neutralize, indicated that IHA-Ab were antibodies.

Attempts to precipitate IHA-Ab with alpha toxin by immunodiffusion in agar gel tests were unsuccessful (Fig. 6) although the wells with IHA-Ab were recharged 11 times. The IHA titre of the preparation used was 64 and the neutralization titre was 128 N.

The nonprecipitating nature of IHA-Ab was further observed when an immune serum that lacked AB-Ab was used in immunodiffusion tests. This serum had an IHA titre of 128 and 12 N. Adsorption with LGCE reduced both the IHA titre and N to zero indicating that AB-Ab was absent from this antiserum.

Similar to the case of AB-Ab, toxin neutralization was dependent on the concentration of IHA-Ab. The degree of protection decreased with decreasing amounts of IHA-Ab (Fig. 16).

Likewise, toxin neutralization was also dependent on the time of addition of IHA-Ab to the toxin-erythrocyte mixture. The longer the erythrocytes were exposed to the toxin

FIGURE 14

Immunodiffusion of IHA-Ab against antiglobulins

Centre well contained undiluted IHA-Ab.

Well 1 contained goat anti-rabbit whole serum.

Well 2 contained goat anti-rabbit IgG.

Well 3 and 4 contained PBS.

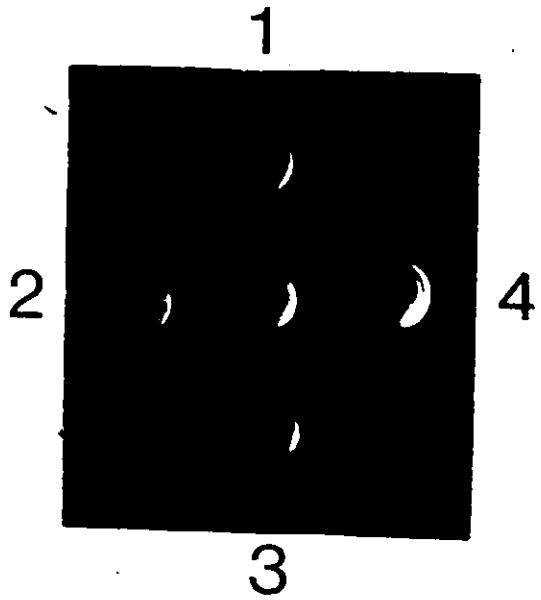

1
2
4
3

TABLE X

IHA-Ab RESPONSES IN RABBITS

RABBIT	LOG_2 IHA TITRES OF WEEKLY SERA							
	0	1	2	3	4	5	6	7
F105	1	7	12	10	7	7	7	7
F106	3	10	10	10	8	8	8	7
F112	1	7	12	12	11	10	9	9
F116	1	6	10	10	9	8	7	6
MEAN	1.5	7.5	11	10.5	8.75	8.25	7.75	7.25
S.E.	0.43	0.75	0.5	0.43	0.74	1.18	0.41	0.54

FIGURE 15

IHA-Ab response in rabbits

Four rabbits (F105, F106, F112, F116) were immunized with alpha toxoid as described in Materials and Methods. The IHA titres of the weekly antisera were averaged as shown in TABLE X.

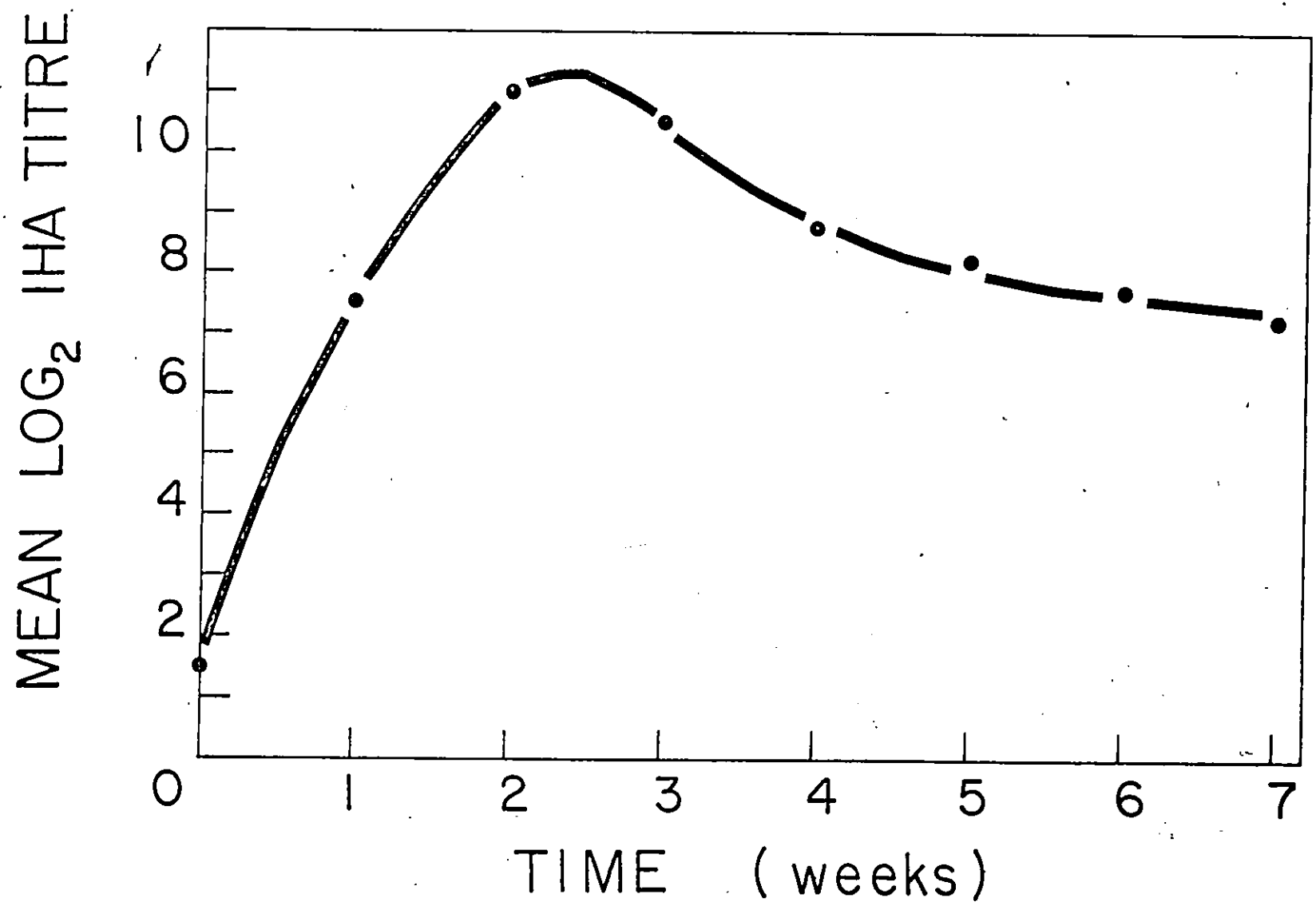

MEAN LOG2 IHA TITRE
0
2
4
6
8
10
0
1
2
3
4
5
6
7
TIME (weeks)

FIGURE 16

The effect of IHA-Ab concentration on neutralization.

Test conditions were as described for FIGURE 6. B = baseline hemolysis. The amount of IHA-Ab in test 1 = 10 µl, test 2 = 50 µl, test 3 = 100 µl.

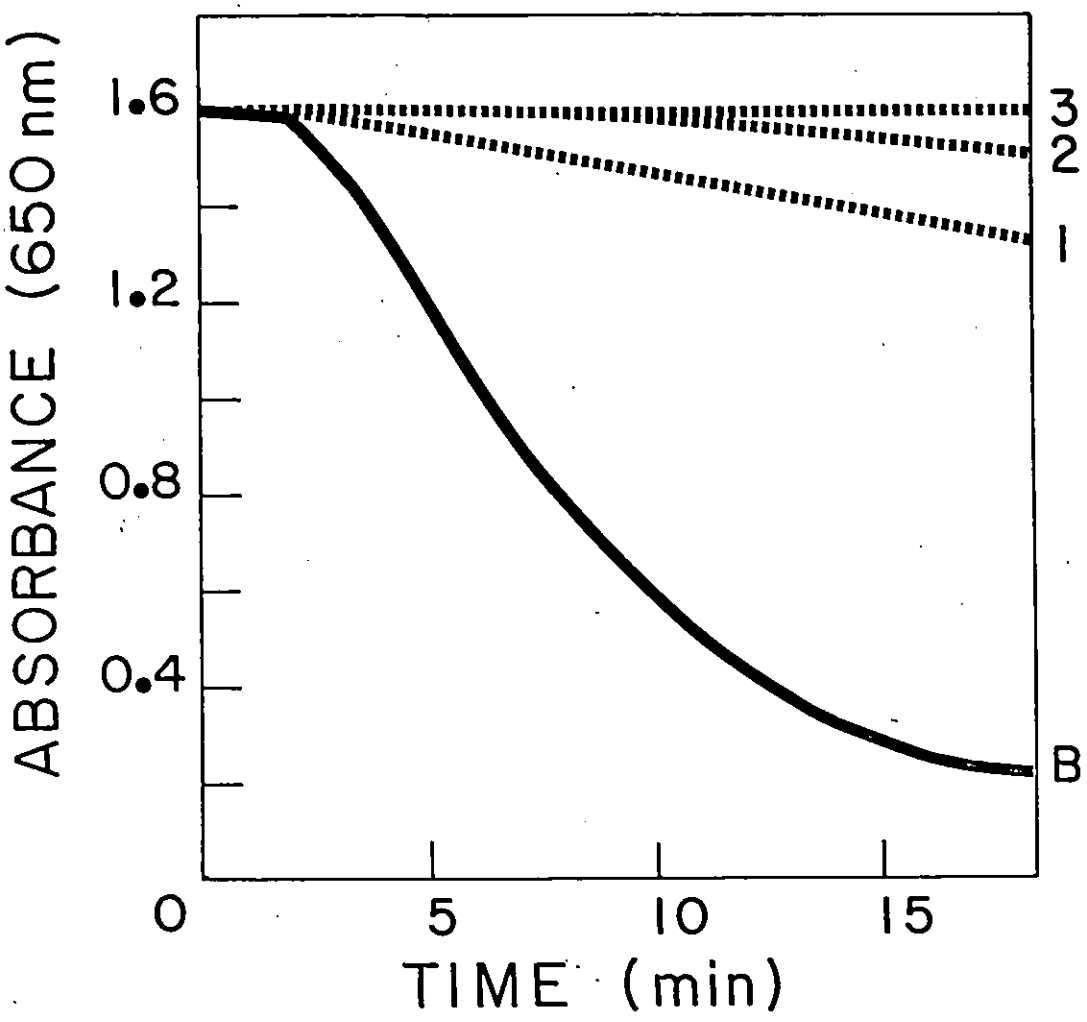
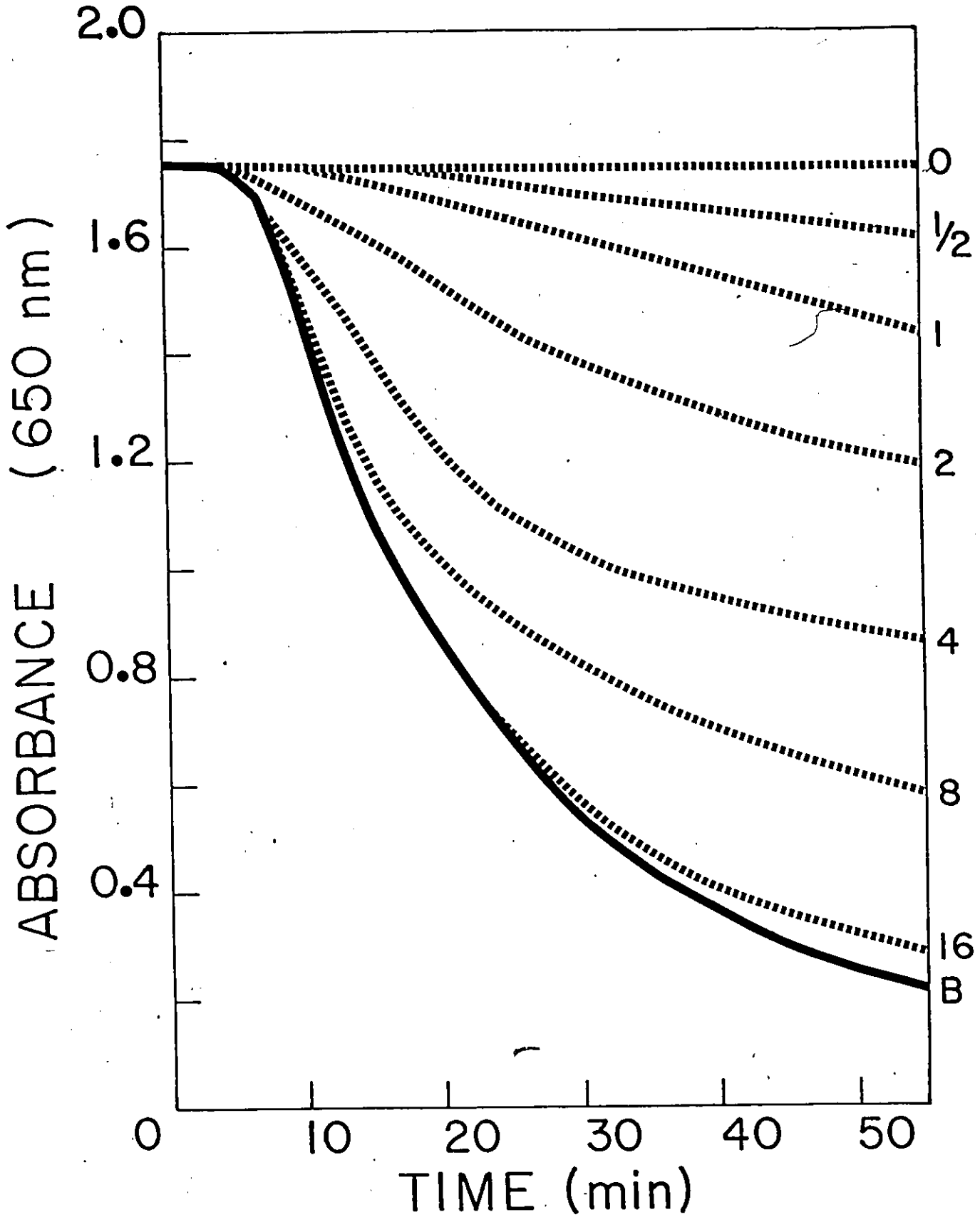

ABSORBANCE (650 nm)
1.6
1.2
0.8
0.4
0
5
10
15
TIME (min)
3
2
1
B

FIGURE 17

The effect of time of introduction of IHA-Ab on neutralization.

Each test (final volume 310 µl) contained erythrocytes in 250 µl of PBS and 10 µl of toxin initally. Then 50 µl of IHA-Ab was added at different times to each test. The time (in minutes) at which IHA-Ab was added was indicated at the end of each line. B = lytic baseline.


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE (650 nm)
10
20
30
40
50
TIME (min)
0
1/2
1
2
4
8
16
B

the less protection the IHA-Ab was able to confer on the unlysed erythrocytes (Fig. 17).

As in the case of AB-Ab, attempts to enhance the degree of protection after time zero by increasing the amount of IHA-Ab were unsuccessful.

B. Mode of Neutralization

The investigations were similar to those described for AB-Ab.

1. Hemagglutination

(i) with bound toxoid

IHA-Ab agglutinated coated erythrocytes or LGCE but not uncoated erythrocytes, indicating that the IHA determinants remained accessible to IHA-Ab after the toxoid was bound to erythrocytes.

(ii) with free toxoid

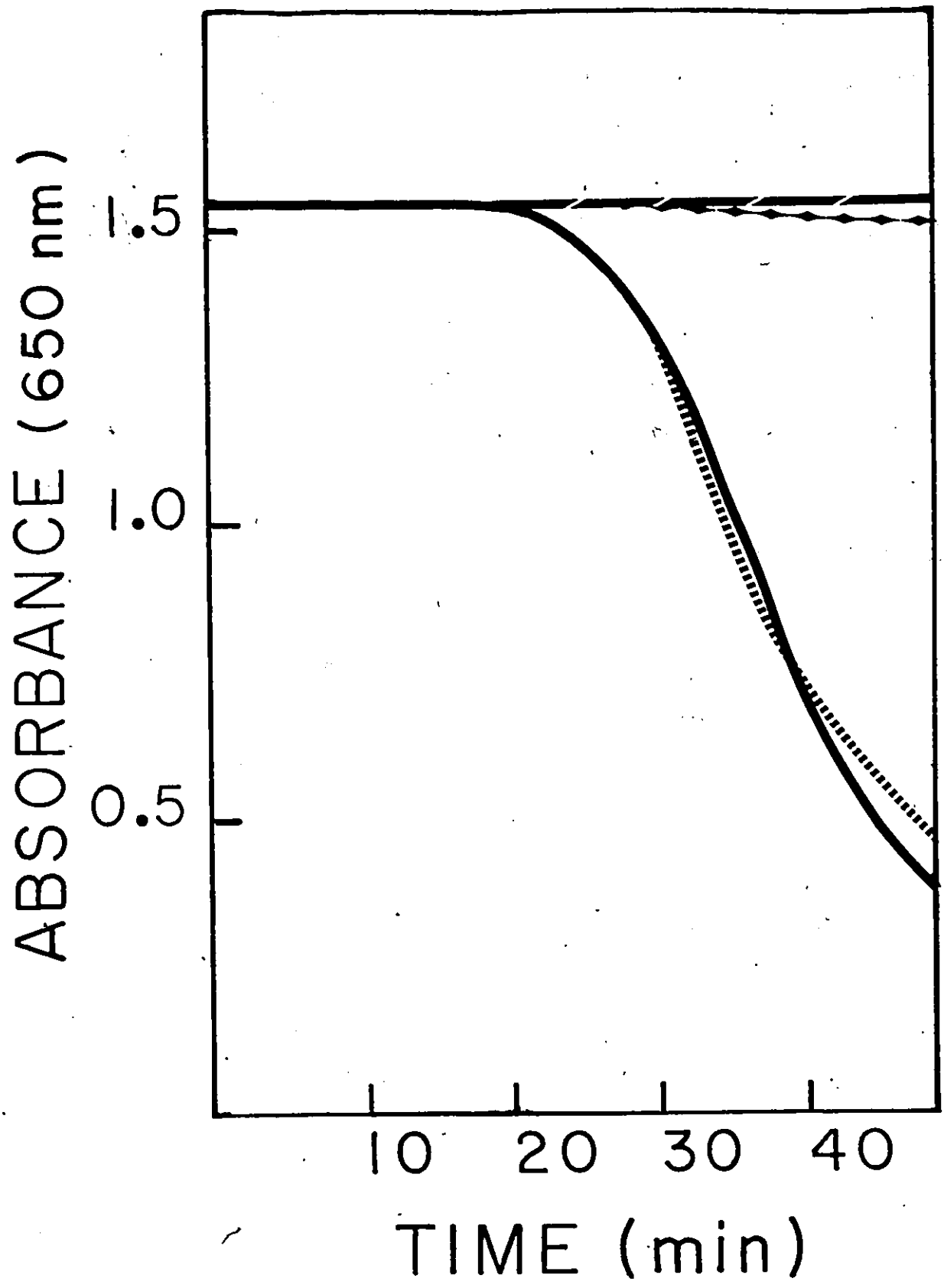
Unlike the case of AB-Ab, when 25µl of soluble toxoid (0.1 mg/ml) was added to a mixture containing 25µl of 2% (V/V) rabbit erythrocytes and 25µl of undiluted IHA-Ab (which had an IHA titre of 128) hemagglutination occurred at both RT or $4^{o}C$ after 1 h. This result indicated that the binding of toxoid to erythrocytes was not prevented by IHA-Ab.

2. Inhibition of Toxoid Protection

Test for inhibition of the toxoid protection phenomenon was repeated with IHA-Ab and compared to AB-Ab. Toxoid, pre-incubated with IHA-Ab offered protection to erythrocytes, but

FIGURE 18

Toxoid-protection test : Line A ▬▬▬ is a spectrophotometric tracing of the course of hemolysis of a suspension of rabbit erythrocytes due to a small amount of alpha toxin. Hemolysis, reflected as the decrease in absorbance of the mixture, with time was measured at 650 nm in a 1 cm light path at RT. Line B ▰▰▰▰ represents toxoid-coated erythrocytes adjusted to the same absorbance and challenged with the same amount of toxin. Protection is evident. Line C ◆—◆—◆—◆ depicts the resistance of erythrocytes coated in the presence of IHA-Ab, washed thrice, and then subjected to the same test. Line D ▪▪▪▪▪▪▪▪▪▪▪▪ describes the loss of resistance when erythrocytes were coated in the presence of AB-Ab, washed thrice and then tested.


ABSORBANCE (650 nm)
1.5
1.0
0.5
10
20
30
40
TIME (min)

not when preincubated with AB-Ab (Fig. 18). Therefore, unlike AB-Ab, IHA-Ab did not prevent binding of toxoid onto erythrocytes.

3. Neutralization on Membranes

Neutralization tests were set up as previously described for AB-Ab (Fig. 13). The following was observed.

(i) neutralization was dependent on concentrations of both toxin and IHA-Ab.

(ii) hemagglutination occurred whenever erythrocytes were protected by IHA-Ab.

4. Indirect Antibody Method

Unlike AB-Ab, indirect immunofluorescence was positive for erythrocytes protected by IHA-Ab in neutralization tests.

Based on these results it was concluded that IHA-Ab neutralized toxin on erythrocyte membranes.

Based on the differences in the mode of neutralization of the two antibodies, one could predict that erythrocytes with toxin on their surfaces would be protected by IHA-Ab but not by AB-Ab. Experimentally, this implied that IHA-Ab should protect more than AB-Ab if added after the toxin, even though

their protections may be equivalent if added before the toxin. Supporting data were derived from KH assays on the time dependencies of antibody neutralization (Fig. 9 and Fig. 17). The percent protection due to antibodies was calculated by the formula

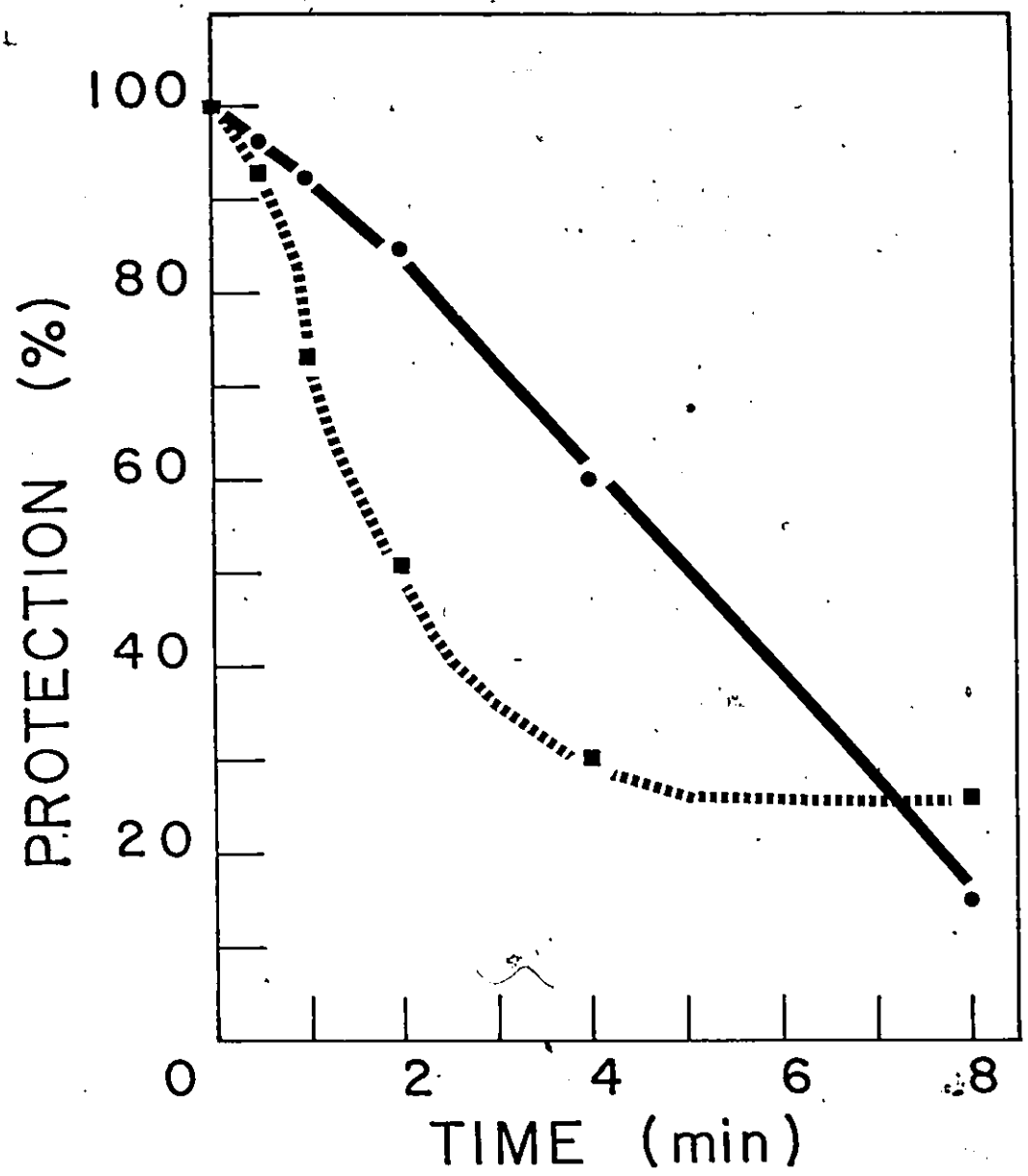
$$\frac{\Delta_{OD}\ \text{Baseline} - \Delta_{OD}\ \text{test}}{\Delta_{OD}\ \text{Baseline}} \times 100\ \%$$

where Δ_{OD} was the change in absorbance per minute and Δ_{OD} Baseline line denoted the maximum rate of hemolysis due to the toxin in the absence of antibodies. In all cases, the minimum amount of antibodies required for 100% protection at time zero with respect to a particular baseline was determined by trials. The neutralization capacities contained in the minimal amounts thus determined for different antibodies were considered to be equivalent at time zero. When the % protection was plotted against time of introduction of the antibodies (Fig. 19) it was apparent that IHA-Ab offered more protection than AB-Ab to a population of erythrocytes that were exposed to alpha toxin. The result strengthened the hypothesis for the different modes of neutralization of the two antibodies.

FIGURE 19

IHA-Ab neutralize better than AB-Ab after time zero.

- % protection due to IHA-Ab introduced at the indicated times.
- % protection due to AB-Ab introduced at the indicated times.


100
80
60
40
20
0
PROTECTION (%)
2
4
6
8
TIME (min)

V. Orientation of Toxin on Different Erythrocytes

Erythrocytes from different animal species vary widely in their sensitivity to alpha toxin (11,33). We sought to establish whether the antigen is oriented differently on different erythrocytes by using the resolving power of the two populations of anti-alpha antibodies.

Erythrocytes of rabbit, rat, sheep, mouse and human were washed three times separately. Each suspension was adjusted to a 10% final concentration in an excess of toxoid (1.5 mg/ml). After 1½ h incubation at RT, the coated erythrocytes were washed twice and adjusted to 1% (V/V) suspensions. IHA titrations were performed with the same antiserum which was previously heterophile adsorbed with erythrocytes from all the five animals. All five coated erythrocytes produced the same titre of 64. Furthermore, AB-Ab obtained by adsorption with coated rabbit erythrocytes was negative in IHA tests with each of the five coated erythrocyte. Likewise, AB-Ab obtained by adsorption with each of the five coated erythrocytes were all negative in IHA tests. Therefore, the two antibodies did not exhibit any shift in characteristics with respect to the erythrocyte species.

For confirmation, coated human erythrocytes were used as an immunoadsorbent to remove IHA-Ab from antiserum. As expected, the adsorbed antiserum (AB-Ab) showed a zero IHA titre with both coated erythrocytes of rabbit and human although human erythrocytes are known to be thousands of times more resistant to alpha hemolysin than rabbit erythro-

cytes. No drastic difference in neutralization capacities was found between AB–Ab obtained by adsorption with human immunoadsorbent and that by rabbit immunoadsorbent. These results (Table XI) indicate that toxoid and toxin bind to erythrocytes of different susceptibility with similar immunologic orientation. Therefore, the membrane binding region of alpha toxin is probably the same for different erythrocyte from different species.

TABLE XI

CHARACTERISTICS OF AB-Ab FROM RABBIT AND HUMAN IMMUNOADSORBENT

AB-Ab OBTAINED BY ADSORPTION WITH	IHA TITRE WITH COATED RABBIT RBC	HUMAN RBC	N UNITS
Coated rabbit RBC	0	0	20
Coated human RBC	0	0	16

RBC = erythrocytes

DISCUSSION

This investigation has shown that anti-alpha toxoid, produced by hyperimmunization of both rabbits and pigs, is comprised of two distinct populations of antibodies, namely, AB-Ab and IHA-Ab. The mechanism of neutralization by these two antibodies has been differentiated: the AB-Ab neutralize by prevention of binding of alpha toxin onto erythrocytes whereas the IHA-Ab can neutralize even after the toxin is membrane bound. A strikingly similar situation was reported for antistreptolysin O. Prigent et al (89) demonstrated two topologically distinct sites on the molecule of streptolysin O: a fixation site (f) which recognises and binds to the receptor (cholesterol) on the cell surface and a lytic site (ℓ) which triggers irreversible cell damage after fixation. The same investigators obtained anti-streptolysin O antisera from hyperimmunized horse (1) and from myeloma patients (93). These antisera have either anti-f antibodies that block binding or anti-ℓ antibodies that block lysis after binding. In our case, antisera were produced in pigs and rabbits. Then, the two populations of antibodies were purified and separated.

In relation to other toxin-antitoxin models, the mode of neutralization of AB-Ab seems to parallel that of the diphtheria antitoxin (86) whereas the IHA-Ab demonstrated that while alpha hemolysin is not a sulphydryl activated toxin, it can likewise be neutralized after attachment to target cells (12). We recognize the possibility that IHA-Ab might neutralize in solution before the immune complex binds to the membrane. If that were the case, the combination of

the IHA-Ab with alpha hemolysin must not hamper binding of the immune complex onto membranes.

As suggested previously, alpha hemolysin is one of the cytolytic toxins that may prove useful in probing biomembrane organization (12,2). This suggestion is sound if alpha toxin can only bind in one specific manner. Moreover, Cassidy and Harshman (29) indicated that alpha toxin binds to membrane by two mechanisms: binding with high affinity to receptors on membranes, and polymerization on membrane surfaces at high concentration of the toxin. Our data establish the immunologic orientation of alpha toxin on membrane. The deduction was as follows.

There are two possibilities in the way alpha hemolysin binds to erythrocyte membranes. The binding could be random in which case all of the surfaces of the hemolysin may be randomly exposed (Fig. 20a). Alternatively, the hemolysin may be oriented on membranes (Fig. 20b) which means that one particular region of the toxin is always involved. This hypothetical region on that toxin will be referred to as the membrane binding region. The immunologic implications are if alpha hemolysin is oriented, antigenic determinants near the membrane binding region would be blocked by the membrane and become inaccessible to the corresponding antibodies (Fig. 21a). Alternatively, if the toxin is randomly oriented when bound to membrane, no single type of antigenic determinant would become inaccessible to antibodies (Fig. 21b).

FIGURE 20

Two possible ways of binding

a. random binding

b. oriented binding

The red lines represent erythrocyte surfaces. The colored circles represent alpha toxin molecules.

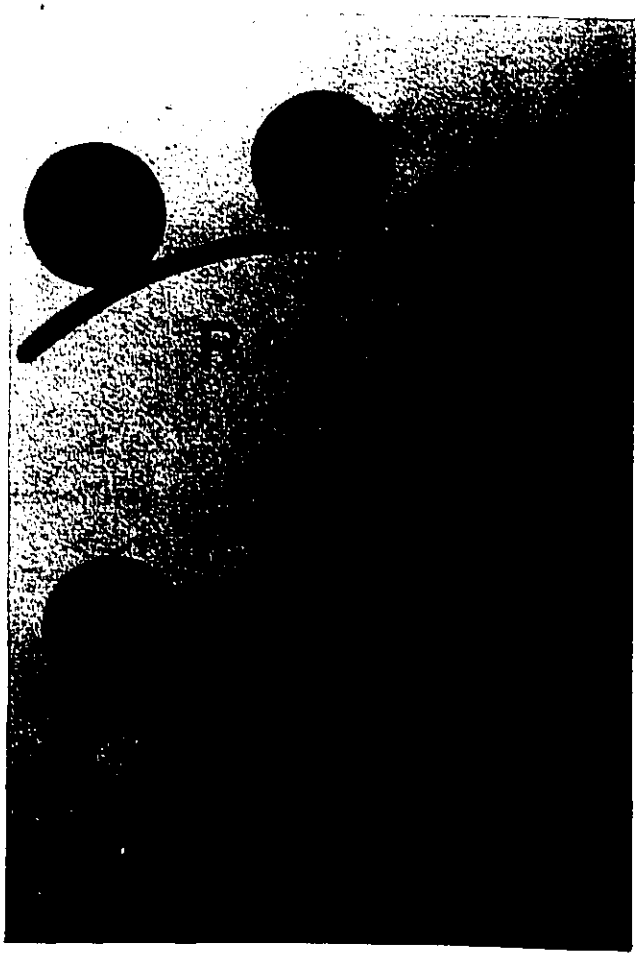

a
b

FIGURE 21

The immunologic implications of random binding versus oriented binding.

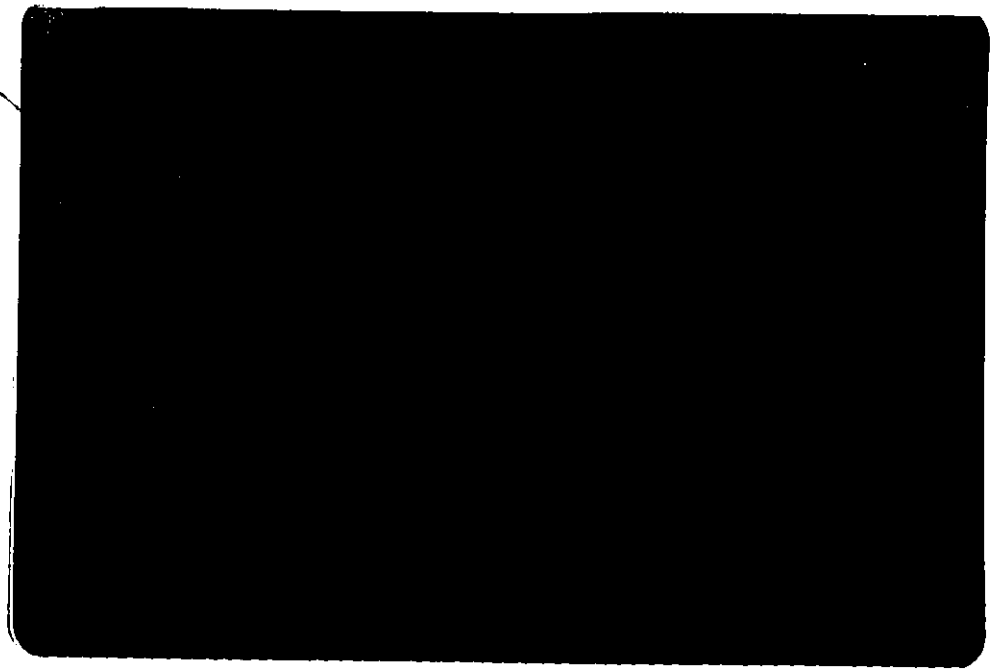
a. Oriented binding secluded the membrane binding regions.

b. Random binding exposes all portions of the toxin.

Antibodies directed to different portions of the alpha toxin molecule (colored circle) are schematically represented by the blocks of the corresponding colors.

a

b



According to this working hypothesis, IHA-Ab and AB-Ab are separable by immunoadsorbent only if toxoid was oriented on membranes. Furthermore, the characteristics of the two antibodies such as hemagglutination via bound or free toxoid, and inhibition of toxoid protection (as reported in the result section), were predictable by this hypothesis. Therefore, alpha toxoid must be immunologically oriented.

The separation of the two antibodies allowed us to investigate the orientation of alpha toxin on erythrocyte surfaces by neutralization tests and antiglobulin tests. Although the antibodies were separated using toxoid coated erythrocytes as the immunoadsorbent, the antibodies showed no detectable shift in characteristics in the toxin-erythrocyte system. Therefore, alpha toxin, like its toxoid, must bind onto rabbit erythrocyte membranes with a specific orientation, involving a specific membrane binding region of the toxin.

The sensitivity of erythrocytes from different animal species correlates with the amount of toxin "specifically" bound, (105,28). This view is not necessarily in conflict with our findings that different erythrocytes when coated produced the same IHA titre with a given antitoxin because coating was performed under condition of toxoid in tremendous excess. At high concentrations, alpha toxin (53,54) and toxoid (5) polymerized on membrane surfaces to form ring structures 10 nm in diameter; this observation was reproduced in our laboratory

(unpublished). Cassidy and Harshman (29) deduced that such structures may not be related to high affinity binding. Based on the limited data presented, we think that the membrane binding region of alpha toxin and toxoid is probably the same for erythrocytes from different species irrespective of the binding affinity. That is, the ring structures are immunologically oriented on the membranes.

Competition between the two populations of antibodies was observed in neutralization tests using unadsorbed antisera. Unadsorbed antisera with IHA titres as high as 4096 caused hemagglutination of protected erythrocytes only at very low sera dilutions (≤ 4). At higher antisera dilutions, neutralization but not hemagglutination was observed, indicating that the IHA reaction was overwhelmed by the anti-binding reaction. Therefore, experimental results obtained by the use of unadsorbed antisera need to be interpreted cautiously. For examples, the outcome of immunofluorescent or immunoferritin studies would depend on the ratio of IHA-Ab to AB-Ab in the antiserum.

Klainer et al (64) using fluorescein labelled anti-antitoxin, observed maximum intensity of fluorescence on erythrocytes during lytic phase. This phenomenon may be reinterpreted in the light of our findings. During the prelytic phase, AB-Ab may prevent fluorescence of erythrocytes to which the binding of toxin is blocked. However, during the lytic phase, most toxin is bound. Thus, IHA-Ab can render the toxin coated

erythrocytes fluorescent.

As stated, numerous attempts to evaluate antitoxin in protection against the onset of infection and in alteration of the course of the disease have met with conflicting results, and some of the explanations for this conflict were given. We may now offer an additional explanation: the ratio of AB-Ab to IHA-Ab varies from serum to serum; thus, the mode of neutralization varies depending on the predominance of a particular population of antibodies.

In the same light, the antigenicity of presently available vaccines could be better defined in terms of these two antibodies. Yet, the refinement of staphylococcal vaccine awaits determination of the relative efficiency and conditions of neutralization by the two antibodies.

Numerous reports show alpha toxin binds to membranes. My findings are consistent with these reports. The hemagglutination reaction brought about by IHA-Ab would not be possible if the toxin is not bound to membranes. Also, the prevention of binding by AB-Ab resulted in no hemagglutination.

With respect to the mode of action of alpha toxin, my investigation suggests that binding per se is nondestructive because the toxin can be neutralized on the membrane by IHA-Ab. This is not to say that binding is unimportant for destruction. Rather, binding is the initial step in a sequence of event(s) leading to hemolysis. If binding per se is nondestructive, some type of activation event(s) must follow.

Our findings do not indicate whether the activation event(s) is "surface activity" or an enzymatic effect, or both. Nonetheless, the AB-Ab isolated the binding step whereas the IHA-Ab arrested the activation event and prolonged protection (Fig. 19). These results add to a growing body of evidence for the existence of destructive event(s) subsequent to binding (76,29,106). Additional data in support of this contention are being generated in this laboratory. We have shown that toxoid binds but does not destroy. Recently, we isolated a mutant that produces a non-hemolytic form of alpha toxin. This material reacts with antibodies, binds to membranes, protects erythrocytes from lysis by the toxin, and is positive in the IHA test. Further study on this mutant should provide valuable information regarding the mode of action of alpha toxin.

Although we assume that AB-Ab blocks the binding of toxin by steric hindrance, AB-Ab could also block by induction of a conformational change in the toxin. However, preliminary evidence suggest that such a conformational change is restricted. The total neutralization capacity of whole serum is less than the sum of the two individual antibodies. This nonadditive effect presumes that a single toxin molecule consumes more than one type of antibody simultaneously. Thus, the combination of AB-Ab with the toxin must not affect the IHA determinant(s).

In the past, the complement fixation test has been used as a standard tool for the study of antigenic determinants of proteins. This method is of limited value for the study of determinants of alpha toxin in spite of the fact that the complement pathway was activated. The major limitation is that the toxin, and the toxin-IHA antibody complex, also binds onto the indicator cell. Although the application of CF tests in vitro is limited, the role of complement in disease processes cannot be ignored.

In comparison to the CF test, the IHA test has the advantage of measuring a defined population of antibodies, namely the IHA-Ab, in a mixture such as whole serum. The value of the IHA test in serodiagnosis and serosurveillance awaits to be determined.

Although much needs to be done, the data presented is a definite foundation upon which the precise antigenic anatomy of alpha toxin shall be built.

SUMMARY

The conclusions derived from this research are the following:

1. Two distinct populations of anti-alpha antibodies were discovered, namely AB-Ab and IHA-Ab.
2. A method was developed to separate the two antibodies in antitoxin.
3. An indirect hemagglutination test was developed to titrate IHA-Ab.
4. The two antibodies exhibited different modes of neutralization. AB-Ab prevent the binding of alpha hemolysin onto erythrocyte membranes. IHA-Ab neutralize membrane-bound hemolysins.
5. Competition between the two antibodies was observed. This competition could affect the outcome of immunologic experiments.
6. Binding of the hemolysin per se is nondestructive to the erythrocyte.
7. A destructive event subsequent to binding was implicated.
8. Alpha hemolysin is immunologically oriented on membranes.
9. The existence of a membrane binding region on the hemolysin is indicated. This implied the existence of corresponding receptors on membranes.
10. The same membrane binding region is probably involved in binding to erythrocytes from different animal species.

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