BINDING OF ^{125}I -ALPHA TOXIN OF *STAPHYLOCOCCUS AUREUS* TO ERYTHROCYTES

G. M. PHIMISTER* AND J. H. FREER

*Department of Microbiology, Anderson College, University of Glasgow,
Glasgow G11 6NU*

SUMMARY. Alpha toxin purified from *Staphylococcus aureus* strain Wood 46 and radioiodinated by the lactoperoxidase method retained full haemolytic activity and was used to study factors affecting binding to rabbit and horse erythrocytes. A relatively fixed percentage of added toxin bound to both cell types; the percentage bound was independent of temperature, pH, cell concentration and toxin concentration. Neither a 50-fold excess of native toxin nor Concanavalin A inhibited the binding of iodinated toxin to erythrocytes. The results suggest that differences in the sensitivity of erythrocytes to haemolysis do not reflect the abundance of high affinity toxin receptors on sensitive cells, but are more probably the result of differences in the intrinsic stability of the membrane and its sensitivity to perturbation by amphiphilic agents.

INTRODUCTION

Amongst the cytolytic protein toxins of bacteria, the α toxin of *Staphylococcus aureus* is one of the most widely studied. Depending upon the dose and route of administration, it can be lethal and dermonecrotising for laboratory animals and cytolytic for various mammalian cells. Its biological activity is usually assessed as haemolytic activity against rabbit erythrocytes, the most sensitive species. Despite considerable effort, the nature of the interaction of the toxin with membranes of sensitive cells is still unclear. From the results of binding studies with toxin which had lost its lytic activity by radioiodination or heating, it was concluded that the high sensitivity of rabbit erythrocytes to toxin-induced lysis was the result of the presence of high specificity toxin receptors on the membrane (Cassidy and Harshman, 1973 and 1976a; Kato *et al.*, 1975a and 1977; Barei and Fackrell, 1979; Lo and Fackrell, 1979), but more recent data support a less specific lytic mechanism involving hydrophobic interaction of toxin with membranes resulting in transmembrane pore formation (Füssle *et al.*, 1981). Our observations on the characteristics of binding of radioiodinated toxin with full haemolytic activity to erythrocytes are reported here.

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* Present address: Department of Bioscience and Biotechnology, Biochemistry Division, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow G4 0NR

MATERIALS AND METHODS

Toxin production and purification. Toxin was purified from culture supernate of *S. aureus* strain Wood 46 (NCTC7121) grown in the medium of Bernheimer and Schwartz (1963). Extracellular protein from 1.5 L of late-exponential-phase culture fluid was precipitated at 4°C by the addition of solid ammonium sulphate to 70% saturation. After stirring for 18 h, the precipitate was recovered by centrifugation at 55 000 *g* for 30 min at 4°C and dialysed against two 4-L volumes of 0.05 M potassium phosphate, pH 6.8 for 6 h at 4°C. The contents of the dialysis sac were separated by chromatography on a controlled-pore glass column (BDH) by the method of Cassidy and Harshman (1976b). The protein peak that was eluted with 1.0 M potassium phosphate, pH 7.5, contained the α -toxin component; and this was concentrated by dialysis against 70% saturated ammonium sulphate. Precipitated protein was then dialysed against several changes of glycine 1% w/v before isoelectric focusing in a linear sorbitol density gradient (5–50% w/v) with a pH range of 5–10 using an LKB 8101 column according to the manufacturers instructions (LKB application note 219). The main toxin component, assayed by haemolytic titration against rabbit erythrocytes (see below), focused at pH 8.5 with minor peaks at pH 8.15 and 9.05. The material with a *pI* of 8.5 was recovered and dialysed at 4°C against 0.15 M NaCl in 0.05M potassium phosphate, pH 7.4 (KPS buffer) before storage at –70°C.

Characterisation of purified toxin. Alpha-toxin was assayed by haemolytic titration against rabbit erythrocytes according to the method of Bernheimer and Schwartz (1963). The level of contamination of the α -toxin fraction with staphylococcal δ lysin was estimated by titration against cod erythrocytes (Birkbeck, Chao and Arbuthnott, 1974; Chao and Birkbeck, 1978) and with staphylococcal β lysin by titration against sheep erythrocytes (Low *et al.*, 1974). Proteolytic activity was assayed against azocasein by the method of Tomarelli, Charney and Harding (1949). The mol. wt and presence of contaminating proteins in purified toxin was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Immunological analysis was done by double diffusion in agar against commercial antiserum to α toxin (Wellcome Reagents Ltd) and against antiserum to purified staphylococcal δ lysin (a gift from Dr L.E.T. Stearne, Department of Microbiology, University of Glasgow).

In titrations against rabbit and cod erythrocytes, the highest dilution of toxin that caused 100% haemolysis was assumed to contain one minimal haemolytic dose (MHD). In titrations against sheep erythrocytes, the difference between the haemolytic titres at 37°C and 4°C (the 'hot-cold' titre) was taken as the titre of β toxin.

Iodination of Toxin. ¹²⁵I-iodination of purified α toxin was based on the method of Hubbard and Cohn (1972). To 1.5 ml of α toxin (c. 1 mg of protein) was added, in sequence, lactoperoxidase (E.C.1.11.1.7 from milk, Sigma) 400 mU, glucose oxidase (E.C.1.1.3.4, Type V, from *Aspergillus niger*, Sigma) 350 mU and carrier-free Na ¹²⁵I (Amersham International plc, Amersham) 1.85 MBq. The reaction was started by the addition of glucose 120 μ g and allowed to proceed at 37°C for 45 min. The reaction mixture was then transferred to acid washed dialysis tubing and dialysed against KPS buffer containing 10^{–5} M Na₂S₂O₃ for 1 h at 21°C. The buffer was replaced by KPS buffer (pH 7.4) alone and dialysis continued for 18 h at 4°C. The buffer was again replaced and dialysis continued for a further 3 h at 4°C. Before storage at –20°C, the haemolytic titre of the radiolabelled toxin was determined against rabbit erythrocytes. The distribution of radioactivity and haemolytic activity in the radiolabelled α -toxin preparation was determined by polyacrylamide disk-gel electrophoresis in the acid gel system of Reisfeld, Lewis and Williams (1962) run at pH 4.3 in acrylamide 7.5% w/v. Protein distribution in the gel was determined by the method of Weber and Osborn (1969). After electrophoresis, pairs of equivalent slices (2mm) were taken from duplicate gels. One of each pair was placed in an automatic β/γ spectrometer (Model NE8312; Nuclear Enterprises, Edinburgh) and γ emissions were counted for 10 min. The other slice was shaken in 0.5 ml of PBS for 2 h, after which the buffer was assayed for haemolytic activity against rabbit erythrocytes.

Toxin-binding assays. Binding of radiolabelled toxin to erythrocytes was assayed in KPS buffer of appropriate pH value. Rabbit or horse erythrocytes were separated from fresh whole citrated blood by centrifugation at 540 *g* for 10 min and removal of the plasma and buffy coat layer by aspiration. The erythrocytes were washed four times by centrifugation in appropriate

buffer before suspension to the required cell concentration (usually 3×10^8 cells/ml, equivalent to c. 3% packed cell volume) which was assessed by a Coulter counter. Dilute cell suspensions were equilibrated for 10 min at the assay temperature before use. Radioiodinated toxin was added to the erythrocyte suspension and, at the end of the incubation period, 1.0-ml samples were removed and centrifuged at 2600 *g* for 1 min. The amount of unbound toxin was calculated from the radioactivity in 0.5 ml of the supernate. Measurement of the γ emission from 1-ml samples of the assay mixture before centrifugation gave the total amount of ^{125}I -toxin present in the assay. Assay mixtures without erythrocytes were used to estimate adsorption of labelled toxin to the vials. In a typical binding assay, the reaction mixture contained 5.0 ml of erythrocyte suspension (c. 1.5×10^9 cells) and 0.7 μg of α toxin ($1.5\text{--}1.8 \times 10^4$ cpm). Each assay was done in duplicate and vials containing erythrocyte suspension alone were used as blanks.

The effect of temperature on binding was measured over the range 1–45°C. Subsequent experiments to measure time course, effect of pH, cell concentration, Concanavalin A (Con A) and the presence of excess unlabelled toxin were performed at 1°C with an incubation period of 30 min except in time-course experiments. In an attempt to saturate putative binding sites on the membrane of rabbit erythrocytes, the effects of simultaneously adding defined mixtures of unlabelled and radiolabelled toxin were measured. The percentage of added toxin bound was calculated from the difference in counts of the total reaction mixture and the cell-free supernate after incubation of the mixture for 30 min at 1°C.

RESULTS

Characterisation of α toxin

Purified α toxin focused at pH 8.5 ($p\text{I} = 8.5$) and had a specific haemolytic activity against rabbit erythrocytes of $(9.3\text{--}11.0) \times 10^3$ MHD/mg of protein. The use of sheep: rabbit or cod: rabbit erythrocyte haemolytic activity ratios to estimate contamination by β or δ lysins indicated that the concentration of these proteins in the purified α toxin was negligible ($<0.01\%$ for β lysin, and $<0.4\%$ for δ lysin, table I). No proteolytic activity ($<0.03\%$ as trypsin) was detectable in the purified α toxin. Whereas immunodiffusion against antiserum to crude α toxin gave a single line, no precipitin line was formed in tests using anti- δ -lysin serum. SDS-PAGE indicated a mol. wt for the toxin of 34 000. At high sample loading ($\sim 20 \mu\text{g}$) trace amounts of components with mol. wts of 24 000 and 17 000 were detected which probably represented proteolytic fragments of the toxin, as reported by Dalen (1976).

Iodinated α toxin had a specific activity of $(2.1\text{--}2.6) \times 10^4$ cpm/ μg of protein and full haemolytic activity. Analysis of the radioiodinated α toxin by PAGE gave a single peak of radioactive haemolytic material which stained with Coomassie blue. Three

TABLE I
Purification of α toxin and the relative haemolytic titres of various fractions against rabbit, sheep and cod erythrocytes as an indication of possible contamination by β , and δ toxins

Fraction	Specific haemolytic activity (MHD/mg) against the erythrocytes of			Haemolytic activity ratio	
	rabbit	sheep	cod	sheep: rabbit	cod: rabbit
Culture supernate	240	7.5	15	0.031	0.062
Eluent from CPG	4700	18	4.5	0.004	0.001
Electrofocussed α toxin	9300	73	<3.6	0.008	<0.0004

MHD = minimal haemolytic dose; CPG = controlled pore glass chromatography.

TABLE II
The effect of excess unlabelled toxin on the binding of radioiodinated α toxin to rabbit erythrocytes
(1.2×10^7 /ml)

Concentration of ^{125}I - α toxin ($\mu\text{g/ml}$)	Concentration of unlabelled α toxin ($\mu\text{g/ml}$)	Total concentration of α toxin ($\mu\text{g/ml}$)	Percentage of ^{125}I - α toxin bound (mean $\pm$ SD)
0.58	—	—	17.4 ± 0.9
0.6	2.2	2.8	17.2 ± 0.4
2.8	2.2	5.0	18.5 ± 1.6
7.1	2.2	9.3	18.7 ± 1.1
0.6	21.2	21.8	15.2 ± 1.2
2.8	21.2	24.0	13.3 ± 0.2
7.1	21.2	28.4	14.7 ± 1.6

minor peaks of radioactivity that were non-haemolytic and did not stain by Coomassie blue were also detected.

Binding studies

Preliminary experiments to assess binding of radiolabelled toxin by the filtration assay described by Cassidy and Harshman (1976a) and by Kato *et al.* (1977) gave unacceptably high levels of non-specific binding to the filters (*c.* 80%) even after saturation of the filters with bovine serum albumin. Hence, an assay based on centrifugation was employed for all binding experiments. Binding of radioiodinated α toxin to rabbit or horse erythrocytes was mostly independent of temperature between 1°C and 45°C. Over this temperature range, 14–22% and 11–14% of added toxin bound to rabbit and horse erythrocytes respectively. Because of this, and the finding that binding but not lysis occurred at temperatures below 14°C (Freer, 1982), all the subsequent binding experiments were done at 1°C, to avoid toxin-induced cell lysis, especially when high toxin concentrations were used. The time-course experiments showed that binding to both species of erythrocyte was rapid, with bound toxin reaching a maximum level after incubation for 5–10 min. The rate of binding was similar for both cell types, and the amount of added toxin bound remained constant throughout the remainder of the incubation period (60 min).

Alteration of the pH of the assay mixture between pH values of 6.8 and 7.5 had no effect on the percentage of added toxin bound to either cell type. Similarly, varying the rabbit erythrocyte concentration from 0.98×10^8 to 5.0×10^8 cells/ml, and horse erythrocytes from 1.4×10^8 to 6.7×10^8 cells/ml did not alter the percentage of added toxin bound to either cell type. When assays were performed in the presence of Con A, a decrease in the level of toxin bound was noted only when the lectin concentration exceeded 10 $\mu\text{g/ml}$, which indicated non-specific effects probably resulting from slight agglutination of the erythrocytes. Neither pre-treatment with, nor simultaneous addition of, unlabelled α toxin affected the binding of radioiodinated α toxin to either cell type (table II). A two-stage binding assay in which the supernate from the first binding assay was used to study binding of toxin to a fresh batch of erythrocytes and a split titration assay in which the supernate from a haemolytic assay was used to assess the residual haemolytic activity (Lominski and Arbuthnott, 1962), showed that not all the toxin capable of binding was bound in the first exposure to erythrocytes, and that

such unbound toxin was capable of haemolysing fresh erythrocytes (results not shown). Over the range of iodinated-toxin concentrations tested, a relatively constant percentage was bound; the percentage depended upon the cell type. An essentially constant percentage of added toxin was bound, which was independent of the initial toxin concentration.

DISCUSSION

In these experiments, α toxin retained full haemolytic activity after radioiodination in contrast with the findings of previous workers. The α toxin used by Cassidy and Harshman (1973, 1976a and 1979) and Kato *et al.* (1977) lost haemolytic activity during iodination, whereas that of Barei and Fackrell (1979) was toxoided by heating. For this reason the previously published binding studies and the work reported here may not be directly comparable. Moreover, the results of attempts to elute membrane-bound toxin by ionic manipulations by Füssle *et al.* (1981) led these authors to conclude that manipulations of the toxin which lead to loss of haemolytic activity may alter membrane-binding activity. In the present studies, binding to rabbit and horse erythrocytes was independent of temperature over the range 1–45°C, as was cell lysis induced by staphylococcal δ lysin, a lytic agent of low specificity whose action is thought to be due to surface-active properties (Chao and Birkbeck, 1978). Cassidy and Harshman (1976a), however, found that binding to rabbit erythrocytes was temperature dependent with maximum binding at 24°C. The binding reported here was independent of pH, which provides evidence against the hypothesis that the receptors are membrane proteins or glycoproteins (Cassidy and Harshman, 1976a; Kato *et al.*, 1977; Maharaj and Fackrell, 1980), because both receptors and the toxin would exhibit different degrees of ionisation over this pH range, which would, in turn, affect binding affinity.

Binding to both species of erythrocyte was rapid; equilibrium was reached 5–10 min after addition of toxin. The binding of a relatively fixed percentage of added toxin over the range of cell concentrations where toxin:cell ratios varied considerably is difficult to explain in terms of high-affinity binding sites. However, it may indicate either that binding sites were always in excess or that membrane-bound toxin could act as a centre for further polymerisation of unbound toxin. The finding that neither pretreatment with, or simultaneous addition of, excess native toxin affected subsequent binding of iodinated toxin suggested that insufficient native toxin was available to block possible binding sites. However, assuming that native toxin and radiolabelled toxin have similar binding properties, at the higher concentrations of native toxin used in the simultaneous binding experiments (table II), each cell would bind *c.* 6×10^6 toxin monomers. Previous work suggests that a rabbit erythrocyte surface would be entirely covered with ring-like polymerised 12S toxin complexes (Freer, Arbuthnott and Bernheimer, 1968; Arbuthnott, Freer and Billcliffe, 1973; Bhakdi, Füssle and Tranum-Jensen, 1981; Füssle *et al.*, 1981) when the level of bound toxin reached $(2-5) \times 10^6$ (Barei and Fackrell, 1979) or 10^7 molecules (Cassidy and Harshman, 1976a). While it is not certain whether ring formation occurs before or after cell lysis (Bernheimer, 1974), recent evidence suggests that ring formation is intimately involved in membrane disruption and cell lysis (Füssle *et al.*, 1981). It is relevant to note here that whereas Cassidy and Harshman (1976a) reported that

specific binding of radioiodinated α toxin to rabbit erythrocytes could be inhibited by native toxin, binding to and formation of 'specific lysin-receptor complexes' on mouse diaphragm muscle cells was unaffected by a 250-fold excess of native (unlabelled) toxin. Such observations are difficult to reconcile with the concept of the presence of toxin-specific receptors on such cells.

Previous reports (Cassidy and Harshman, 1976a; Barei and Fackrell, 1979) have claimed that human erythrocytes, which are of similar sensitivity to lysis by α toxin as those of the horse (Cooper, Madoff and Weinstein, 1966), lack α -toxin binding sites yet are lysed by α toxin. This conflicts with the results presented here, where similar amounts of toxin bound to both cell types. Also, Füssle *et al.* (1981) found that similar amounts of α toxin bound to rabbit and to human erythrocytes, and to human or sheep erythrocyte resealed ghosts. They proposed that differences in haemolytic sensitivity were due to complex factors involving membrane composition and organisation. The relatively fixed percentage of toxin bound to erythrocytes reported here may be due to characteristics of both the toxin and the cell membrane, and initial toxin binding may not be the only step involved in dictating the final amount of toxin bound. A second step involving toxin polymerisation on the cell surface or binding of free toxin to cell-bound toxin could also be involved. While the initial step would be toxin-dependent, the second step may be cell-membrane dependent. Polymerisation of toxin monomer to the 12S polymer occurs both after contact with natural and artificial membranes, and after contact with lipid dispersions or detergent micelles (Freer *et al.*, 1968; Freer, Artbuthnott and Billcliffe, 1973; Bhakdi *et al.*, 1981; Füssle *et al.*, 1981).

Several investigations have indicated the presence of receptors on rabbit erythrocyte membranes, but there is disagreement as to their nature and occurrence. The receptor is sensitive to pronase (Kato *et al.*, 1975b; Cassidy and Harshman, 1976a; Maharaj and Fackrell, 1980), and estimates of the number of copies per cell range from 3.5×10^3 to 1.25×10^5 (Cassidy and Harshman, 1976a; Kato *et al.*, 1977; Maharaj and Fackrell, 1980). Bernheimer and Avigad (1980) showed that glycophorin, a major glycoprotein of the erythrocyte membrane, inhibited the cytolytic activity of α toxin, whereas Maharaj and Fackrell (1980) proposed that a second major glycoprotein component of the erythrocyte membrane, band 3 detected by several methods, was the receptor in question. Band 3 is a ubiquitous component of erythrocyte membranes with 5×10^5 copies per human erythrocyte (Bretscher, 1973) and the resistance to lysis by α toxin was explained in terms of masking of the receptor molecule by other surface components. It is interesting to note, however, that only the cytoplasmic part of human erythrocyte band 3 is immunogenic in rabbits (England, Gunn and Steck, 1980). This suggests that the portion of band 3 exposed at the exterior of the cell is immunologically similar or identical in human and rabbit erythrocytes, and may indicate that it is exposed on the surface of both cell types. Freer (1982) noted that band 3 of rabbit erythrocytes contains much less sialic acid than that of human erythrocytes (Skutelsky *et al.*, 1977) which may result in a relatively lower net negative surface charge than those of human or horse erythrocytes. It is likely that initial interaction of the toxin with the membranes involves charge interactions of the basic toxin with acidic groups on the membrane surface. Differences in charge density on the cell surfaces may determine the degree of mobility of the bound toxin, and the lower charge density of the rabbit erythrocyte may allow sufficient flexibility in the membrane-bound toxin for subsequent reorientation at the surface followed by

insertion of part of the toxin into the hydrophobic domain of the membrane. It is known that the binding of basic proteins to acidic phospholipids in liposomes can result in penetration of the protein into the hydrophobic lipid domain resulting in increased ion permeability (Kimmelberg and Papahadjopoulos, 1971).

The fact that binding of toxin to both rabbit and horse erythrocytes is independent of temperature, but that lysis is strictly temperature-dependent (Freer, 1982) with each cell type showing a different temperature-sensitivity profile (lysis of rabbit cells occurs only above 12°C whereas lysis of horse cells occurs only above 20°C), is in keeping with a lysis mechanism that depends upon toxin penetration and the state of fluidity of the lipid region of the membrane. Evidence for a temperature-dependent change in the state of the membrane of the rabbit erythrocyte at about 15°C comes from the work of Inoue *et al.* (1974, 1977), who showed that agglutination of rabbit erythrocytes by Con A occurred only at temperatures above 15°C, whereas rabbit reticulocytes were agglutinated by Con A at temperatures below 10°C.

In conclusion, it appears that toxin can bind relatively non-specifically to natural and model membranes, initial binding being followed by events which may lead to or result from disruption of the membrane by penetration of the hydrophobic region by the toxin. The primary event depends upon the characteristics of the toxin whereas the subsequent events depend upon the characteristics of the membrane.

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