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Binding of *Cerebratulus* cytolysin A-III to human erythrocyte membranes

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Binding of *Cerebratulus lacteus* cytolysin A-III to intact human erythrocytes and erythrocyte membranes has been investigated. Binding to ghosts is essentially complete within 2.5 min of mixing which is slightly faster than the rate of hemolysis measured with intact cells. Approximately $4 \cdot 10^4$ binding sites per cell, exhibiting a $K_{0.5}$ of 0.7 μM exist; this compares with 50% hematocrit of about 0.3 μM for A-III. Binding is absent in ghosts extracted with Nonidet P-40, but is unaffected by pretreatment of ghosts with either trypsin or elastase.

Introduction

The Atlantic coast heteronemertine *Cerebratulus lacteus* produces and secretes a number of proteins of biological interest, including a family of highly potent, structurally homologous cytolysins which have been designated A toxins [1]. The most abundant member of this group, toxin A-III, has been thoroughly characterized with respect to structure [2,3] and has been shown to cause complete lysis of human erythrocytes and Ehrlich ascites cells in the concentration range of 1 to 10 μg per ml. Toxin A-III has been shown to lack both phospholipase A [1] and proteinase (Blumenthal, K.M., unpublished data) activities and is therefore presumed to function via a non-enzymic mechanism. Recently, we demonstrated that A-III is capable of directional insertion across the phospholipid bilayer of sphingomyelin-containing liposomes, with at least the amino terminal tridecapeptide becoming exposed on the interior of the liposome [4]. In addition to amino-terminal sequences, there exists a requirement for at least a portion of a carboxy-terminal amphipathic helix for full expression of lytic activity, as shown by removal of C-terminal sequences in proteinase nicking experiments [15].

In the present communication, the binding of A-III to intact erythrocytes and erythrocyte ghosts is described. The concentration-dependence and kinetics of binding correlate well with the dose-response curve and time-course for hemolysis, suggesting that the measured binding occurs at a functionally relevant site. Indirect evidence indicates that this site may be protein in nature.

Experimental procedures

Materials. Toxins A-III and A-IV were purified as described by Kem and Blumenthal [1]. Trypsin (TPCK-treated trypsin) and elastase were obtained from Worthington, lactoperoxidase, pronase, subtilisin, pepstatin, PMSF and 1,10-phenanthroline from Sigma, iodoacetamide from Pierce, and $Na^{125}I$ (17 Ci/mg) from ICN. Fresh human erythrocytes, obtained by venipuncture, were washed extensively with Ca^{2+}-free, Tris-buffered saline prior to use and were used within two days. Unsealed erythrocyte ghosts were prepared as described by Steck and Kant [6] and stored under nitrogen at

Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

−70°C. Purified A-III was iodinated in 50-μg portions using the lactoperoxidase-H_2O_2 system as described elsewhere, and the iodinated protein was separated from by-products by gel filtration on Bio-Gel P-6 in the presence of 0.2 mg/ml bovine serum albumin. The product protein was routinely labeled to a specific activity of $(5–7.5)\cdot 10^5$ cpm per μg. We have previously demonstrated that iodination is without effect on the activity of toxin A-III [4].

Binding assay. Binding of toxin A-III to intact human red blood cells was measured in a two step process. Initially, native A-III was added to a 1% suspension of cells in normal saline and incubated for 60 min at 0°C. Cells, containing bound toxin, were removed by centrifugation and the supernatant divided into three 1.0 ml portions. After measurement of A_{540}, 1.0 ml of a standard suspension of human red cells was added to each ml of supernatant, and hemolysis was measured after 60 min at 37°C. A parallel series of tubes allowed direct measurement of percent hemolysis at identical concentrations of A-III. The number of toxin molecules bound per cell at each toxin concentration could then be estimated by difference.

Direct binding of ^{125}I-labeled A-III to erythrocyte ghosts was measured using rapid filtration through glass-fiber filters (Whatman GF/C). Membranes suspended in 10 mM Tris-HCl (pH 7.4) containing 145 mM NaCl and 2 mM $CaCl_2$ (Tris-saline) were incubated for 20 min at 4°C in the presence of 1.2 μM pepstatin, 1 mM 1,10-phenanthroline, 1 mM iodoacetamide, and 0.1 mM PMSF prior to the addition of toxin. For measurement of A-III binding to proteinase-treated ghosts, membranes were pretreated for 60 min at 37°C with the desired enzyme and then mixed with proteinase inhibitors. For detergent-extracted ghosts, the membranes were first treated with inhibitors, then extracted with 0.5% Nonidet P-40 as described by Yu et al. [7,8].

Binding was initiated by addition of ^{125}I-labeled A-III to membrane suspensions and was terminated after the desired time at either 0 or 37°C by rapid filtration of duplicate aliquots of the reaction mixture. Filters were washed with three 5-ml portions of cold Tris-saline and counted to determine total A-III binding. Nonspecific binding to membranes was initially measured in the presence of 500 μg per ml of unlabeled toxin A-III; subsequently, it was noted that a like concentration of the homologous toxin A-IV provided equal protection; toxin A-IV was henceforward employed for measurement of nonspecific binding. All binding data are also corrected for binding of ^{125}I-labeled A-III to filters, both in the presence and absence of 500 μg per ml A-IV. Specific binding was determined as the difference between total and nonspecific.

Other methods. Toxin concentrations were routinely measured spectrophotometrically [1] and occasionally checked by amino acid analysis. The hemolytic potency of native or modified A-III was assessed using the hemolysis assay described elsewhere [1]. Analysis of the protein compositions of ghost and Nonidet P-40-treated ghost membranes was done using the SDS gel electrophoresis system described by Fairbanks et al. [9].

Results

Binding of ^{125}I-labeled A-III to ghosts

The results of typical binding experiments are shown in Fig. 1a and are transposed to a Scatchard plot in Fig. 1b. Over the A-III concentration range of 1–10 μg per ml, specific binding represents 85–90% of the total; at higher concentrations, nonspecific binding increases drastically, precluding a significant demonstration of saturation. From the slope and intercept of the Scatchard plot, a $K_{0.5}$ of 7.2 μg per ml (0.72 μM) and a binding site density of 41 000 molecules per cell equivalent of membranes are estimated. Binding data are in excellent agreement with a theoretical curve generated using these parameters (Fig. 1a), at the subsaturating concentrations used.

Binding of A-III was essentially undetectable when ghosts were not pretreated with proteinase inhibitors. This appears to be an effect on binding site stability rather than being due to protection of the toxin, as shown by the data in Table I. Iodinated A-III (5 μg per ml) was incubated at 37°C with either ghosts or ghosts pretreated with proteinase inhibitors. At various times, aliquots were precipitated with trichloroacetic acid and the supernatants and washed precipitates were counted. Essentially no conversion of labeled toxin to a trichloroacetic acid-soluble form was observed

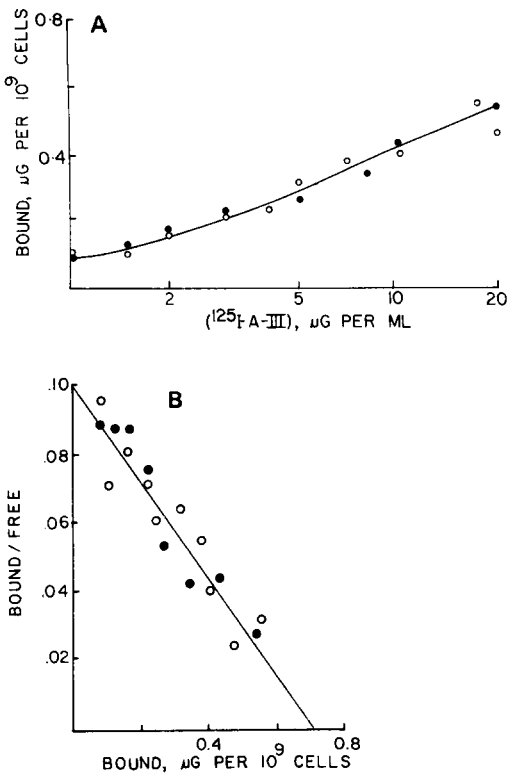

Fig. 1. (a) Specific binding of ^{125}I-labeled A-III to proteinase inhibitor-treated erythrocyte ghosts at either 0°C (○) or 37°C (●) was measured by rapid filtration after 20 min incubation in Tris-saline buffer (pH 7.4). Specific binding was determined as the difference between total binding and that in the presence of 500 μg per ml toxin A-IV. The solid line is a theoretical curve for a $K_{0.5}$ of 0.7 μM and a B_{max} of 41000 molecules per cell, or 0.7 μg per 10^9 cells. (b) Scatchard analysis of the equilibrium binding data presented in (a).

at any time up to 4 h. Analysis of the 4 h pellets by SDS-gel electrophoresis and autoradiography (not shown) revealed that all the counts migrated with a band of molecular weight 10000, identical to the known molecular weight of A-III. Since ^{125}I-labeled A-III is not degraded by ghosts, and because proteinase inhibitors are observed to enhance its binding, it is tentatively concluded that the A-III binding site is sensitive to an erythrocyte membrane proteinase.

In order to further characterize the nature of the A-III binding site, ghosts were pretreated with 5 μg per ml of either trypsin or elastase and their A-III binding capacity was measured as usual.

TABLE I

STABILITY OF A-III IN THE PRESENCE OF GHOSTS

Iodinated A-III (5 μg per ml) was incubated with ghosts at 37°C. At the indicated times, samples were removed and precipitated with 10% trichloroacetic acid (TCA) in the presence of 200 μg bovine serum albumin. Precipitates were washed twice with 10% trichloroacetic acid, and the initial supernatants and washed pellets were counted. Proteinase inhibitor ghosts were pre-incubated for 30 min in the presence of 0.1 mM PMSF/1.2 μM pepstatin/1 mM iodoacetamide/1 mM 1,10-phenanthroline.

Time (h)	% cpm TCA-precipitable	
	Ghosts	Proteinase-inhibitor ghosts
0	93.6	96.5
1	95.0	96.9
2	94.4	92.7
3	93.8	93.1
4	91.0	91.6

Surprisingly, neither proteinase caused a significant reduction in binding (Table II), suggesting that the site is either not a protein or that it is resistant to the soluble proteinases used.

Yu et al. [7,8] have shown that treatment of ghosts under controlled conditions with 0.5% Non-

TABLE II

A-III BINDING TO PROTEINASE-TREATED GHOSTS

Ghosts, equivalent to $1.08 \cdot 10^9$ human erythrocytes were treated for 60 min at 37°C with 2.5 μg of the indicated proteinase. Proteinase inhibitors were then added, and binding of ^{125}I-labeled A-III was measured as usual.

A-III (μg/ml)	A-IV (μg/ml)	Proteinase	cpm bound	Specific binding (μg per assay)
1.0	0	none	7918	0.11
1.0	500	none	2450	
1.0	0	trypsin	8315	0.12
1.0	500	trypsin	2543	
1.0	0	elastase	9062	0.12
1.0	500	elastase	3037	
5.0	0	none	24252	0.37
5.0	500	none	5860	
5.0	0	trypsin	23248	0.34
5.0	500	trypsin	6000	
5.0	0	elastase	25327	0.38
5.0	500	elastase	6200	

idet P-40 results in solubilization of band 3, PAS1-3, and the bulk of the membrane phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Gel analyses of our Nonidet P-40-treated ghosts are generally consistent with their observations except that in our hands, PAS-3 shows a slight preference for remaining membrane-associated (data not shown). Binding of A-III to Nonidet P-40-treated ghosts is absent at A-III concentrations less than 20 μg per ml (data not shown). It is interesting that PAS-2, present at about 70000 copies per cell [10] is, like the A-III site, both insensitive to external trypsin [10] and extracted from ghosts by 0.5% Nonidet P-40 [8].

Kinetics of binding

The kinetics of A-III binding to ghosts was measured at a cytolysin concentration of 2 μg per ml and 37°C. As shown in Fig. 2, binding is essentially complete within 2 min of mixing which is slightly faster than the time-course of hemolysis at this toxin concentration [1]. Bound toxin does not dissociate measurably within 60 min at 37°C (not shown). This is not unexpected, in view of the ability of A-III to insert across the bilayer under these conditions [4].

Binding of A-III to human erythrocytes

Native A-III (4.8 μg per ml) was mixed with varying numbers of human red blood cells, from 0 to $18.86 \cdot 10^9$ cells, incubated 60 min at 0°C, and cells and supernatants were separated by centrifugation. None of the supernatants showed greater than 2.5% hemolysis, despite the fact that the concentration of A-III used is known to cause greater than 75% hemolysis at 37°C. We have previously observed (Blumenthal, K.M., unpublished data) that A-III is an ineffective hemolysin at 0°C, even after incubations of 3–4 days.

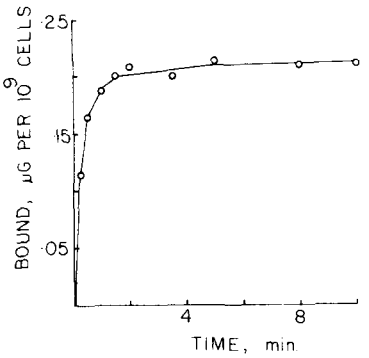

Fig. 2. Association of ^{125}I-labeled A-III to proteinase inhibitor-treated ghosts. ^{125}I-labeled A-III (2 μg per ml) was mixed with ghosts derived from $1.08 \cdot 10^9$ cells at zero time and 37°C. At the indicated times, aliquots were removed, filtered and washed and the filters were counted. Data presented represent association to specific binding sites, as defined in the legend to Fig. 1.

After centrifugation, three 1.0-ml aliquots of the supernatant were mixed with 1.0 ml each of a fresh 1% erythrocyte suspension. This dilution would give a toxin concentration of 2.4 μg per ml if no A-III were removed at 0°C. Following incubation for 60 min at 37°C, the percent hemolysis was measured as usual. As shown in Table III, incubation of 4.8 μg per ml A-III with $1.89 \cdot 10^{10}$ red cells at 0°C gives a supernatant having a A-III concentration of 1.95 μg per ml, as measured by reference to a standard hemolysis curve. Little or no removal of A-III is observed using smaller numbers of cells.

The amount of A-III removed during incubation at 0°C was calculated by subtracting the amount measured by hemolysis (6 ml at 1.95 μg per ml = 11.7 μg) from that initially added (4.8 μg per ml in 3 ml = 14.4 μg). Thus, 2.7 μg A-III have been bound by $1.89 \cdot 10^{10}$ erythrocytes; this amounts to 8625 molecules of A-III per cell.

TABLE III

BINDING TO INTACT ERYTHROCYTES

3.0-ml samples containing the indicated concentration of erythrocytes and 4.8 μg per ml A-III were incubated 60 min at 0°C and cells were removed by centrifugation. To each ml of supernatant was added 1.0 ml standard red cells and after 60 min at 37°C, % hemolysis was determined as usual. The concentration of A-III in the 0°C supernatant was then calculated from a dose-response curve constructed with an A-III solution of known concentration.

Cells ($\times 10^{-9}$)	% Hemolysis		A-III (μg per ml)
	0°C	37°C	
0	0	66.3	2.4
3.19	1.8	61.4	2.26
6.37	1.2	62.7	2.30
9.56	0.8	63.3	2.32
12.74	2.3	63.0	2.31
15.93	1.1	60.4	2.23
18.86	2.5	50.4	1.95

If it is assumed that A-III binding follows a Michaelis-Menten type isotherm, and that $K_{0.5}$ is about 7 μg per ml (as found in direct binding studies) then the maximal A-III site density in human erythrocytes can be calculated to be 33781 molecules per cell, in good agreement with data obtained in the ghost experiments.

Discussion

Binding of *Cerebratulus* toxin A-III to membranes of intact human erythrocytes or ghosts has been studied. For ease of comparison, binding data are reported in terms of molecules per cell, although obviously no cells are present in the ghost experiments; in these studies, cell number is based on the number of cells used to prepare the ghosts. It will be noted that the binding capacity of the ghosts appears to be slightly higher than that of intact cells (41000 vs. 34000). This discrepancy may be due to exposure of new sites during ghosting or may simply reflect losses of membrane during purification of the ghosts. Binding is characterized by a $K_{0.5}$ of 0.7 μM and shows a concentration-dependence similar to that for hemolysis, although complete hemolysis is observed at an A-III concentration giving only 50% saturation of binding. Binding occurs slightly faster than hemolysis, at a toxin concentration of 2 μg per ml, and bound toxin fails to dissociate within 60 min at 37°C. Jolivet-Reynaud and Alouf [14] have described similar results, i.e., rapid initial binding and little or no dissociation, in studies of *Clostridium perfringens* Δ-toxin binding to sheep erythrocytes. The increase in nonspecific binding at cytolysin levels greater than 10 μg per ml may be due to a weaker interaction of A-III with membrane phospholipid [5,13], possibly mediated by C-terminal helical sequences [2].

The binding site is tentatively a protein, although it is not destroyed upon treatment of ghosts with either trypsin or elastase. This conclusion is primarily based on the relatively low number of copies present per cell and the protection of binding by proteinase inhibitors under conditions where A-III itself is not digested (Table I). The absence of binding to Nonidet P-40 treated ghosts may also be suggestive of a proteinaceous binding site.

One other observation arising from these studies should be noted. We have consistently noted that detection of A-III binding to ghosts requires pretreatment of the membranes with the proteinase inhibitor cocktail described in Experimental procedures, suggesting the presence of an erythrocyte membrane proteinase. Pant et al. [11] have proposed the existence of such a proteinase in rat erythrocyte membranes based on a Ca^{2+}-induced proteolysis of spectrin and band 3 in rat ghosts. It is interesting that Posner and Kem [12] have reported that A-III induced depolarization of cardiac Purkinje fibers is inhibited by 50 mM Ca^{2+}. We have observed (Blumenthal, K.M., unpublished data) that A-III-dependent hemolysis is likewise inhibited by 50 mM Ca^{2+} and that this inhibition is abolished by pretreatment of red cells with our proteinase inhibitor cocktail. We therefore consider it unlikely that the inhibition observed by Posner and Kem represents a direct effect of Ca^{2+} on toxin A-III but suggests, rather, the presence of a Ca-activated proteinase in human erythrocyte membranes. This enzyme probably degrades the A-III binding site, since the toxin itself is not measurably degraded after 4 h in the presence of ghosts.

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References

1 Kem, W.R. and Blumenthal, K.M. (1978) J. Biol. Chem. 253, 5752–5757
2 Blumenthal, K.M. and Kem, W.R. (1980) J. Biol. Chem. 255, 8266–8272
3 Blumenthal, K.M. (1980) J. Biol. Chem. 255, 8273–8274
4 Blumenthal, K.M. (1982) Biochemistry 21, 4229–4233
5 Blumenthal, K.M. and Kreider, N.B. (1982) Fed. Proc. 41, 6553 Abstr.
6 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–180
7 Steck, T.K. and Yu, J. (1973) J. Supramol. Struct. 4, 220–232

8 Yu, J., Fishman, D.A. and Steck, T.K. (1973) J. Supramol. Struct. 4, 233–248
9 Fairbanks, G., Steck, T.K. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2616
10 Furthmayr, H. (1978) J. Supramol. Struct. 9, 79–95
11 Pant, C., Virmani, M. and Gallant, P.E. (1983) Biochem. Biophys. Res. Commun. 117, 372–377
12 Posner, P. and Kem, W.R. (1978) Toxicon 16, 343–349
13 Blumenthal, K.M. (1984) Biochem. Biophys. Res. Commun 121, 14–18
14 Jolivet-Reynaud, C. and Alouf, J.E. (1983) J. Biol. Chem 258, 1871–1877
15 Dumont, J.A. and Blumenthal, K.M. (1984) Arch. Biochem Biophys. 207, in the press