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Binding of a radiolabeled sea anemone cytolysin to erythrocyte membranes

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Stichodactyla helianthus **cytolysin III, a 17 kDa basic polypeptide isolated from a Caribbean sea anemone, is one of the most potent hemolysins yet found in a living organism. This toxin has been reported to form new ion channels in artificial lipid bilayer membranes. The ability of this toxin to attack cell membranes is greatly enhanced by the presence of sphingomyelin. In order to investigate the mechanism by which the cytolysin causes cell lysis, we have prepared a highly active [^{3}H]cytolysin derivative by reductive methylation with sodium cyanoborohydride and [^{3}H]formaldehyde. A dimethylated toxin derivative was used to investigate the basis for the differential lytic activity of this polypeptide upon erythrocytes from six mammalian species. Using both direct [^{3}H]toxin binding and indirect (Thron method) binding techniques, we found that the interspecies differences are due to variable membrane susceptibilities toward the bound toxin, rather than to differences in membrane affinity for the toxin. Similarly, we showed the enhanced lytic activity of the toxin for rat erythrocytes at elevated pH to be caused by enhanced activity of the bound toxin.**

Introduction

Bernheimer and Avigad [1] isolated an extremely potent hemolytic protein from a Caribbean sea anemone, *Stichodactyla helianthus*. Erythrocytes from a variety of mammalian species displayed considerable variation in susceptibility to this toxin. Two subsequent investigations of the action of this toxin upon artificial lipid bilayers showed that the toxin increases membrane permeability, apparently as a result of several toxin monomers aggregating to form a stable ion channel complex [2,3]. The ability of the toxin to lyse cells seemed dependent upon the presence of sphingomyelin, a neutral phospholipid, in the target cell membrane, since bacterial protoplasts and sphingomyelinase-treated erythrocytes were resistant to the toxin [1]. However, the ability of the toxin to act upon certain artificial bilayer membranes seemed less dependent upon the presence of sphingomyelin [2–4].

Recently, this protein cytolysin was further purified under conditions (4 M urea) inhibiting aggregation of toxin monomers; two major and two minor variants of the toxin were thus resolved [5]. Cytolysin III, the most abundant and potent variant, has been sequenced [6]. The partial amino acid sequence [7] of equinatoxin, another sea anemone cytolysin, was found to be very similar to the sequence for cytolysin III. Additional cytolysins with similar molecular sizes, amino acid compositions, basicities, and sphingomyelin preferences have now been found in a number of sea anemone species representing several taxonomic families [8–12].

In order to investigate the mechanism of cell disruption and lysis caused by this type of toxin, it was highly desirable to obtain a radiolabeled, biologically-active toxin derivative which could be used to directly measure toxin binding to membranes. In this paper, we report the preparation and characterization of an active [^{3}H]toxin derivative and utilize it to provide new insight regarding: (a) the differential susceptibilities of mammalian erythrocytes to the toxin, and (b)the pH dependence of toxin-mediated lysis of rat erythrocytes.

Materials and Methods

Chemicals

Cytolysin III was purified by Sephadex G-50 gel chromatography and Whatman CM52 cellulose ion exchange chromatography in the presence of 4 M urea, as previously reported [5]. [^{3}H]Formaldehyde, 100 mCi/mmol, was purchased from New England Nuclear Corporation while sodium cyanoborohydride was purchased from Sigma Chemical Company. All other chemicals were at least reagent grade.

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Reductive alkylation of cytolysin III

The toxin was dissolved in 0.05 M sodium phosphate (pH 7.0) and incubated with 2 moles of [^{3}H]formaldehyde and 4 moles of sodium cyanoborohydride per toxin amino group for 1 h at 22°C. The resulting products were separated on a CM52 cellulose column developed with an ammonium acetate gradient (Fig. 1). Each 2-ml eluate fraction was assessed for radioactivity (by liquid scintillation), hemolytic activity, conductivity, and protein concentration (by measuring toxin fluorescence at 340 nm upon 280 nm excitation). Fractions containing the same toxin peak were pooled, freeze-dried, and stored at −20°C.

Preparation of erythrocyte suspensions

Blood obtained from male Sprague-Dawley rats either by cardiac puncture or by decapitation was immediately diluted 20-fold in ice-cold, calcium-free saline (148 mM NaCl, 10 mM Tris-HCl, pH 7.4). The cells were then centrifuged three times at 3000 × *g* for 10 minutes, each time resuspending the cells in standard saline (145 mM NaCl, 2 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.4). It was important to thoroughly remove plasma proteins, since the toxin avidly binds to lipoproteins. Blood from the other species was obtained by venipuncture using heparin as anticoagulant. The erythrocytes were separated from plasma proteins and heparin by washing at least four times with the standard saline, as described above. The concentration of erythrocytes was routinely standardized by preparing erythrocyte suspensions which would yield a specified 540 nm absorbance when completely lysed with Triton X-100 (0.1% v/v). A suspension yielding an absorbance of 1.0 corresponded approximately to a 1% suspension of blood. The actual cell concentration of these erythrocyte suspensions was later determined with a Coulter counter.

Hemolysis assays

The percent of cells undergoing lysis was determined after incubating a 2.0 ml suspension (540 nm absorbance = 1) of erythrocytes with the desired toxin concentration for 1 h at 37°C [13]. Immediately after adding the toxin, the suspension was vortexed in order to achieve good equilibration. The tubes were vortexed again briefly at 30 and 60 min. They were then pelleted by centrifugation at 3000 × *g* for 10 min and the 540 nm absorbance of the supernatant was determined. Spontaneous lysis in the absence of toxin as well as 100% lysis in the presence of 0.1% Triton X-100 was measured. All measurements were done in triplicate. Percent hemolysis (% H) was calculated as follows:

$$\%\ H = \frac{A_{\text{toxin}} - A_{\text{notoxin}}}{A_{\text{T-X-100}} - A_{\text{notoxin}}} \times 100$$

Indirect method for measuring toxin binding

The affinity and efficacy (ability of bound toxin to lyse the cell) of the toxin's interaction with the erythrocyte membrane were assessed using an equation developed by Thron [14]:

$$(\text{Toxin})_{\text{Total,50\% Lysis}} = a_{50\%} N + (\text{Toxin})_{\text{Free,50\% Lysis}}$$

The term on the left is the median hemolytic concentration (HC_{50}); $a_{50\%}$ is the amount (ng) of toxin bound per cell at 50% lysis, N is the number of cells per ml, and $(\text{Toxin})_{\text{Free,50\%}}$ is the free concentration of toxin at 50% lysis. By plotting the HC_{50} as a function of erythrocyte concentration, the free toxin concentration at 50% lysis can be obtained from the *y*-intercept. The efficacy of bound toxin in causing the lytic process will be inversely proportional to the slope, the number of toxin molecules required to produce 50% lysis. The affinity of the toxin for the membrane is directly proportional to the ratio, $a_{50\%}/(\text{Toxin})_{\text{Free,50\%}}$.

Binding of ^{3}H-labeled Stichodactyla helianthus cytolysin III to erythrocytes

An $A_{540} = 1$ suspension of rat erythrocytes, washed free of plasma constituents three times, was incubated with various concentrations of [^{3}H]toxin for 1 h at 37°C in the standard saline. Each suspension was then filtered with an Amicon Vacuum Manifold. The sample was placed on a Gelman glass fiber prefilter (25 mm diameter) which covered a Whatman glass microfiber filter; this reduced lysis of the cells. Both filters were placed in a scintillation vial and counted with a Beckmann LS7000 counter which corrected for quenching caused by hemoglobin. Each assay was done in triplicate. The number of toxin molecules required to lyse 50% of the cells was calculated by multiplying the total bound radioactivity (dpms) by the specific radioactivity of the toxin (dpms per mole) and then dividing by the number of erythrocytes per ml, as determined with a Coulter counter. The 540 nm absorbance of the filtrate was measured to determine the percent of cells lysed, as described above.

Results

Radiolabeling of toxin

Several preliminary experiments were carried out to determine suitable conditions for the reductive alkylation of cytolysis III, without destroying its lytic activity. Increasing the reactant concentrations or the reaction time caused more extensive labeling, but this was accompanied by loss of activity. Four protein peaks could be resolved by CM cellulose chromatography (Fig. 1). Although peaks III and IV possessed the lowest specific radioactivities, their lytic activities were similar to that of the native toxin (Table I). We only used peak III [^{3}H]toxin for the binding measurements presented in

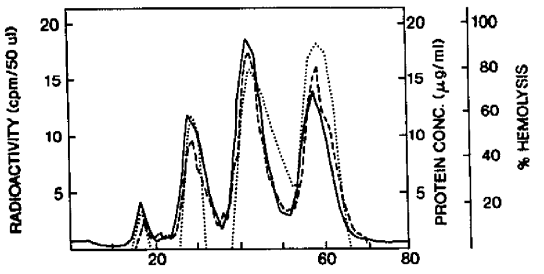

Fig. 1. Carboxymethylcellulose column separation of four [^{3}H]cytolysin derivatives resulting from reductive alkylation with [^{3}H]formaldehyde. Each fraction was assayed for radioactivity (———) protein concentration (— — —), and hemolytic activity at a 400-fold dilution (·····).

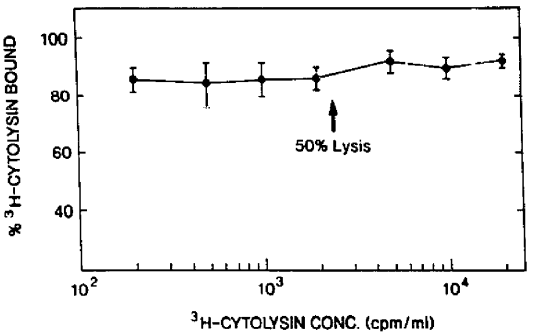

Fig. 2. Relationship between [^{3}H]cytolysin binding to rat erythrocytes and total [cytolysin]. Labeled toxin was added to rat erythrocytes (A_{540} = 2) and after 1 h at 37°C the percent of toxin bound to the cells determined. In this experiment, the concentration of toxin which produced 50% lysis was 51 ng/ml. Each point is the mean value of four estimates ± the standard error.

this paper, since it retained the most toxicity. The isoelectric points (9.8) of the peak III and IV toxin derivatives were indistinguishable from that of the native toxin.

Binding of the labeled toxin to erythrocytes

In a recent review (Ref. 15, Fig. 1), preliminary data was presented, showing that the median hemolytic concentration (HC_{50} of this cytolysin was linearly proportional to the red cell concentration. In addition, the *y*-intercept (a measure of free toxin concentration at 50% hemolysis) was in all cases not significantly different from zero, which indicated that only a small fraction of the toxin molecules initially added to the tube remained in free solution at the time of the hemolysis. Feline erythrocytes, the most sensitive cells, were about 32-times more sensitive to the toxin than were rat erythrocytes, the least sensitive cells. Since only the slopes differed significantly among the erythrocyte species, the observed differences in toxin potency must be due to differences in the ability of the membrane-bound toxin to exert its action in these erythrocytes. From the Thron equation, the number of toxin molecules bound per cell at 50% hemolysis could be calculated, assuming $3.54 \cdot 10^{10}$ molecules of toxin per nanogram (mol. wt. 16968, Ref. 6). The number of cells per ml (N) was estimated with a Coulter counter. Considering the extremes in erythrocyte susceptibility, approx. 700 toxin molecules were bound per cat erythrocyte at 50% lysis, compared with 22000 molecules for the rat erythrocyte (Table II).

The binding of [^{3}H]cytolysin III was also investigated upon the same erythrocytes at cell concentrations equivalent to approximately 1% of whole blood (Table II). Analysis of the concentration dependence of [^{3}H]toxin binding to rat erythrocytes indicated that the toxin bound was relatively constant over the entire concentration range investigated (Fig. 2), which included concentrations below as well as above the median hemolytic concentration. Due to the previously established detergent property of higher concentrations [16] of this toxin, we did not attempt to determine whether the addition of an excess concentration of unlabeled toxin would reduce binding of the labeled toxin.

The number of molecules required for lysing a particular erythrocyte as determined by [^{3}H]toxin binding, is also presented in Table II. There was good agreement with the results obtained by the Thron method.

TABLE I

Characteristics of the separated [^{3}H]cytolysin III fractions resulting from reductive alylation with [^{3}H]formaldehyde

Peak Number	Specific radioactivity (Ci/mmol)	CH_3 per molecule	Percent of total [a]	Hemolytic activity [b]
I	765	7.6	4	25
II	386	3.8	16	38
III	247	2.4	40	90
IV	217	2.1	40	65

[a] Percent of total labeled toxin.

[b] Hemolytic activity of the product relative to an equal amount of unlabeled toxin.

pH dependence of hemolysis and toxin binding

The hemolytic activity of the toxin was quite dependent upon the extracellular pH. In Fig. 3A, it was observed that the HC_{50} progressively decreased as the pH increased. This dependence was more thoroughly investigated at each pH, using several erythrocyte concentrations. As observed in Fig. 3B, the slope (an inverse measure of efficacy) decreased as the pH increased. At all pH values tested, the *y*-intercept HC_{50} was indistinguishable from zero, indicating that an effect of pH upon toxin binding was unlikely to be responsible for the observed pH dependence.

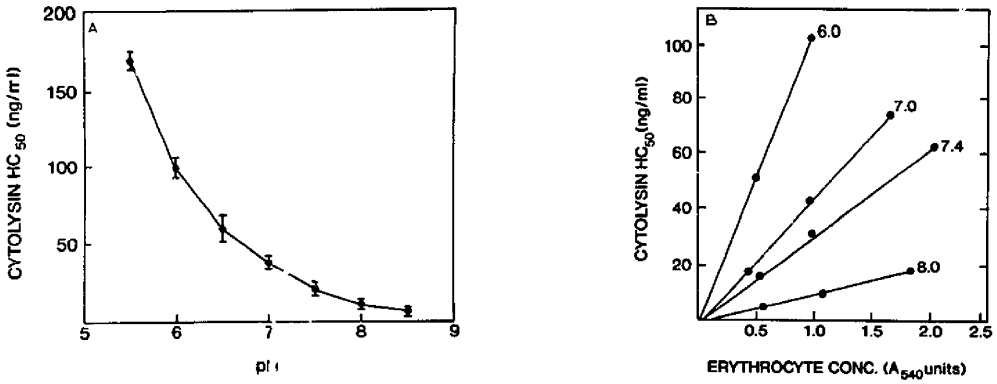

Fig. 3. Dependence of the median hemolytic concentration upon external pH. (A) The effect of pH was measured using an erythrocyte concentration which yielded a 540 nm absorbance of 1.0 when completely lysed. Each point is the mean value for three tubes ± the standard error. (B) The HC_{50} is plotted as a function of erythrocyte concentration at each pH tested. Each point is the average of two estimates.

TABLE II

Relative susceptibilities of mammalian erythrocytes to Stichodactyla helianthus cytolysin III

Species	[Sphingomyelin] [a] (% of total)	HC_{50} [b] (ng/ml)	$MBPC_{50}$ [c]		Ratio $\frac{MBPC_{50}}{HC_{50}}$
			[^{3}H]toxin	Thron Method	
Rat	12.8	26.8	18200	21562	742
Horse	13.5	14.7	9821	10190	680
Human	25.8	9.8	7316	9163	841
Cat	26.1	1.2	680	685	569
Goat	45.9	10.6	7896	10337	860
Sheep	51.0	6.9	4686	4730	683

[a] Expressed as of total lipid (Ref. 20).

[b] Median hemolytic concentrations for cytolysin III when incubated with red cell suspensions which, when completely lysed, produced a 540 nm absorbance of 1.0. The data in this column were obtained from the experimental data shown in Fig. 1 of Ref. 15.

[b] $MBPC_{50}$ = molecules bound per cell at 50% lysis.

Fig. 4 demonstrates that toxin binding is essentially independent of pH over the range of 6–8. In Fig. 5, it is shown that smaller numbers of toxin molecules are required per cell for lysis at the higher pH values.

Discussion

Radiolabeling of cytolysin III

Initially we attempted to iodinate the toxin using three different methods (lactoperoxidase, Iodogen, and chloramine T). However, iodination always caused considerable loss of toxicity. Cytolysin III possesses 11 tyrosines, so it is presumed that iodination of one or more phenolic groups inactivates the toxin. Subsequently we turned to the milder, reductive alkylation procedure. Methylation only alters the pK_a of the reacted amino group to a small degree, usually less than

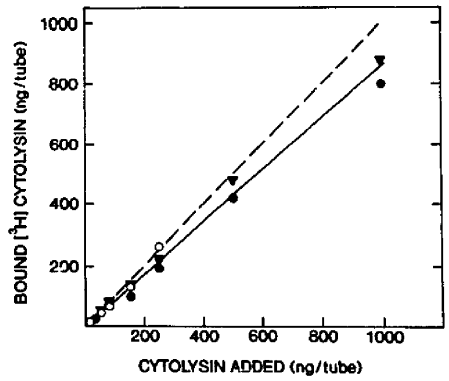

Fig. 4. Relationship between initial [^{3}H]cytolysin concentration, expressed as ng/2 ml tube, and erythrocyte binding of the toxin (pH 6.0, ▼; pH 7.0, ●; pH 8.0, ○). The dashed line indicates the expected relation, assuming 100% binding of the tritiated cytolysin by the erythrocytes. The solid line is the observed relation. Each point is the mean estimate for triplicate samples.

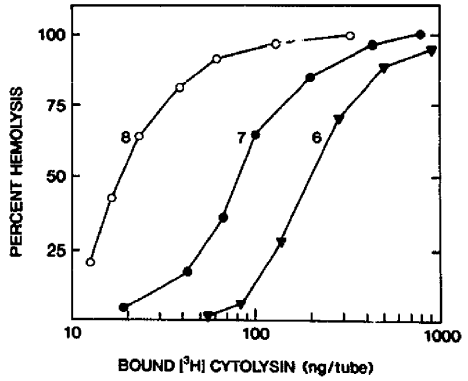

Fig. 5. Relationship between ^{3}H-labeled *Stichodactyla* cytolysin III binding to rat erythrocytes and hemolysis (pH 6.0, ▼; pH 7.0, ●; pH 8.0, ○). The data in this figure are from the same experiment as those shown in Fig. 4. Each point is the mean value for triplicate tubes. One nanogram of bound toxin corresponds to 49 cpm per tube.

0.5 pH unit [17]. Assuming that the N-terminal amino acid α-amino group has a normal pK_a, one would expect that this group would be more reactive than most ε-amino groups at pH 7.0. We have not yet identified which amino groups are modified, but using HPLC, we have found that radioactivity is primarily associated with one tryptic peptide (Kem and Doyle, unpublished results).

The two [^{3}H]cytolysin derivatives containing two methyls per molecule showed only a minor loss of hemolytic activity, whereas the first two ion exchange peaks showing greater [^{3}H]methyl incorporation were clearly less toxic. Since a wide variety of carbonyl-containing groups can be added to amino groups by reductive alkylation, it should be possible, in the future, to investigate the roles of the most reactive amino groups of this toxin using this chemical modification reaction.

Binding of the toxin to erythrocytes

Stichodactyla helianthus cytolysin III very quickly (within several seconds) reaches binding equilibrium with the erythrocyte membrane (Kem and Doyle, in preparation). In this paper, we are only considering the steady-state binding properties of this toxin. Over the concentration range we investigated, toxin binding was always proportional to the initial concentration of toxin added (Figs. 2 and 4). From these figures and Table II, it can be seen that the toxin partitioned in a relatively constant ratio between the membrane and aqueous phases, as if no specific membrane acceptor site was present. An alternate explanation is that such sites exist but are present in great excess. Bernheimer and colleagues [1,4] have reported considerable evidence for specific interaction between the neutral phospholipid sphingomyelin and the toxin. All of the mammalian erythrocytes we studied possess considerable membrane concentrations of sphingomyelin [18]. Even in the rat erythrocyte, which contained the lowest relative fraction of sphingomyelin, the number of sphingomyelin molecules in the outer half of the membrane exceeds by at least three orders of magnitude the number of toxin molecules bound at 50% lysis. Thus the linear binding relation for the cytolysin cannot be considered as evidence against sphingomyelin (or some other common membrane molecules) serving as an acceptor site, for the toxin.

In a recent monolayer study, we found that *Stichodactyla helianthus* cytolysin III had a high affinity for nonpolar interfaces [19]. The toxin displayed no selectivity for sphingomyelin relative to dipalmitoylphosphatidylcholine monolayers, suggesting that it initially binds to membranes in a nonselective fashion dependent upon their hydrophobic character. Consistent with this interpretation was our observation (results not shown) that the binding of the [^{3}H]cytolysin to rat erythrocytes is essentially irreversible.

Relationship between hemolysis and binding

Although toxin binding was proportional to the initial toxin concentration, hemolysis varied in a sigmoidal fashion with toxin concentration [5]. The sigmoidal shape indicates that there is a threshold (critical) number of toxin molecules which must interact with the cell to cause lysis. Another factor probably contributing to the sigmoidal shape is variation in erythrocyte sensitivity to lysis. This is not surprising, as previous investigations of erythrocyte lysis have shown that variations in susceptibility to lysis exist among cells that are primarily related to their age.

That the toxin HC_{50} was always directly proportional to the red cell concentration also indicates that a critical number of toxin molecules must be bound to produce lysis. Even in the most sensitive type (cat) of erythrocyte, about 700 molecules of toxin were required to cause cell lysis. This is somewhat surprising since data from the artificial bilayer studies predict that the ion conductance generated by formation of a single toxin ion channel complex should be sufficient to cause an electrolyte imbalance sufficient to cause lysis of the erythrocyte. Thus the probability of forming the toxin ion channel complex must be quite small in erythrocyte membranes. Further investigation of the relative susceptibility of erythrocytes to the toxin is desirable, using cells or cell-derived vesicles made deficient in certain membrane lipids and proteins. In this manner, it may be possible to assess the possible modulatory influence of such constituents. For instance, it is conceivable that the toxin has a high affinity for certain membrane proteins, but when bound to them is unable to form an ion channel.

The indirect (Thron) method for measuring toxin binding to lysing cells yielded results which were quite consistent with the [^{3}H]toxin direct binding results. One advantage of the Thron approach is that it doesn't require radiolabeled toxin. However, it cannot be used to measure specific ligand binding. Nevertheless, it should be quite useful for investigating the binding of other cytolysins to biological membranes.

pH dependence of toxin action

Hydrogen ions could affect the ability of the cytolysin to lyse the cells in a number of ways by changing ionization-related properties of either the toxin or the target cell membrane. Further investigation is required to acquire a physical understanding for this pH effect upon the toxins action. However, both binding methods have clearly established that the observed pH dependence of lysis is related to a change in the ability of the membrane-bound toxin to cause cell damage. Since Varanda and Finkelstein [3] showed that the conductance and cation selectivity of the cytolysin channel is markedly enhanced as the pH is raised in the same range, it seems probable that much of the pH effect we

have observed is due to this phenomenon. Normal mammalian erythrocytes have a relatively high anion permeability but small cation permeability [9]. The toxin would be less likely to generate an electrolyte imbalance as long as it only slightly enhanced erythrocyte anion permeability; on the other hand, a nonselective increase in cation permeability would allow sodium and calcium ions to freely enter the cell at rates which could not be counteracted by their respective active transport systems. This would result in colloid osmotic lysis. In a future paper, we will present electrolyte flux and other data consistent with this interpretation (Kem and Doyle, in preparation).

The process of ion channel formation by toxin monomer aggregation within the cell membrane may also be affected by external pH. Further analysis of this aggregation phenomenon will depend upon development of suitable techniques for specifically measuring this process.

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