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Inhibitory Effect of Flavin Mononucleotide on the Hemolysis of Rabbit Erythrocytes by Staphylococcal Alpha-Toxin

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Flavin mononucleotide diminished the hemolytic action of staphylococcal alpha-toxin on rabbit erythrocytes by competitive inhibition, probably by its interaction with the alpha-toxin binding site on the cell membrane.

In our previous papers (6, 7), alpha-toxin from the Wood-46 strain of *Staphylococcus aureus* was isolated in a crystalline form, and the physicochemical properties and biological activities were described. Among the many biological activities, alpha-toxin has a lytic effect on rabbit erythrocytes and artificial membranes (1, 2). Although the specific mechanism of the action of alpha-toxin is still unknown, Cassidy et al. (4), using [^{125}I]alpha-toxin and liposomes, have recently suggested that specific receptor substances for alpha-toxin may exist in rabbit erythrocyte membranes. In this study we present data indicating that the inhibition of alpha-toxin hemolysis by flavin mononucleotide (FMN) is due to competition with staphylococcal alpha-toxin for a site on the rabbit erythrocyte membranes.

The crystalline staphylococcal alpha-toxin was obtained from the procedures of the previous paper (6, 7). [^{125}I]alpha-toxin was prepared by the method of Kato and Watanuki (5). Iodinated toxin retained 82% of its original hemolytic activity for rabbit erythrocytes as assayed by the method of Bernheimer and Schwartz (3).

Table 1 shows the relative sensitivity of enzyme-treated and normal erythrocytes to hemolysis by alpha-toxin. Pronase-treated erythrocytes (15% protein, 10% sugar components released) were more resistant to the hemolytic activity than those treated with amino sugar-splitting enzymes. The binding of [^{125}I]toxin to the Pronase-treated cells was greatly decreased to less than 3% of that of nontreated, normal cells. Neuramidase treatment (10% sialic acid released) caused no alteration in the sensitivity to hemolysis by the alpha-toxin or the extent of binding of [^{125}I]toxin (Table 1). A comparison of the concentrations needed for 50% hemolysis inhibition shows that FMN is an order of magnitude more active than riboflavin or flavin adenine dinucleotide. Riboflavin is at least an order of magnitude more active than the phospholipids lecithin, phosphorylcholine, and phosphatidyl ethanolamine (Table 2).

The effect of FMN upon alpha-toxin hemolysis was studied further to ascertain the nature of the inhibition. Reciprocal plots of hemolysis against alpha-toxin concentrations (Fig. 1) suggested that the inhibition of the initial rate of

TABLE 1. *Relative sensitivity of enzyme-treated and normal rabbit erythrocytes to hemolysis by alpha-toxin*

Enzyme treatment[a]	Sensitivity to hemolysis relative to normal cells[b]	[^{125}I]toxin bound (counts/min)[c]	Percent [^{14}C]riboflavin bound relative to normal cells[d]
None	100	376	100
Pronase (1 mg/ml)	3	9	31.7
Trypsin (0.3 mg/ml)	20	64	42.3
Subtilin (0.5 mg/ml)	20	66	43.4
Bromelin (5 mg/ml)	50	210	66.6
Ficin (5 mg/ml)	53	240	68.9
β-Galactosidase (2 U/ml)	62	298	79.1
β-Glucosidase (2 U/ml)	61	294	78.8
Neuramidase (0.1 μg/ml)	91	372	96.6

[a] Rabbit erythrocytes (2%) were incubated with each enzyme for 60 min at 37 C. The washed erythrocytes (6 × 10^8 cells/ml) were suspended in 0.05 M phosphate-buffered saline solution (pH 7.0).

[b] Hemolytic assays were performed by the method of Bernheimer (3). Serially diluted alpha-toxin was mixed with 1 ml of the cells and incubated at 28 C for 30 min.

[c] The suspended erythrocytes (6 × 10^8 cells/ml) were incubated with 1 μg of [^{125}I]toxin (5,000 counts/min) at 28 C for 5 min. The counting technique was carried out by the previous procedure (5).

[d] The suspended erythrocytes (6 × 10^8 cells/ml) were incubated with 2 μCi of [^{14}C]riboflavin per ml (28.8 mCi/mmol) at 28 C for 30 min in a dark room.

TABLE 2. *Effect of flavin mononucleotide and various inhibitors on the hemolysis of rabbit erythrocytes by alpha-toxin*

Compound tested[a]	Concn required for half-maximal inhibition[b]	[^{125}I]toxin bound to cells (counts/min)[c]
None		380
FMN	10^{-7} M	76
Riboflavin	2×10^{-6} M	93
FAD	8.5×10^{-6} M	108
Alloxazine	10^{-3} M	246
Hydroquinone	10^{-3} M	281
Lecithin	10^{-5} M	229
Phosphorylcholine	7×10^{-4} M	204
Phosphatidyl ethanolamine	5×10^{-4} M	192
β-Cholesterol, sphingosine, stearic acid, androsterone, ergosterol and stigmasterol	10^{-3} to 3×10^{-3} M	
cAMP	5×10^{-5} M	266
Dibutyryl cAMP	6.5×10^{-5} M	278
ATP, poly(A), GTP, cGMP, cIMP, and NAD	10^{-3} to 3×10^{-3} M	
N-acetylgalactosamine	2×10^{-2} M	341
N-acetylglucosamine	2×10^{-2} M	339
Chondroitin sulfate A	6×10^{-2} M	
Heparin	2×10^{-2} M	

[a] Abbreviations: FAD, flavin adenine dinucleotide; cAMP, cyclic adenosine 5′-monophosphate; poly(A), polyadenylic acid; GTP, guanosine 5′-triphosphate; cGMP, cyclic guanosine 5′-monophosphate; cIMP, cyclic inosine 5′-monophosphate; NAD, nicotinamide adenine dinucleotide.

[b] Rabbit erythrocytes (2%) were incubated with each compound for 30 min at 37 C. The washed and suspended erythrocytes (8×10^8 cells/ml) were incubated with 3 hemolytic units of alpha-toxin per ml at 28 C for 39 min. The values were obtained from inhibition curves, all showing hyperbolic shapes.

[c] The washed and suspended cells were incubated with 1 μg of [^{125}I]toxin (5,000 counts/min) at 28 C for 5 min. Values represent total counts per 0.9 ml of washed, resuspended erythrocytes.

the hemolysis by FMN was competitive in nature. If FMN (10^{-7} M) was preincubated with 2% rabbit erythrocytes for 30 min at 37 C, the binding of [^{125}I]toxin to the washed erythrocytes was greatly inhibited (Table 2). Furthermore, the binding of [^{14}C]riboflavin instead of labeled FMN, which was not available, to the Pronase-treated erythrocytes was decreased to about 32% of that of the nontreated cells (Table 1). In the preliminary experiments, the released glycoproteins from the Pronase-treated erythrocytes inhibited alpha-toxin hemolysis when preincubated with the toxin and were also capable of binding to [^{14}C]riboflavin preferentially.

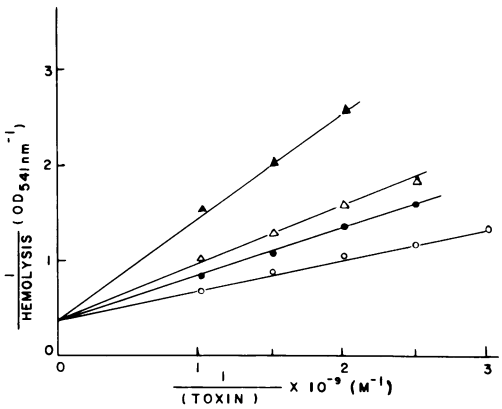

FIG. 1. *Competitive inhibition of hemolysis by staphylococcal alpha-toxin by FMN. Hemolytic assays were performed by the method of Bernheimer (3) utilizing crystallized alpha-toxin having a molecular weight of 3.6 × 10⁴ and one hemolytic unit per 0.019 μg. Serially diluted alpha-toxin was mixed with various concentrations of FMN. An equal volume of 2% rabbit erythrocytes was added and incubated at 28 C for 30 min. Concentrations of FMN: 0.1 μM (●), 0.5 μM (△), 1 μM (▲); and absence of FMN (○).*

Since [^{14}C]riboflavin does not bind the alpha-toxin and the alpha-toxin treated with FMN does not change the hemolytic activity, the mechanism proposed for the inhibitory effect of FMN on the hemolytic activity of the staphylococcal alpha-toxin is the interaction of FMN with the specific glycoprotein in the erythrocyte cell membrane which is the binding site for the alpha-toxin.

LITERATURE CITED

1. Arbuthnott, J. P. 1970. Staphylococcal alpha-toxin, p. 189-236. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
2. Bernheimer, A. W. 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27-50.
3. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal alpha-toxin. J. Gen. Microbiol. **30**:455-468.
4. Cassidy, P., H. R. Six, and S. Harshman. 1974. Biological properties of staphylococcal alpha-toxin. Biochim. Biophys. Acta **332**:413-423.
5. Kato, I., and A. Watanuki. 1970. Effect of diphtheria toxin on lysosome activity of leukocytes and Ehrlich ascites tumor cells. Jpn. J. Exp. Med. **40**:87-100.
6. Watanabe, M., and I. Kato. 1974. Purification and some properties of staphylococcal alpha-toxin. Jpn. J. Exp. Med. **44**:165-178.
7. Watanabe, M., and I. Kato. 1975. Chemical structure and biochemical action of staphylococcal alpha-toxin. Toxicon **13**:129-130.