INFECTION AND IMMUNITY, Jan. 1980, p. 97-101
0019-9567/80/01-0097/05$02.00/0

Vol. 27, No. 1

Binding of Cholesterol by Sulfhydryl-Activated Cytolysins

MARY K. JOHNSON,[1]* CHRISTIANE GEOFFROY,[2] AND JOSEPH E. ALOUF[2]

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112,[1] and Unité des Antigènes Bactériens (ERA CNRS 794), Institut Pasteur, 75724 Paris Cedex 15, France[2]

The binding of cholesterol by pneumolysin, alveolysin, and streptolysin O has been demonstrated. The properties of the cytolysin-cholesterol interaction parallel those of cytolysin-erythrocyte interaction in that the reaction is rapid, temperature independent, decreased at elevated pH, and shows the same specificity with respect to other related sterols. However, oxidized or *p*-hydroxymercuribenzoate-treated toxin showed no decrease in cholesterol-binding activity, whereas the ability of cytolysin to bind to erythrocytes was modified by such treatment.

One of the several properties common to that group of cytolytic agents known as the sulfhydryl (or thiol)-activated toxins is sensitivity to inhibition by cholesterol and certain related sterols (see review by Bernheimer, 4). These agents act only on cells containing cholesterol (3), and it has been shown with one member of the group, cereolysin, that the interaction of toxin with liposomes depends on their cholesterol content (6). The work cited above supports the hypothesis that target cell membrane cholesterol is the receptor for the sulfhydryl-activated toxins. Although several reports of in vitro interactions between purified sulfhydryl-activated toxins and sterol dispersions have appeared (7, 18, 21), there has been no direct demonstration of the binding of pure toxin with cholesterol. In the present communication we present evidence for such a binding and describe some properties of the system.

MATERIALS AND METHODS

Reagents. Cholesterol (cholest-5-en-3β-ol) was obtained from Sigma Chemical Co. and from Steraloids, Ltd, [^{3}H]cholesterol with a specific activity of 50 Ci/mmol from New England Nuclear Corp. and the Commissariat de l'Energie Atomique (France), epicholesterol (cholest-5-en-3α-ol) and 7-dehydrocholesterol (cholsta-5,7-dien-3β-ol) from Schwartz/Mann; bovine serum albumin from Pentex Biochemical; cytochrome *c* from Mann Research Laboratories; *p*-hydroxymercuribenzoate (pHMB) from Sigma Chemical Co.; oxidized dithiothreitol (DTT) from Calbiochem; and reduced DTT from Koch Light Laboratories.

Purification of cytolysins. Pneumolysin was purified and titrated as described previously (11, 12) except that concentration of column eluates was accomplished by the use of the Amicon Corp. Stirred Cell with a PM-30 membrane. The specific activity was 10^6 hemolytic units (HU) per milligram of protein. Streptolysin O was purified on activated thiol Sepharose 4B as described previously (19) and had a specific activity of 2×10^5 HU/mg. Alveolysin was purified by the same technique from cultures grown as described previously (1) and had a specific activity of 6×10^5 HU/mg.

Cholesterol binding assay. Unless otherwise stated, the binding of cholesterol was measured as follows. To 0.5 ml of sodium phosphate buffer (0.1 M, pH 7.4) contained in a polystyrene tube (12 by 75 mm) were added 10 μl of cholesterol dissolved in propylene glycol/dioxane (2:1), containing 0.5 μg of unlabeled and 0.5 μCi of [^{3}H]cholesterol, and from 2 to 10 μl of cytolysin appropriately diluted in phosphate-buffered saline (0.075 M sodium phosphate with 0.075 M NaCl at pH 6.8). The solution was blended in a Vortex mixer after each addition. At this concentration, cholesterol is soluble, but self-associates to form micelles (10). Approximately 15% of the cholesterol was retained on the walls of the reaction tube. (In experiments performed at submicellar concentrations, the radioactive cholesterol was used without the addition of unlabeled cholesterol; 5 μl of [^{3}H]cholesterol in ethanol containing 0.5 μCi and 3.86 ng of cholesterol was added to the 0.5-ml reaction mixture). The solution was incubated 2 min in an ice bath and then 100 μl was transferred with a Pipetman to a column of Sephadex G-25 (1 ml bed volume contained in a Pasteur pipette) at room temperature. The top of the pipette was broken off to allow addition of the sample directly to the gel bed, thus minimizing contact of the cholesterol solution with the walls of the pipette. The cytolysin-bound cholesterol was eluted with 1 ml of phosphate-buffered saline, and the eluate was collected in a scintillation vial. Two samples of 100 μl of the eluate were transferred to other vials and counted in an Intertechnique liquid scintillation counter after the addition of 10 ml of scintillation cocktail (Instagel, Packard Instrument Co., Inc.). The values were corrected for the small amount of radioactivity eluted in the absence of cytolysin. The unbound cholesterol remaining in the column could be eluted with 2.5 ml of 1% Triton X-100 in water, yielding a total recovery of 98 to 101% added radioactivity. All assays were performed in duplicate, and each experiment was repeated at least two times.

Oxidation and reduction of pneumolysin. Pneumolysin was oxidized and then reduced with

DTT, by using a modification of the method of Creighton (8). Eighteen micrograms of cytolysin was added to 360 μl of tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.1 M, pH 8.7) containing 0.015 M oxidized DTT (or in the same buffer without DTT in the case of the control). After 90 min of incubation at 37°C, the test and control preparations were titrated, and duplicate 40-μl samples were used for determination of cholesterol binding activity. For reduction of the oxidized pneumolysin, 130 μl of the above solution was added to 200 μl of tris(hydoxymethyl)aminomethane-hydrochloride buffer (0.2 M, pH 8.7) containing 0.1 M reduced DTT. The preparation was titrated at the end of 30 min of incubation at room temperature.

Treatment of pneumolysin with pHMB. Fifty microliters of lysin (16.5 μg) was preincubated with 20 μl of 0.01 M glycyl-glycine buffer with or without 0.03 M pHMB for 5 min at room temperature, after which 7 μl was withdrawn for use in the standard binding assay.

Gel filtration on Sephacryl. A 0.5-ml reaction mixture prepared as described for the cholesterol binding assay was added to a column of Sephacryl-200 superfine (20 by 0.9 cm) at 4°C or at room temperature and eluted with phosphate-buffered saline. Sephadex G-200 was employed in a similar manner. The cholesterol remaining on the column was eluted with Triton X-100, and the columns were reused after thorough rinsing.

Protein determination. The protein content of concentrated cytolysin solutions was determined by the method of Lowry et al. (17) and that of column eluates was determined by the method of Schaffner and Weissman (20), with amido black.

RESULTS

The results presented in Fig. 1 show the pattern of elution of cholesterol from G-25 in the presence and absence of pneumolysin. Cholesterol was eluted to an appreciable extent only in the presence of cytolysin. Assay of hemolytic activity in the reaction mixture with 1,500 to 3,000 HU of lysin showed an inhibition of 95 to 99%, confirming that the cholesterol was bound to the lysin. The same elution pattern was observed when alveolysin was substituted for pneumolysin in the reaction mixture.

The character of the cytolysin-cholesterol complex was further investigated by chromatography of the reaction mixture on Sephacryl-200 (Fig. 2). The cholesterol (and associated protein, confirmed by the amido black method) was eluted in the excluded volume, whereas cytolysin alone was, as expected from its molecular weight of approximately 63,000 (16), eluted later in a well-separated peak. Similar elution patterns were observed when alveolysin or streptolysin O was substituted for pneumolysin, when the chromatography was performed on Sephadex G-200, when the reaction mixture was incubated at room temperature and the chromatography performed at this same temperature (on Sephacryl-200), and when a submicellar (7.72 ng/ml) concentration or a dispersion (66 μg/ml) of cholesterol was used in the reaction mixture.

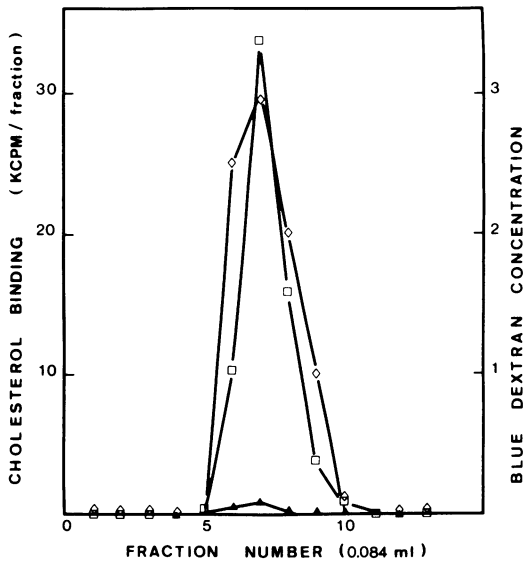

FIG. 1. *Elution of pneumolysin-cholesterol complex from Sephadex G-25: ▲, control (cholesterol alone); ■, with pneumolysin; ◆, blue dextran (relative units). One tenth milliliter of reaction mixture (containing 0.5 μg of cholesterol plus 0.5 μCi of [^{3}H]cholesterol plus 3 μg of pneumolysin per 0.5 ml of reaction mixture) was applied to column. KCPM, Counts per minute in thousands.*

The effect of temperature on cholesterol binding is demonstrated in Fig. 3. At 0°C a single rapid reaction was observed, whereas at room temperature this was followed by a further binding which continued at a rate much slower than that of the initial phenomenon. The lower temperature was selected for further experiments as this reaction corresponds to the rapid and temperature-independent binding of lysin to erythrocytes (2, 9).

The relationship between cytolysin concentration and cholesterol binding activity is shown in Fig. 4 to be linear. Heated cytolysin was inactive and the binding activity of the other proteins tested was much less than that of pneumolysin (Table 1).

The specificity of the cholesterol binding assay with respect to other sterols was investigated by adding unlabeled sterol before the addition of radioactive cholesterol (Table 2). Epicholesterol, which does not inhibit hemolysis, and 7-dehydrocholesterol, a potent inhibitor of hemolysis (18), were tested. The addition of epicholesterol caused only a small decrease in binding of [^{3}H]cholesterol. However, 7-dehydrocholesterol

inhibited [^{3}H]cholesterol binding to an extent equal to that observed with unlabeled cholesterol.

The effect of pH on the binding reaction was tested by using 0.1 M tris(hydroxymethyl)-aminomethane-hydrochloride buffer at pH 7.5 and pH 8.7 in place of the phosphate buffer used in the standard assay. Activity at the higher pH was only 27% of that observed at pH 7.5 (1,020 versus 3,820 cpm/0.1 ml of eluate).

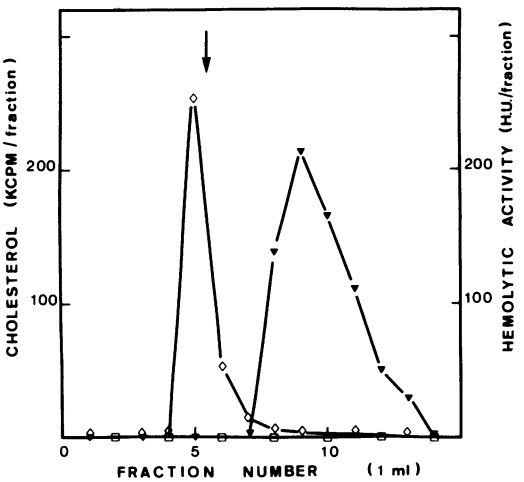

FIG. 2. *Elution of pneumolysin and pneumolysin-cholesterol complex from Sephacryl-200. ▼, Pneumolysin alone, 1.5 μg, (hemolytic activity); ■, cholesterol alone (radioactivity); ◆, pneumolysin plus cholesterol (radioactivity). Five-tenths milliliter of reaction mixture (composition as in Fig. 1) applied to column. Arrow indicates peak of blue dextran elution. KCPM, Counts per minute in thousands.*

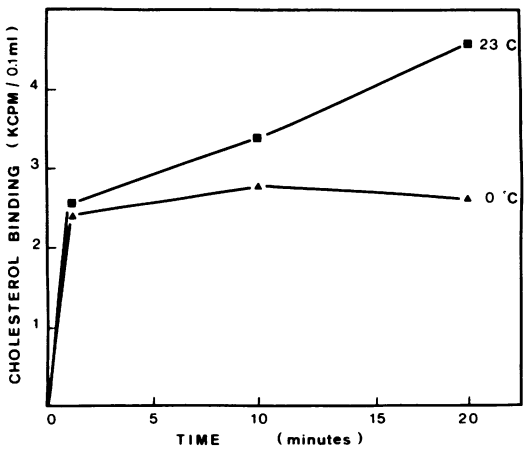

FIG. 3. *Effect of temperature of incubation of reaction mixture on cholesterol binding by pneumolysin. At indicated time, 0.1 ml of the reaction mixture (containing 1 ml buffer, 1 μg cholesterol, 1 μCi [^{3}H]cholesterol, and 2.5 μg pneumolysin) was applied to the G-25 column. Values for cholesterol binding represent 0.1-ml column eluate. KCPM, Counts per minute in thousands.*

TABLE 1. *Activity of other proteins in cholesterol binding assay*

Protein[a]	Cholesterol binding activity (cpm/0.1 ml of eluate)
Control (pneumolysin)	3,079
Heated pneumolysin (75°C, 10 min)	74
Bovine serum albumin	172
Cytochrome *c*	50

[a] 1.65 μg/0.5 ml of reaction mixture.

TABLE 2. *Effect of other sterols on cholesterol binding*

Unlabeled sterol added[a]	Cholesterol binding activity (cpm/0.1 ml of eluate)	% Decrease
Control	5,704	0
Cholesterol	1,723	70
Epicholesterol	4,876	15
7-Dehydrocholesterol	1,793	69

[a] To 0.5 ml buffer were added 3 μl of pneumolysin (1 μg), 0.5 μg of unlabeled sterol, and 0.5 μCi of [^{3}H]cholesterol (3.86 ng).

The binding activity of cytolysin oxidized with DTT is shown in Table 3 to be equal to that of the native pneumolysin. The results of the titrations performed demonstrate that the loss of hemolytic activity observed after treatment with oxidized DTT was indeed due to oxidation of the lysin, as substantial recovery of activity occurred after rereduction.

Since reagents reacting with sulfhydryl groups have been shown by many investigators to inhibit hemolysis by the members of this group of toxins, the effect of pHMB on cholesterol binding activity was determined.

Toxin incubated with pHMB (at a concentration of 8.6 mM during preincubation and 1.2 mM in the binding assay) showed no decrease in cholesterol binding activity (4,591 cpm/0.1 ml of eluate in the control preparation and 4,983 for pHMB-treated lysin). In contrast, preincubation of this level of lysin with as little as 0.06 mM pHMB caused an 80% inhibition of hemolysis. It has been shown previously (11) that this inhibitor acts by preventing fixation of lysin to erythrocytes.

DISCUSSION

The present communication appears to be the first direct demonstration of cholesterol binding by pure proteins derived from procaryotic cells

(Smyth and Duncan [21] refer to unpublished data on [^{14}C]cholesterol binding to cereolysin and streptolysin O). Studies of cholesterol binding are hampered by the limited solubility of this compound and its tendency to form micelles and to stick to the walls of glassware and other materials (10). In the present work, advantage was taken of the fact that when solutions of cholesterol in phosphate buffer are added to Sephadex columns, the free cholesterol sticks to the Sephadex and only cytolysin-bound cholesterol is eluted with buffer. A similar procedure was employed by Johnson and Shah to study cholesterol binding by brain proteins (14). The existence of a cytolysin-cholesterol complex is evidenced by the fact that after combination of cytolysin and cholesterol the properties of cholesterol were changed in that it was readily eluted (Fig. 1) from the Sephadex, and the properties of the cytolysin changed in that it was no longer hemolytic.

The results obtained (Fig. 2) with Sephacryl-200 indicate that the cytolysin-cholesterol complex is a large one (in that it was excluded) and must consist of more than one molecule of lysin and one molecule of cholesterol. The micelles of cholesterol are in some instances very large (10), but this does not explain these results since the same pattern was observed when submicellar concentrations of cholesterol were employed. Cowell et al. (7) discuss the occurrence in several of the thiol-activated cytolysins of protein aggregates, the formation of which is enhanced by cholesterol, and it is possible that such aggregates are present in our reaction mixture and account for the apparently large size of the protein-cholesterol complex.

Because of solutions of cholesterol used in these experiments contained micelles, it was not possible to establish a molar ratio for cytolysin-cholesterol interaction. Binding also occurred at submicellar concentrations (line 1, Table 2) but the difficulties of working at this level are considerable. Only concentrations below 25 nM can be utilized (10), and the problems of sticking and concentration of cholesterol in the air-water interface become important. It was impossible under these conditions to examine the binding reaction over a range of concentrations of toxin and cholesterol sufficient to establish molar ratios or affinities.

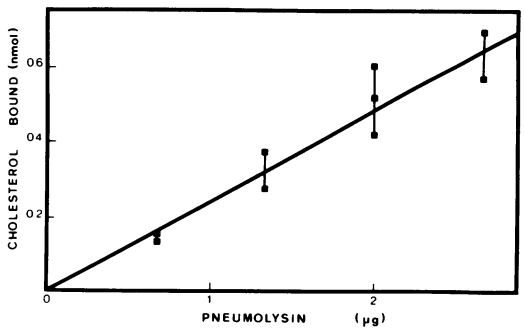

FIG. 4. *Relationship of pneumolysin concentration to cholesterol binding activity expressed as total cholesterol bound per 0.5 ml of reaction mixture containing indicated level of pneumolysin.*

TABLE 3. *Effect of oxidation of pneumolysin on cholesterol binding activity*

System	Titer (HU/0.5 ml of reaction mixture)	Cholesterol binding activity (cpm/0.1 ml of eluate)
Control	2,600	2,933
Oxidized[a]	260	2,981
Rereduced[a]	2,000	

[a] Oxidation and reduction effected with DTT as described in Materials and Methods.

Many of the properties of the cytolysin-cholesterol interaction which we have studied are similar to those described for the interaction of sulfhydryl-activated cytolysins and erythrocytes, which is generally perceived to occur in two steps, the first involving fixation of lysin to erythrocyte and the second involving lysis of the target cell (2). The binding of cholesterol to pneumolysin is very rapid and independent of temperature (Fig. 3), as is the case in the fixation of cytolysin by erythrocytes (2, 9). The effect of pH on the cholesterol binding by pneumolysin (decreased at pH 8.7) also parallels the effects observed with the fixation of pneumolysin and streptolysin O to erythrocytes (13). In addition, the specificity of the system toward other sterols (that is, epicholesterol noncompeting and 7-dehydrocholesterol strongly competing; Table 2) reflects exactly the pattern described by Prigent and Alouf (18) with respect to their effects on hemolytic activity of streptolysin O. The above-described similarities in properties indicate that the cholesterol-cytolysin interaction which we have studied is relevant to the erythrocyte-cytolysin interaction.

In the experiments designed to explore the effects of modification of the sulfhydryl group on the ability of pneumolysin to bind cholesterol, we found that neither oxidation of the lysin nor treatment with the sulfhydryl reagent pHMB affected the cholesterol binding activity. It has been found by numerous investigators that the activity of thiol-activated toxins is inhibited by oxidation and by agents reacting with the sulfhydryl group. In general, the fixation step has been found to be the sensitive one (13). There has been, however, no direct demonstration that the

sulfhydryl group itself participates in the fixation reaction, and it is possible that oxidation of the group or its substitution produces a steric hindrance to fixation to the cholesterol which is embedded in the erythrocyte membrane. Our results (i.e., lack of the effect of oxidation or substitution on binding) might be explained by a lack of this steric hindrance in the case of binding to free cholesterol. In his study of the interaction of streptolysin O with cholesterol in agar gels, Prigent (D. Prigent, thesis, Univ. of Paris, 1975) also found oxidized lysin to be active. On the other hand, Cohen et al. (5) found that oxidized pneumolysin reacted with cholesterol more slowly than did the reduced form, but he also concluded that the sulfhydryl group remained free after the interaction of cholesterol with reduced toxin. In the studies of Kanbayashi et al. (15) it was reported that both the oxidized and reduced forms of streptolysin O were adsorbed by cholesterol particles although the "affinity" of the oxidized toxin was less than that of the reduced toxin.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant EY 00424 from the National Eye Institute, and by the Centre National de la Recherche Scientifique (ERA 794). This collaboration was facilitated by grants from NATO and the Phillippe Foundation.

LITERATURE CITED

1. **Alouf, J. E., M. Kiredjian, and C. Geoffroy.** 1977. Purification de l'hemolysine thiol-dépendante extracellulaire de Bacillus alvei. Biochimie **59**:329–336.
2. **Alouf, J. E., and M. Raynaud.** 1968. Action de la streptolysine O sur les membranes cellulaires. I. Fixation sur la membrane érythrocytaire. Ann. Inst. Pasteur **114**: 812–827.
3. **Bernheimer, A. W.** 1974. Interaction between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27–50.
4. **Bernheimer, A. W.** 1976. Sulfhydryl activated toxins, p. 85–97. *In* A. W. Bernheimer (ed.), Mechanisms of bacterial toxinology. John Wiley & Sons, Inc., New York.
5. **Cohen, B., H. Shwachman, and M. E. Perkins.** 1937. Inactivation of pneumococcal hemolysin by certain sterols. Proc. Soc. Exp. Biol. Med. **35**:586–591.
6. **Cowell, J. L., and A. W. Bernheimer.** 1978. Role of cholesterol in the action of cereolysin on membranes. Arch. Biochem. Biophys. **190**:603–610.
7. **Cowell, J. L., K-S. Kim, and A. W. Bernheimer.** 1978. Alteration by cereolysin of the structure of cholesterol-containing membranes. Biochim. Biophys. Acta **507**: 230–241.
8. **Creighton, T. E.** 1974. Renaturation of the reduced bovine pancreatic trypsin inhibitor. J. Mol. Biol. **87**:563–577.
9. **Fehrenbach, F. J.** 1977. Interaction of streptolysin O with natural and artificial membranes. Z. Naturforsch. Teil C **32**:101–109.
10. **Haberland, M. E., and J. A. Reynolds.** 1973. Self-association of cholesterol in aqueous solution. Proc. Natl. Acad. Sci. U.S.A. **70**:2313–2316.
11. **Johnson, M. K.** 1972. Properties of purified pneumococcal hemolysin. Infect. Immun. **6**:755–760.
12. **Johnson, M. K., and J. H. Allen.** 1975. The role of cytolysin in pneumococcal ocular infection. Am. J. Ophthalmol. **80**:518–521.
13. **Johnson, M. K., and K. S. Aultman.** 1977. Studies on the mechanism of action of oxygen-labile hemolysins. J. Gen. Microbiol. **101**:237–241.
14. **Johnson, R. C., and S. N. Shah.** 1976. Binding of squalene, lanosterol, desmosterol, and cholesterol to proteins in brain and liver 105,000 g supernatant fractions: evidence for specific binding sites. Lipids **11**:645–651.
15. **Kanbayashi, Y., M. Hotta, and J. Koyama.** 1972. Kinetic study on streptolysin O. J. Biochem. (Tokyo) **71**: 227–237.
16. **Kreger, A. S., and A. W. Bernheimer.** 1969. Physical behavior of pneumolysin. J. Bacteriol. **98**:306–307.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:263–275.
18. **Prigent, D., and J. E. Alouf.** 1976. Interaction of Streptolysin O with sterols. Biochim. Biophys. Acta **443**:288–300.
19. **Prigent, D., C. Geoffroy, and J. E. Alouf.** 1978. Purification de la streptolysine O par chromatographie covalente sur gel de thiolagarose. C. R. Acad. Sci. Ser. D **287**:951–954.
20. **Schaffner, W., and C. Weissman.** 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. **56**:502–514.
21. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins. p. 129–193. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and membranes. Academic Press Inc., New York.