Membrane Glycoprotein Receptor and Hole-Forming Properties of a Cytolytic Protein Toxin†

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ABSTRACT: Aerolysin, a cytolytic bacterial exotoxin, was radioiodinated by using the Iodogen reagent. Binding of the labeled toxin to rat erythrocytes was inhibited by the native protein and by anti-aerolysin antibody. Toxin, once bound, was not removed by the addition of a large excess of free aerolysin. Binding of the radioactive toxin to erythrocytes of different species paralleled the hemolytic specificity of the unlabeled toxin. Pretreatment of the rat erythrocytes with trypsin, which removed a major membrane glycoprotein, resulted in a dramatic decrease in binding, whereas chymotrypsin treatment had no effect. Binding was inhibited by a glycoprotein fraction isolated from these cells but not by a total rat erythrocyte glycolipid preparation. Aerolysin caused the formation of holes in erythrocytes which were sized by measuring the release of labeled molecular weight markers. Glucagon (molecular weight 3550) and smaller molecules entrapped in human or rat erythrocytes were released by treatment with aerolysin, whereas methoxyinulin (molecular weight 5500) and larger molecules were not. Aerolysin also caused the release of glucose from large unilamellar lipid vesicles. The results indicate that a specific glycoprotein receptor facilitates the interaction of aerolysin with erythrocyte membranes. Binding is followed by the formation of discrete holes or pores, and this results in cell rupture by a colloid-osmotic process.

Many bacterial species release proteins which can cause disruption of eucaryotic cells by means of selective interaction with their plasma membranes. Most of these cytolytic toxins are produced by Gram-positive bacteria; however, those released by Gram-negative bacteria are receiving increasing attention because of their potential roles in pathogenesis. Although some of these toxins have been purified, including the hemolysins of *Escherichia coli* (Noegel et al., 1979), *Aeromonas hydrophila* (Buckley et al., 1981), *Pseudomonas aeruginosa* (Lutz, 1979), and several members of the *Vibrio* genus (Miyamoto et al., 1980; Honda & Finkelstein, 1979), there is little or no information on their modes of action. Many of the cytolytic proteins of Gram-positive bacteria have been at least partially characterized, and they can be divided into several groups based on the mechanisms whereby they disrupt cell membranes. The first group, perhaps best characterized by staphylococcal β-toxin (Möllby, 1976), contains enzymes which degrade membrane components. The second group consists of surfactants such as streptolysin S (Alouf, 1980) which disrupt the membrane lipid bilayer. The third group is represented by proteins which form discrete membrane holes. The family of thiol-activated toxins, which selectively interact with membrane cholesterol, falls into this category (Smyth & Duncan, 1978). So may staphylococcal α-toxin (Harshman, 1979), which apparently binds to a specific membrane glycoprotein prior to hole formation (Maharaj & Fackrell, 1980). Although members of this group are thought to produce holes of defined sizes, no accurate estimations of their dimensions have been carried out.

Thelestam & Möllby (1979) have constructed a general classification of cytolysins based on leakage patterns of three molecular weight markers from human fibroblasts. Their groupings were somewhat arbitrary, however, and the procedures did not permit accurate measurement of hole sizes nor did they provide any information on the nature of specific membrane binding components.

Recently we have purified the potent cytolytic toxin aerolysin, released by the Gram-negative pathogen *A. hydrophila* (Buckley et al., 1981). This protein has a characteristic hemolytic spectrum (Bernheimer et al., 1975) which suggests its action is mediated by a specific membrane binding component. Its activity has been shown to bear some relationship to membrane phosphatidylcholine content (Bernheimer et al., 1975); however, the nature of its receptor and the mode of membrane disruption are unknown. In this communication we show that aerolysin binds to a membrane glycoprotein and that it forms discrete holes of defined size in erythrocyte membranes.

Materials and Methods

Materials. Aerolysin was purified as previously described (Buckley et al., 1981). Bovine serum albumin, glucagon, dicetyl phosphate, phosphatidylcholine (PC),[1] lysozyme, glucose-6-phosphate dehydrogenase, and hexokinase were from Sigma Chemical Co. 1,3,4,6-Tetrachloro-3α,6α-diphenylglucoluril (Iodogen) was from the Pierce Chemical Co. Leucyltyrosylleucine and α-melanocyte-stimulating hormone (α-MSH) were products of Vega Biochemicals. D-[U-^{14}C]-Glucose (328 mCi/mmol) and [^{3}H]$NaBH_4$ (10 Ci/mmol) were obtained from Amersham Corp. [^{3}H]Methoxyinulin (237.6 mCi/g) and $Na^{125}I$ (17 Ci/g) were purchased from New England Nuclear. Rat erythrocyte membrane glycoprotein was isolated as described by Marchesi & Andrews (1971). Total rat erythrocyte glycolipid was obtained following the procedure of Laine et al. (1974). All blood samples were obtained immediately prior to use.

Iodination of Aerolysin. Radiolabeling of aerolysin was carried out by using the methods of Fraker & Speck (1978) and Markwell & Fox (1978). Iodogen (20 μg) in chloroform was dried under nitrogen onto the bottom of a test tube. The tube was rinsed with PBS, pH 7.0, and 400 μL of a solution containing 300 μg of aerolysin and 1 mCi of carrier-free $Na^{125}I$

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[1] Abbreviations: PC, phosphatidylcholine; PBS, phosphate-buffered saline; HU, hemolytic unit; PAS, periodic acid–Schiff; α-MSH, α-melanocyte-stimulating hormone.

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was added. After occasional agitation for 15 min at 4 °C, the reaction was terminated by removal of the solution from the test tube. The preparation was passed through a Sephadex G-25 column equilibrated in 20 mM Tris (pH 7.4)–0.85% NaCl to remove unreacted iodine. Radiolabeled aerolysin preparations of 0.2–0.4 μCi/μg were obtained in this manner.

Binding Assays. The binding of iodinated aerolysin to erythrocytes was followed by γ counting of supernatants obtained by brief centrifugation at 12000*g* after timed incubation at 37 °C. The assay mixtures contained, in a total volume of 1.4 mL, 20 mM Tris, 0.85% NaCl, pH 7.4, 0.1% BSA, 0.3 M sucrose, and the indicated quantities of erythrocytes and aerolysin. All results are expressed as means of duplicate or triplicate assays, and controls were carried out (in which no erythrocytes were added to the assay mixture) for each set of experiments. The number of erythrocytes added in each set of assays was estimated by hemocytometer counts of stock blood solutions.

Labeling of Marker Peptides. Samples (0.1 mg) of lysozyme, glucagon, α-MSH, and leucyltyrosylleucine were labeled by reductive alkylation according to the method of Rice & Means (1971) except that [^{3}H]$NaBH_4$ was used rather than [^{14}C]formaldehyde. Unreacted borohydride was removed by gel filtration on Sephadex G-10.

Release of K^+, Glucose, and Hemoglobin from Intact Human Erythrocytes. [^{14}C]Glucose (10 μCi) was loaded by equilibrium exchange into 2 mL of packed human erythrocytes as described by Eilam & Stein (1974). The cells were then washed 3 times with PBS and diluted in the same buffer. One-milliliter samples containing 20 μL of packed cells were incubated at 37 °C for various time periods with or without 2 HU of aerolysin, followed by centrifugation for 1 min at 14000*g*. Where indicated, 0.3 M sucrose (final concentration) was present during incubation. Aliquots of supernatant were taken for spectrophotometric determination of hemoglobin at 545 nm, measurement of potassium by flame atomic absorption spectrophotometry (Varian AA475), and scintillation counting of [^{14}C]glucose (Beckman LS8100).

Entrapment of Radioactive Markers in Resealed Erythrocyte Ghosts. Rat or human resealed erythrocytes containing marker molecules were prepared as described by Giavedoni et al. (1979). One milliliter of freshly drawn washed packed cells was diluted with 9.0 mL of 5.0 mM Tris (pH 7.3)–10 mM glucose–0.15 mM $CaCl_2$–0.5 mM $MgCl_2$, containing approximately 10 μCi of the required markers (i.e., [^{14}C]-glucose and one of the tritiated markers). After 5 min at 4 °C, isotonicity was restored via the addition of 5.0 mM Tris (pH 7.3)–0.15 mM $CaCl_2$–0.5 mM $MgCl_2$–2.9 M NaCl, and after a further 5 min the cells were incubated at 37 °C for 50 min. The resealed cells were then washed 3–4 times in the isotonic buffer and used immediately.

Release of Radioactive Marker Molecules from Resealed Erythrocytes by Aerolysin. Aliquots of resealed ghosts (50 μL) in a total volume of 1.0 mL of 5.0 mM Tris (pH 7.3)–0.15 mM $CaCl_2$–0.5 mM MgCl were incubated at 37 °C. An amount of aerolysin was added such that 100% of the reference marker ([^{14}C]glucose) but none of the residual hemoglobin in the ghosts was released into the medium in 20 min. At the indicated times, samples were withdrawn and briefly centrifuged, and the supernatants were counted to determine the quantity of each marker released. To ensure that marker molecules were not adsorbing to the ghosts (i.e., could potentially be released), we subjected ghost samples to three cycles of rapid freezing and thawing and centrifuged them and measured the radioactivity released into the supernatant. Values obtained in this way were taken to represent maximum possible release of the markers and were normally >90% of the total marker present. Quench correction using quenched standards was applied to all data, and leakage values obtained from duplicate samples without added aerolysin were subtracted from the aerolysin-induced release values. Data on the release of each molecular weight marker are expressed as the ratio of its release to that of the reference marker, [^{14}C]glucose (97–100% in each experiment).

Preparation of Liposomes. Large unilamellar vesicles containing either PC–cholesterol–dicetyl phosphate (70:20:10 mol/mol/mol) or PC–dicetyl phosphate (90:10) were prepared by the solvent vaporization procedure of Schieren et al. (1978). Petroleum ether solutions of the lipid mixtures were injected into phosphate-buffered saline, pH 7.3, containing 0.2 M glucose at 60 °C. After injection the aqueous phase was vortexed 1 min and passed through a Sepharose 2B column (K16 × 40; Pharmacia) in the same buffer to remove untrapped glucose.

Release of Glucose from Liposomes. The release of glucose from lipid vesicles was followed at 37 °C in a double-beam recording spectrophotometer by using the system described by Kinsky (1974). The reaction mixture contained 1 mM ATP, 0.5 mM $NADP^+$, 1 mM $MgCl_2$, 0.015 mg/mL (212 IU/mg) hexokinase, 0.013 mg/mL (76 IU/mg) glucose-6-phosphate dehydrogenase, and 50 μL of liposomes (190 μM phospholipid), in 1.0 mL of PBS. The increase in absorbance at 339 nm was monitored after the addition of various quantities of aerolysin. Values were corrected for absorbance due to untrapped glucose and to light scatter from the liposomes themselves. Values for 100% release were obtained by the addition of Triton X-100 to a final concentration of 1.0%.

General Procedures. Samples were prepared for electrophoresis and separated in $NaDodSO_4$ on 7.5% or 12% polyacrylamide gels according to the methods described by Hancock & Carey (1979) in their modification of Neville's (1971) method. Proteins were stained by using Coomassie blue, and carbohydrate-containing components were detected with periodic acid–Schiff stain following the procedure of Fairbanks et al. (1971), after de-O-acetylation as reported by Sarris & Palade (1979). The protein content of membrane-containing samples was measured by the Lowry procedure as modified by Markwell et al. (1978) and otherwise by the method of Bradford (1976). Phospholipid phosphorus was estimated as described by Bartlett (1959) following digestion with perchloric acid. Titers were measured as described by Bernheimer & Avigad (1974) except that human or rat erythrocytes were used and dilutions were made in 20 mM Tris (pH 7.4)–0.85% NaCl containing 0.1% albumin.

Results

Iodination of aerolysin resulted in preparations which retained 25% or more of the hemolytic activity of the native protein. Specific activities of 0.2–0.4 μCi/μg of protein were obtained, indicating the addition of one to two atoms of iodine to each molecule of aerolysin. A single radioactive band was obtained by $NaDodSO_4$ electrophoresis which corresponded to native aerolysin in molecular weight (Figure 1).

Binding of ^{125}I-labeled aerolysin to rat erythrocytes was time dependent (Figure 2). It was completely inhibited by preexposure of the erythrocytes to the unlabeled toxin or by prior treatment of the labeled protein with anti-aerolysin antiserum (Table I). The radioactive toxin, once bound, could not be removed by treatment with a large excess of the native protein (Table I). The ability of aerolysin to bind to erythrocytes corresponded to the hemolytic sensitivity of cells from

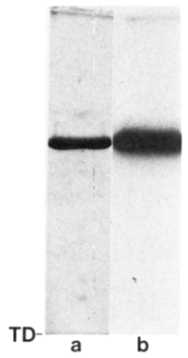

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of ^{125}I-labeled aerolysin. Lane a, native aerolysin (approximately 10 μg of protein); lane b, ^{125}I-labeled aerolysin (approximately 2×10^4 cpm). TD = tracking dye.

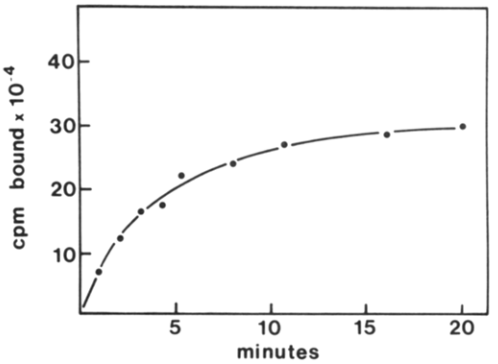

FIGURE 2: Time dependence of ^{125}I-labeled aerolysin binding to rat erythrocytes. Aerolysin (1.3 μg) and 10 μL of a 33% suspension of rat erythrocytes were incubated at 37 °C in a total volume of 1.4 mL of binding assay buffer. After the indicated times, aerolysin binding was determined as described in the text.

Table I: Effect of Native Aerolysin and Anti-aerolysin Antibody on ^{125}I-Labeled Toxin Binding to Rat Erythrocytes

addition	time of addition	μg of ^{125}I-labeled aerolysin bound	% maximal ^{125}I binding
control [a]		0.85	100
75 μg of native aerolysin	10 min before ^{125}I-labeled aerolysin	0	0
75 μg of native aerolysin	10 min after ^{125}I-labeled aerolysin	0.80	94
anti-aerolysin antiserum [b]	preincubated at 37 °C, 5 min, with aerolysin	0	0

[a] Binding to rat erythrocytes (10 μL of 33% packed cells) was measured after 10 min as described in the text. [b] 85 anti-HU of antiserum was used.

different species as reported by Bernheimer et al. (1975) except that mouse erythrocytes bound more aerolysin than those of rat (Figure 3). At the highest concentration of ^{125}I-labeled aerolysin tested, approximately 9.0×10^5 molecules were bound per mouse cell, indicating that the binding component was present in high copy numbers in murine erythrocytes.

Treatment of rat erythrocytes with trypsin resulted in a time-dependent decrease in the binding of ^{125}I-labeled aerolysin and a parallel decrease in their sensitivity to hemolysis (Table II and Figure 4). Similar treatment with chymotrypsin had no effect on either binding or hemolysis. In contrast, both binding of aerolysin and hemolytic sensitivity were increased when human erythrocytes were exposed to either trypsin or chymotrypsin (Table II).

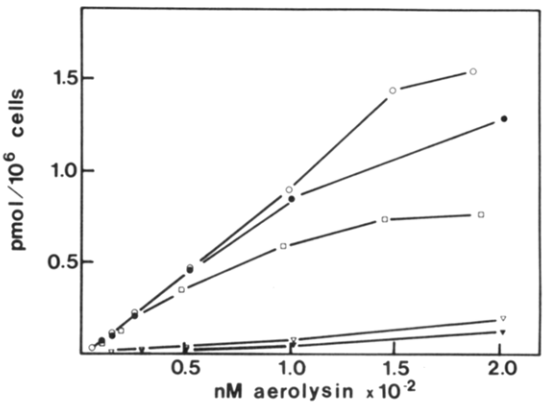

FIGURE 3: Species specificity of ^{125}I-labeled aerolysin binding to erythrocytes. The indicated concentration of aerolysin was incubated with 10 μL of a 33% erythrocyte suspension in 1.4 mL of binding assay buffer, and binding was determined after 10 min at 37 °C. (▼) Human; (▽) rabbit; (□) guinea pig; (●) rat; (○) mouse.

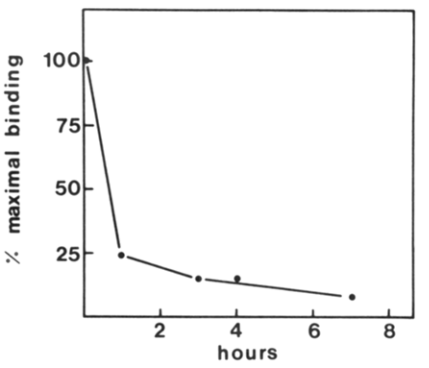

FIGURE 4: Effect of trypsin on aerolysin binding. Rat erythrocytes (20% v/v) were incubated with 1 mg/mL trypsin. At the indicated times, cells were washed with 1.0% albumin and 2 mg/mL trypsin inhibitor, and ^{125}I-labeled aerolysin binding was measured.

Table II: Aerolysin Binding and Hemolytic Sensitivity of Erythrocytes after Treatment with Trypsin or Chymotrypsin

erythrocytes	protease	conditions	% of control [a] hemolytic sensitivity	% of control binding
rat	0.2 mg of chymotrypsin/mL	2 h; 37 °C	100	115
rat	0.2 mg of trypsin/mL [b]	2 h; 37 °C	60	36
rat	1 mg of trypsin/mL	6 h; 25 °C	15	30
human	1 mg of trypsin/mL	6 h; 25 °C	270	200
human	1 mg of chymotrypsin/mL	6 h; 25 °C	270	230

[a] Control cells were treated with trypsin and trypsin inhibitor (1:2 w/w). [b] Trypsin inhibitor (1:2) was added after treatment with trypsin and prior to aerolysin addition.

The results of $NaDodSO_4$–polyacrylamide electrophoresis indicated no major changes in membrane protein staining profiles after treatment of intact rat erythrocytes with either of the two proteases (Figure 5A). In addition, neither of the major PAS-positive proteins appeared to be affected by chymotrypsin (Figure 5B). Exposure to trypsin, however, completely removed one of these proteins, indicating that it contains the binding site for aerolysin (Figure 5B). The differential sensitivity to proteases suggested that the protein might be analogous to the human red cell glycoprotein glycophorin,

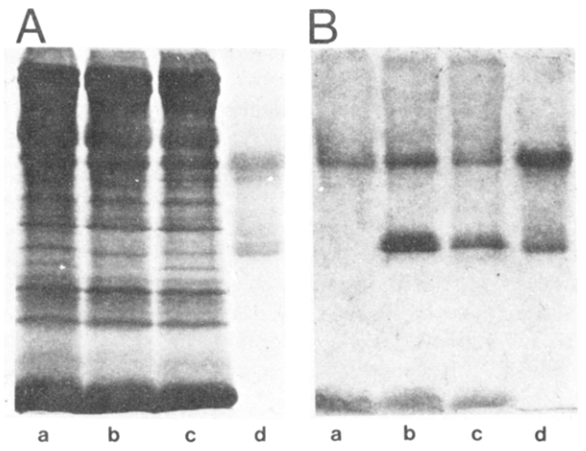

FIGURE 5: Effect of trypsin and chymotrypsin on rat erythrocyte membrane proteins and glycoproteins. Cells were incubated with 1 mg/mL trypsin or 1 mg/mL chymotrypsin. Control cells were incubated with 1 mg/mL trypsin and 2 mg/mL trypsin inhibitor. After 1 h, the cells were washed with 10 mg/mL albumin and membranes were prepared. (A) Coomassie blue staining proteins; (B) PAS-positive membrane components; (a) trypsin-treated cells; (b) controls; (c) chymotrypsin-treated cells; (d) rat erythrocyte glycoprotein, 18 μg. 100 μg of membrane protein was applied in lanes a–c.

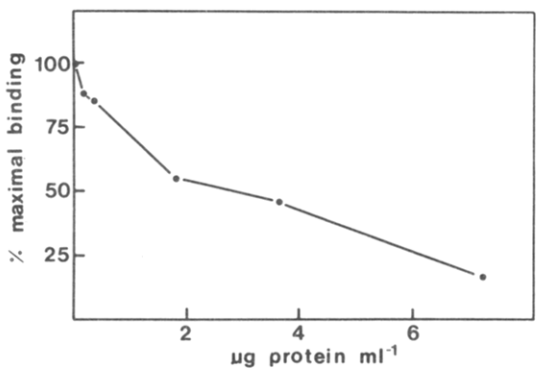

FIGURE 6: Inhibition of ^{125}I-labeled aerolysin binding by rat erythrocyte glycoprotein. Cells (7×10^7/mL) were preincubated with the specified concentration of glycoprotein for 10 min at 37 °C before addition of 0.3 μg of ^{125}I-labeled aerolysin. Binding was determined after a further 10-min incubation at 37 °C.

which is known to be sensitive to trypsin (Steck et al., 1976; Marchesi et al., 1976) but not chymotrypsin in intact cells (Bretscher, 1973; Cabantchik & Rothstein, 1974). When the procedure of Marchesi & Andrews (1971), first used to purify human glycophorin, was applied to rat erythrocyte membranes, a preparation which contained both major membrane glycoproteins was obtained (Figure 5). It was extremely effective as an inhibitor of rat erythrocyte hemolysis by aerolysin and as an inhibitor of aerolysin binding to rat erythrocytes (Figure 6). In contrast, a total glycolipid fraction from rat erythrocytes had no effect on either hemolysis or binding (data not shown).

In order to determine if binding to a membrane glycoprotein was absolutely required for membrane disruption, we determined the action of aerolysin on artificial lipid dispersions. Even very high concentrations of aerolysin (500 HU/μmol of total lipid) did not release glucose from small unilamellar PC–cholesterol vesicles prepared by sonication or from multilamellar vesicles prepared by vortexing. Large unilamellar vesicles containing similar amounts of lipid were disrupted however. Liposomes containing cholesterol appeared to be slightly more sensitive to the action of aerolysin, but clearly the presence of cholesterol was not a prerequisite for disruption (Figure 7). On the basis of total lipid content, the amount

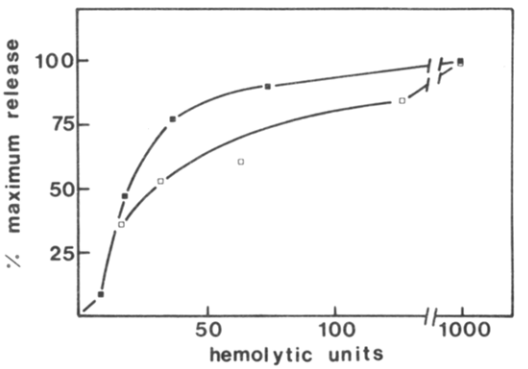

FIGURE 7: Aerolysin-induced release of glucose from large unilamellar lipid vesicles. Release of glucose was measured after 15-min incubation as described in the text. (■) PC–cholesterol–dicetyl phosphate liposomes (7:2:1); (□) PC–dicetyl phosphate (9:1). The aerolysin used was 16.5 HU/μg of protein, measured by using human erythrocytes.

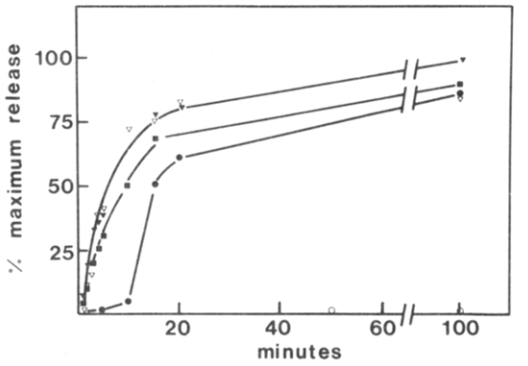

FIGURE 8: Time dependence of aerolysin-induced release of intracellular erythrocyte components. (▼) K^+ release; (■) glucose; (●) hemoglobin; (▽) K^+ release in the presence of 0.3 M sucrose; (○) hemoglobin release with 0.3 M sucrose. Release of glucose in the presence of 0.3 M sucrose was the same as in its absence. Maximal release was measured after 3× rapid freezing and thawing in hypotonic buffer.

of aerolysin required to release half the glucose from cholesterol-containing liposomes (400 HU/μmol of lipid) was similar to the amount required for 50% lysis of human erythrocytes (70 HU/μmol of lipid).

Thelestam & Möllby (1979) observed that aerolysin caused the selective release of low molecular weight markers from human fibroblasts. Similarly, leakage of both potassium and glucose preceded hemoglobin release from both human and rat erythrocytes (Figure 8). Sucrose prevented the release of hemoglobin from cells of both species without affecting leakage of the smaller molecules (either potassium or glucose) (Figure 8). These results indicated that aerolysin causes cell disruption by a colloid-osmotic mechanism in which small holes or pores are produced in the membrane. For estimation of the size of these holes, the release of molecular weight markers from resealed human and rat erythrocytes was measured. Each of the markers chosen was readily entrapped by the resealing procedure, and very little remained associated with the membranes after repeated freezing and thawing. In each case, total release of ^{3}H-labeled marker was compared to the release of [^{14}C]glucose. The results are presented in Table III. [^{3}H]Glucagon (M_r 3500) and smaller molecules were released by aerolysin whereas [^{3}H]carboxyinulin (M_r 5500) and larger molecules were retained by both human and rat erythrocytes. As the effective diffusion radius of the inulin used is approximately 1.5 nm (Pappenheimer, 1951), these observations indicate that the pores formed by aerolysin must be smaller than 3 nm in diameter.

Table III: Aerolysin-Induced Release of Molecular Weight Markers from Resealed Erythrocytes

marker	molecular weight	release factor[a] human	release factor[a] rat
glucose	180	1	1
leucyltyrosylleucine	444	0.94	ND[b]
α-MSH	1650	0.79	ND
glucagon	3550	0.95	0.77
methoxyinulin	5500	0.07	0.09
cytochrome *c*	12684	0.01	ND

[a] This is a ratio of the percent marker release to percent glucose release, determined as described in the text. [b] ND, not determined.

Discussion

The results obtained in this study provide significant insight into the action of aerolysin. It would appear that the toxin binds to a specific glycoprotein receptor in the cell membrane. Binding is followed by the formation of discrete holes in the lipid bilayer which results in cell disruption.

Several bacterial toxins including diphtheria toxin (Draper et al., 1978), choleragen (Cuatrecasas, 1973), and α-toxin (Kato & Naiki, 1976; Maharaj & Fackrell, 1980) are known to bind to specific glycolipid or glycoprotein receptors. This, however, is the first cytotoxin from a Gram-negative bacterium for which a receptor has been identified. Species-specific differences in the binding of the iodinated toxin to erythrocytes (Figure 3) paralleled differences in sensitivity to hemolysis observed by Bernheimer et al. (1975) except that mouse erythrocytes bound more aerolysin than would be predicted by the data of these authors. The observations that the binding component is present in high numbers in erythrocytes of several species (approximately 9.0×10^5/cell for mouse), that it is removed by trypsin treatment (Table II), and that a major erythrocyte glycoprotein is also removed by trypsin (Figure 5B) all suggest that an analogue of human glycophorin in these erythrocytes is responsible for binding. It seems likely that the binding site is a specific carbohydrate determinant which is characteristic of these species. The determinant is more complex than a single monosaccharide residue, however, as none of the sugars and sugar derivatives known to exist in mammalian erythrocyte glycoproteins affected binding at concentrations up to 5 mM (data not shown).

Rabbit and human erythrocytes appear to contain little or none of the binding determinant (Figure 3), and hemolysis of these cells may be due to direct interaction of the toxin with their lipid bilayers. The fact that similar toxin concentrations were required to lyse liposomes and human erythrocytes lends some support to this conclusion. In addition, Bernheimer & Avigad (1980) have shown that human glycophorin has no effect on the hemolytic activity of aerolysin, and this is supported by the absence of a trypsin-induced decrease in hemolytic sensitivity of human erythrocytes found in this study (Table II). It is not possible to make a direct comparison of cells and liposomes because the liposome populations were not characterized in terms of size distribution and surface area. Changes in either of these parameters would significantly affect the apparent sensitivity of any liposome population and thus account for the observed insensitivity of small unilamellar and multilamellar liposomes. In addition, such changes may explain the difference observed between liposomes which contained cholesterol and those which did not (Figure 6). Differences between the sensitivities of cells which lack the binding determinant such as human and rabbit may be due to differences in the chemical properties of their bilayers. This could account for the tenuous relationship between hemolytic sensitivity and membrane phosphatidylcholine content observed by Bernheimer et al. (1975). Differences in the accessibility of individual lipid bilayers could also affect sensitivity. Thus, the results in Table II, in which trypsin and chymotrypsin are shown to more than double human erythrocyte sensitivity, may have been due to removal of part of the exterior glycoprotein, thus increasing the ease with which aerolysin could approach the bilayer.

Many bacterial toxins are known to produce defined holes in the membranes of eucaryotic cells, and several of these including staphylococcal α-toxin (Madoff et al., 1964) and streptolysin S (Duncan & Mason, 1976) cause colloid-osmotic cell lysis. This is the first toxin, however, for which hole size has been accurately determined. The results indicate that the holes are large enough to permit molecules of 3500 daltons to pass through but too small to allow the passage of molecules of 5500 daltons (Table III). The results of Thelestam & Möllby (1979), based on less accurate leakage data, group aerolysin with the thiol-activated toxins. The current data indicate that this grouping is incorrect since thiol-activated toxins permit leakage of molecules as large as hemoglobin (Duncan, 1974).

The binding component, when present, does not appear to influence pore size as leakage patterns were similar for both human and rat erythrocytes (Table III). Its function may be to increase the effective concentration of aerolysin at the bilayer surface although further experiments are required to determine if it influences insertion or perhaps aggregation of the toxin, leading to pore formation. Binding is apparently irreversible as toxin, once bound, is not released by the addition of a large excess of unlabeled toxin. Similar results have been reported by Cassidy & Harshman (1976) for the binding of α-toxin.

The size of the holes produced in erythrocyte membranes by aerolysin is within the range reported for several outer membrane hole-forming proteins, or porins, notably those of *P. aeruginosa* (Hancock & Nikaido, 1978), which shares many properties with *Aeromonas* sp. It is tempting to speculate that aerolysin and perhaps other Gram-negative hemolysins are modified porins. Once released by the bacteria, they would be effective cell disrupters, providing the bacteria with intracellular nutrients. We are currently examining the structural and immunological relationships between aerolysin and *Aeromonas* porins.

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Lateral Diffusion of Epidermal Growth Factor Complexed to Its Surface Receptors Does Not Account for the Thermal Sensitivity of Patch Formation and Endocytosis†

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ABSTRACT: The patching and endocytosis of EGF (epidermal growth factor) bound to A-431 cells (a human epidermoid carcinoma line) are temperature-sensitive processes which are completely inhibited at 4 °C. Receptor-mediated endocytosis generally occurs through coated regions, and EGF bound to its membrane receptor must diffuse laterally to these points of internalization. In this work we investigated the thermal sensitivity of the lateral diffusion of EGF receptor complexes and the thermal sensitivity of the patching and endocytosis of the hormone receptor complexes. Using the fluorescence photobleach recovery technique, we measured the lateral diffusion coefficients of a fluorescent derivative of EGF as a function of temperature. The lateral diffusion coefficient (D) increased gradually from 2.8×10^{-10} cm^2/s at 5 °C to 8.5×10^{-10} cm^2/s at 37 °C, and no phase transition was detected. Neither was a phase transition detected when we measured the diffusion coefficient of fluorescent lipid probes over this temperature range. From a calculation of the collision frequency of the occupied EGF receptors with coated regions using our measured values of D at 5 and 37 °C, we conclude that diffusion is not the rate-limiting step for either endocytosis or patching.

Epidermal growth factor (EGF)[1] is a 6045-dalton polypeptide which binds to specific membrane receptors on various epidermal, epithelial, and fibroblastic cells (Carpenter & Cohen, 1979). EGF initiates rapid responses such as the uptake of metabolites, phosphorylation of membrane proteins (Carpenter & Cohen, 1979; Carpenter et al., 1979; Ushiro & Cohen, 1980), and changes in the cytoskeleton (Schlessinger & Geiger, 1981) and in cell morphology (Chinkers et al.,

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[1] Abbreviations: EGF, epidermal growth factor; R-EGF, EGF labeled with tetramethylrhodamine; EGF-RC, EGF receptor complex; FPR, fluorescent photobleach recovery; PBS, phosphate-buffered saline; WW591, 5-[4-[3-(γ-sodium sulfopropyl)-6,7-benzo-2(3*H*)-benzoxazolylidene]-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid; diI, 3,3′-dioctadecylindocarbocyanine iodide.

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