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MEMBRANE DAMAGE OF LIPOSOMES BY THE MUSHROOM TOXIN PHALLOLYSIN

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We have investigated the membrane-damaging effect of phallolysin on liposomes varying in phospholipid composition, net charge and physical constitution. Liposomes were prepared from lipids extracted from bovine or human erythrocyte ghosts. The liposomes composed of bovine lipids (the intact cell showing little sensitivity to phallolysin) were found comparably sensitive to those prepared from lipids of human red cells (these cells being of high sensitivity). In addition, artificial mixtures of lipids were used for the preparation of liposomes, consisting of (a) negatively charged phospholipids such as dicetyl phosphate or phosphatidylserine, (b) cholesterol, and (c) either sphingomyelin (as the major component of erythrocytes from ruminants) or phosphatidylcholine (as the major component of erythrocytes from non-ruminants). Again, we found only little difference in the susceptibilities of sphingomyelin- and phosphatidylcholine-containing liposomes. On the other hand, the susceptibility depended on the presence of phospholipids with negative net charges. Omittance of phosphatidylcholine or dicetyl phosphate, or replacement by the positively charged stearylamine, decreased the susceptibility by a factor of more than 20. Finally, we prepared liposomes from dicetyl phosphate, cholesterol and phosphatidylcholine in two physical states: large unilamellar and smaller multilamellar liposomes. The unilamellar liposomes were about 10-times more sensitive to phallolysin. We conclude: (1) Phallolysin damages phospholipid-membranes in the absence of receptor proteins, but high concentrations of the toxin are required. (2) Membrane damage takes place with liposomes containing phosphatidylcholine as well as those containing sphingomyelin. (3) Phallolysin damages only liposomes containing phospholipids with a negative net charge.

Introduction

The toxic mushroom *Amanita phalloides* produces, besides the heat-resistant peptides like amatoxins and phallotoxins [1], three heat-labile proteins with cytolytic activity, differing in their isoelectric points [2,3]. These protein components are designated phallolysin, and cause disruption of various mammalian cells including erythrocytes [4–6]. The erythrocytes of various animal species differ extensively in their susceptibility to the toxin [3,7]. For example, erythrocytes of cattle or sheep are approx. 1000-times less sensitive to phallolysin than erythrocytes of man or rabbit. In its specificity, phallolysin differs from some other cytolytic toxins such as pleurotolysin [8], staphylococcal α-toxin [9] or the toxin of the sea anemone

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Stoichactis helianthus [10]. For example, pleurotolysin and sea anemone toxin preferentially destroy erythrocytes of ruminant animals, which resist phallolysin.

Selectivity of pleurotolysin and *S. helianthus* toxin for ovine and bovine erythrocytes was attributed to the high sphingomyelin content of ruminant red blood cells. For one of these toxins, *Stoichactis* toxin, this view was substantiated by showing that liposomes containing sphingomyelin were disrupted by this toxin more easily than those containing phosphatidylcholine [11]. Similarly, the specificity of phallolysin for human and rabbit erythrocytes was claimed to depend on the high concentration of phosphatidylcholine in these cells [12].

In this study, we wished to resolve the apparent inconsistency that phallolysin activity was shown to depend on specific membrane proteins [13], but in other experiments was able to release markers from liposomes composed of lipids only [12]. Furthermore, we wished to prove that phallolysin activity depends on the phospholipid composition, particularly on a high content of phosphatidylcholine, while the higher content of sphingomyelin causes the resistance of erythrocytes of ruminant. Finally, we wanted to substantiate our recent finding that positively charged amino acid residues in phallolysin are involved in cytolytic activity (unpublished results). We therefore investigated liposomes with negative and positive net charges.

Experimental procedures

Toxin. The purification procedure involves protein fractionation with ammonium sulfate, ion-exchange chromatography with DEAE-Sephadex and CM-Sephadex, and exclusion chromatography with Bio-Gel P-100. In the final step of purification, which removes a contaminant and increases the specific activity significantly, phallolysin is collected in the void volume during chromatography on *N*-acetylglucosamine immobilized to polyacrylamide. The final product is lyophilized and stored refrigerated at 0–4°C. Definition of the specific activity of phallolysin and details of the purification procedure are described elsewhere [7].

Radioisotopes. [^{14}C]Glucose with a specific activity of 5 mCi/mmol was purchased from New England Nuclear Chemicals, Dreieich, F.R.G.

Lipids. Egg phosphatidylcholine, bovine sphingomyelin, dicetylphosphate, stearylamine and cholesterol were purchased from PL Biochemicals Inc., WI, U.S.A., as lipid mixtures of defined molar ratio in chloroform solutions (Lipo Prep); phosphatidylserine was obtained from Sigma Chemicals, München, F.R.G.

Other chemicals. Sephasorb HP, Ultrafine, was obtained from Pharmacia, Uppsala, Sweden. All the other chemicals were of reagent grade.

Preparation of erythrocyte ghosts. Human erythrocytes (A_2, Sangocell) were obtained from Behring Werke, Marburg/Lahn, F.R.G. Bovine erythrocytes were collected in standard saline citrate solution from animals immediately after they had been slaughtered. Before use, both human and bovine erythrocyte suspensions were washed three times with 5 mM sodium phosphate (pH 8.0)/154 mM NaCl (phosphate-buffered saline). Ghosts were prepared by the method of Steck [14].

Preparation of lipids from erythrocyte ghosts. Lipids were extracted from ghosts according to the method described by Roelofsen and Zwaal [15].

Preparation of multilamellar liposomes. Liposomes were prepared essentially by the method of Freer et al. [16]. In brief, a chloroform solution with 3 μmol of lipids of defined molar ratio was dried in vacuo, and then resuspended in 3 ml of a 0.308 M [^{14}C]glucose solution. After shaking the flask, further dispersion of the lipids was accomplished by ultrasonification for 90 s. The liposome suspension was allowed to equilibrate for 2 h. Untrapped glucose was removed by extensive dialysis against 0.154 M NaCl/5 mM Hepes (pH 7.0).

In those experiments in which lipids were extracted from bovine or human erythrocytes to prepare liposomes, 3 μmol phospholipid, as determined by total phosphorus content [17], were used as the starting material. Electron microscopic examination revealed multilamellar structures of liposomes.

Preparation of unilamellar macroliposomes. Vesicles were prepared by a method adapted from that described by Rögner et al. [18]. A mixture of 1.2 ml of a phospholipid solution (20 μmol lipids of defined molar ratio, 2% cholate, 1% deoxycholate, 300 mM glucose solution mixed with 50 μCi

[^{14}C]glucose, and 5 mM Hepes (pH 8.0)) and 0.4 ml of a washed Sephasorb suspension (50 mg/ml) was dialyzed against 500 ml of 300 mM glucose and 5 mM Hepes (pH 8.0) at 30°C for 16 h. Untrapped glucose was removed by dialysis against 0.154 M NaCl and 5 mM Hepes (pH 7.0) at room temperature. The dialyzed mixture was centrifuged at $10000 \times g$ for 15 min. The resulting pellet contained the macroliposome fraction and was suspended in 1.6 ml of the dialyzing buffer. Electron microscopy showed liposomes of 0.5–1.5 μm diameter.

Assay for phallolysin activity on liposomes. Varying concentrations of phallolysin were added to dialysis bags containing the liposome suspension in 0.154 M NaCl/5 mM Hepes (pH 7.0) (total volume 300 μl). The bags were shaken in a 1 ml solution of the dialyzing buffer. After 30 min of incubation at room temperature with gentle shaking, the amount of [^{14}C]glucose released was determined from an aliquot taken from the solution outside the dialysis bag. The background value was determined from a sample incubated without phallolysin. Total [^{14}C]glucose release was caused by the addition of Triton X-100 (1% final concentration). The following formula was used to express the relative glucose release:

$$\% \text{ glucose release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Spontaneous release did not exceed 10%.

Results and Discussion

Susceptibility of multilamellar liposomes containing native lipids of human and bovine erythrocytes

In order to prove the involvement of membrane proteins in the cytolytic process induced by phallolysin, we prepared liposomes composed of lipid mixtures as extracted from erythrocyte ghosts. For these experiments, human erythrocytes were chosen, these being among the most sensitive cells and, above that, easily available. For comparison, we prepared liposomes of lipids extracted from bovine erythrocytes, which are known to be nearly insensitive toward the attack of phallolysin. Thus, varying concentrations of the toxin were incubated for 30 min at room temperature with multilamellar liposomes composed of either of the lipid extracts. Fig. 1 shows that the release of [^{14}C]glucose from both of the two liposome preparations starts at a concentration of 20 μg phallolysin per ml, while a 50% release of the internal marker was shown graphically to occur at concentrations of 64 and 74 μg/ml, respectively.

From these results we conclude that membranes composed solely of lipids satisfy the requirements for disruption by phallolysin. The observation is in line with recent studies of Seeger and Wachter [12], who observed the leakage of different marker molecules from liposomes composed of mixtures of artificial lipids after their exposure to phallolysin.

The second conclusion we can draw is that phallolysin does not exhibit significant selectivity for one of the two liposome systems. Rather, the data suggest that the difference between the lipid composition of bovine and human erythrocytes cannot account for the different susceptibilities of the intact cells toward phallolysin. In no case will the small difference observed explain the factor of approx. 1000 between the sensitivities of human and bovine red cells.

If not the lipid composition, what else determines the different sensitivities of the cells? We feel that the answer to this question must take into

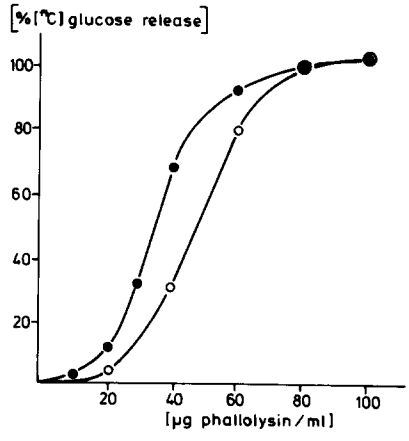

Fig. 1. Release of [^{14}C]glucose from multilamellar liposomes composed of natural lipid extracts of human (●) and bovine (○) erythrocytes.

account the very different toxin concentrations required for destroying liposomes and bovine erythrocytes on the one hand, and sensitive cells like human or rabbit erythrocytes on the other. Taking for granted that the membrane damage proceeds via lipids or phospholipids only, as has been shown in Ref. 12 and in this study, an effective concentration of the toxin in the membrane seems to be attained for sensitive cells at low toxin concentrations, but requires high concentrations for bovine cells or liposomes. One plausible explanation for that would be that receptor proteins with high affinity for phallolysin were present in some cell membranes and achieve an accumulation of the toxin, even at low toxin concentrations in the medium, while for other, more resistant, cells such receptors are lacking. Consequently, the resistant cells require high concentrations of the toxin in the medium in order to attain an effective toxin concentration in the membrane. The presence of such specific receptor proteins (or glycoproteins) has recently been shown for erythrocytes of different animal species (Ref. 19 and our unpublished data).

Susceptibility of multilamellar liposomes containing artificial phospholipids

Erythrocytes of ruminants show an extremely high content of sphingomyelin compared to the sensitive erythrocytes from non-ruminant species, which prefer phosphatidylcholine as the major phospholipid component. We therefore wanted to confirm the above results by assaying liposomes prepared from mixtures of artificial lipids, containing either sphingomyelin or phosphatidylcholine, as the major component. As shown in Fig. 2, both liposome systems are about equally sensitive to phallolysin. This confirms the result obtained with the native mixtures and shows that in fact sphingomyelin and phosphatidylcholine do not represent the membrane components determining the sensitivity of a cell.

The results further show that the involvement of cholesterol in the cytolytic process is negligible. This can be concluded from the relation of the two dose-response curves in the artificial system (Fig. 2) and in the native system (Fig. 1) which are very similar even though the content of cholesterol varies widely (10–30% in Fig. 2 and 50% in Fig. 1).

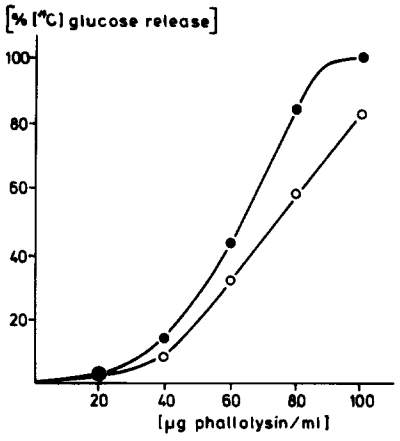

Fig. 2. Release of [^{14}C]glucose from multilamellar liposomes of various lipid composition by phallolysin. (●) phosphatidylcholine/dicetyl phosphate/cholesterol (7 : 2 : 1); (○) sphingomyelin/dicetyl phosphate/cholesterol (30 : 6.6 : 15).

Effect of phallolysin on liposomes with different net charges

Suspecting that the negative charge of the membrane may be of importance for the lytic process, we prepared liposomes in which the negatively charged dicetyl phosphate was replaced by the likewise negatively charged component phosphatidylserine, which is particularly common in erythrocyte membranes, while in other liposomes, dicetyl phosphate was either omitted or replaced by the positively charged stearylamine. As shown in Fig. 3, phallolysin is active also toward liposomes containing phosphatidylserine. A 50% [^{14}C]glucose release was measured at a phallolysin concentration of approx. 100 µg/ml. This value is 3-times higher than the concentration required for lysis of liposomes containing dicetyl phosphate. The difference is possibly explained by the fact that phosphatidylserine has one negative net charge instead of two in dicetyl phosphate.

In accordance to that the liposomes composed of phosphatidylcholine and cholesterol only (molar ratio, 5 : 1), showed no [^{14}C]glucose release even with phallolysin concentrations up to 1 mg/ml (Fig. 3). The same was true for liposomes in which dicetyl phosphate was replaced by stearylamine.

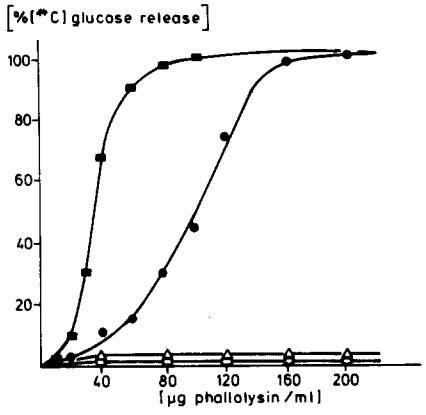

Fig. 3. Release of [^{14}C]glucose form multilamellar liposomes with different net charges. ●, phosphatidylcholine/phosphatidylserine/cholesterol (7:2:1); △, phosphatidylcholine/cholesterol (5:1); □, phosphatidylcholine/stearylamine/cholesterol (30:6.6:15); ■, phosphatidylcholine/dicetyl phosphate/cholesterol.

We conclude that liposomes have to have a negative net charge in order to be attacked by phallolysin. The importance of negative net charges for the activity of phallolysin had become obvious from recent experiments in our laboratory showing that arginine and lysine residues present in phallolysin must be involved in the toxin's activity (unpublished results). When the positive charges of these side-chains were removed by specific reagents, the lytic activity of phallolysin was lost. We understand that the negatively charged components of the membranes may interact with the positive charges of arginine and lysine residues of the toxin. It should be emphasized, however, that the site of toxin activity on liposomes, probably a negatively charged phospholipid component, should be distinguished from the high-affinity binding sites present in membranes of some very sensitive species of erythrocytes, which are believed to be proteins or glycoproteins (for details see Ref. 7).

Susceptibility of multilamellar liposomes and unilamellar macroliposomes toward phallolysin

As pointed out, leakage in multilamellar liposomes was caused only with relatively high toxin concentrations (Table I), when compared with the toxin concentrations causing hemolysis in nonruminant erythrocytes [7]. Actually, the effective toxin concentration required for lysis of the liposomes was $(1.2–3)\cdot 10^{-6}$ M, while that for the sensitive human erythrocytes was about 1000-times lower ($(2.5–3)\cdot 10^{-9}$ M). Similar data have been reported for staphylococcal α-toxin: Cassidy and Harshman [21] found that the toxin concentration necessary to induce leakage in multilamellar liposomes was $(0.6–1.2)\cdot 10^{-6}$ M while the effective hemolytic concentration causing lysis of the sensitive rabbit erythrocytes was only $(3–6)\cdot 10^{-9}$ M.

Part of this large difference may be caused by the fact that the above data were obtained with multilamellar liposomes, while cell membranes are unilamellar. We expected that multilamellar structures might exhibit a higher resistance to the toxin than unilamellar ones. We therefore prepared unilamellar liposomes composed of phosphatidylcholine, dicetyl phosphate and cholesterol in the same ratio as used for the multilamellar liposomes. When employed in the same assay system, we found that phallolysin is in fact about 10-times more active toward unilamellar than toward the multilamellar liposomes (Table I). The fact that the unilamellar liposomes are still much less sensitive to phallolysin than, for example, human erythrocytes, provides one more piece of evidence that protein receptors on the cell surface are involved in the cytolytic process [7].

Evidence for a myelin structure of multilamellar liposomes is given in Fig. 4a by means of electron microscopy. The lamellar structure observed in this preparation could never be detected in the unilamellar preparations (Fig. 4b). We therefore conclude that liposomes prepared according to the dialysis method described by Rögner et al. [18] were in fact unilamellar. These liposomes have a diameter (about 1000 nm) which exceeds that of multilamellar liposomes (150–200 nm) by a factor of 5–7. The larger surface of unilamellar liposomes is certainly an additional factor contributing the increased sensitivity of the unilamellar liposomes.

Phallolysin and staphylococcal α-toxin show some strong parallels with respect to cytolytic activity, but also to physical properties. These common features of the two toxins have recently been discussed in detail [7]. One similarity is that both toxins are able to damage protein-free lipid vesicles. Another similarity of the two toxins is the sigmoidal shape of their dose-response curves. We

TABLE I

SENSITIVITY OF LIPOSOMES (50% GLUCOSE RELEASE) TO PHALLOLYSIN AS DEPENDENT ON LIPID COMPOSITION AND PHYSICAL STATE OF THE LIPOSOMES

State and size: A, unilamellar, 500–1500 nm; B, multilamellar, 100–200 nm.

Phospholipid composition	Physical state; size	Phallolysin (μg/ml)	Phallolysin (M)
Phosphatidylcholine/dicetyl phosphate/ cholesterol (7:2:1)	A	4	$1.2 \cdot 10^{-7}$
Phosphatidylcholine/dicetyl phosphate/ cholesterol (7:2:1)	B	36	$1 \cdot 10^{-6}$
Sphingomyelin/dicetyl phosphate/ cholesterol (30:6.6:15)	B	47	$1.4 \cdot 10^{-6}$
Extract of human erythrocytes	B	64	$1.9 \cdot 10^{-6}$
Extract of bovine erythrocytes	B	74	$2.2 \cdot 10^{-6}$
Phosphatidylcholine/phosphatidylserine/ cholesterol (7:2:1)	B	103	$3.0 \cdot 10^{-6}$
Phosphatidylcholine/cholesterol (5:1)	B	> 1000	$> 3.0 \cdot 10^{-5}$
Phosphatidylcholine/stearylamine/ cholesterol (30:6.6:15)	B	> 1000	$> 3.0 \cdot 10^{-5}$

argue that the lag time between binding and rupture of the cell may represent the formation of complex structures composed of toxin oligomers and/or lipid components, acting as pores in the membrane. Such structures were described by Freer et al. [16], who found staphylococcal α-toxin in ring-like hexamers in electron micrographs. However, no comparable structures were detected in our liposomes treated with phallolysin (Fig. 4). The morphological changes revealed for

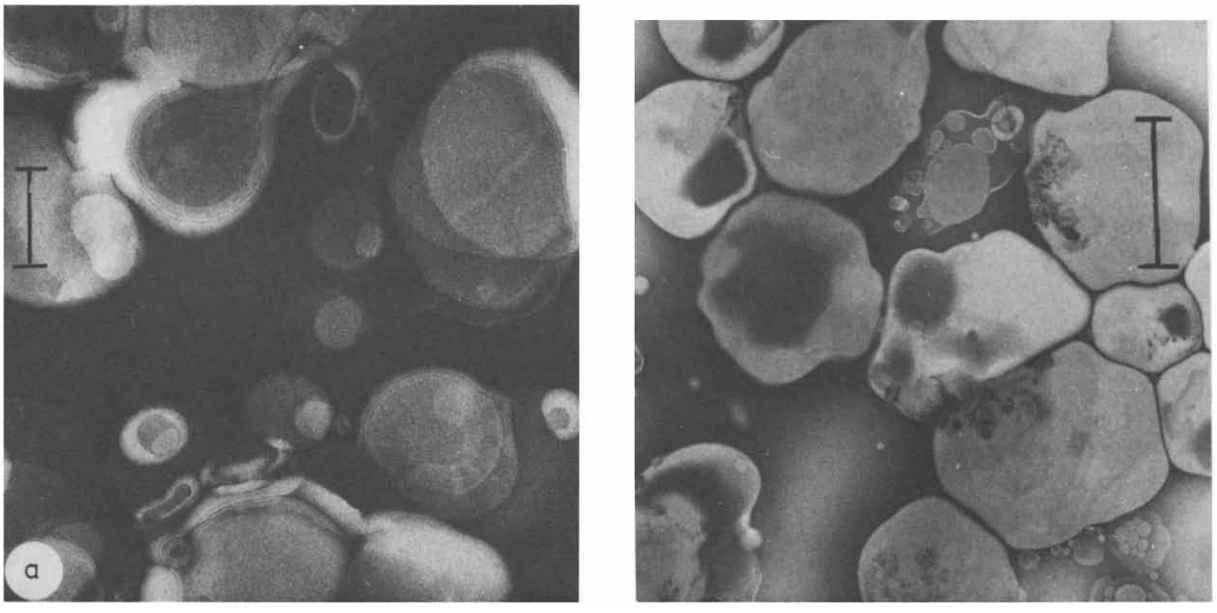

Fig. 4. Electron microscopy of (a) multilamellar and (b) unilamellar liposomes. Vesicles were stained with 2% ammonium molybdate and 2% ammonium acetate. In (a), the bar indicates 100 nm; (original magnification, 150000×). In (b), the bar indicates 1000 nm; (original magnification, 23750×).

freeze-etchings in erythrocyte membranes treated with phallolysin (decrease in intramembrane particles in P- and M-faces, and formation of numerous troughs [22]), probably correspond to a subsequent redistribution of proteins, or phospholipid areas in the membrane, rather than to the formation of toxin or phospholipid toxin aggregates.

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