Biochimica et Biophysica Acta, 686 (1982) 177–181
Elsevier Biomedical Press

BBA 71082

EFFECTS OF CHOLESTEROL EVULSION ON SUSCEPTIBILITY TO PERFRINGOLYSIN O OF HUMAN ERYTHROCYTES

KEN'ICHIRO MITSUI, YOSHIKO SAEKI and JUN'ICHI HASE

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama-shi 930-01 (Japan)

(Received July 20th, 1981)

Key words: Cholesterol content; Phosphatidylcholine; Perfringolysin O; (Erythrocyte membrane)

Human erythrocytes preincubated with a phosphatidylcholine suspension (preincubated cells) showed decreased susceptibility to perfringolysin O, the decrease being strongly affected by preincubation time and temperature, and the phosphatidyl choline concentration. The binding of the toxin to the preincubated cells also decreased with the preincubation time and reached minimum at 37°C for 6 h. Through this preincubation, about 30% of cholesterol was removed from cells without lysis. The susceptibility of preincubated cells to the toxin seemed to be affected by the amount of cholesterol removed from cells, but not by the cholesterol content of cell membranes. This indicates that most of the cholesterol interactive with the toxin is removable from cell membranes by preincubation with phosphatidylcholine suspension, and that the residual cholesterol is firmly constituted in the membrane structure and cannot interact with the toxin. After cholesterol evulsion by the preincubated plasma method (Murphy, J.R. (1962) J. Lab. Clin. Med. 60, 86–109 and 60, 571–578), cells also exhibited lower susceptibility to the toxin and to saponins, but higher susceptibility to lysophosphatidylcholine.

Introduction

Perfringolysin O (*Clostridium perfringens* θ-toxin) is an oxygen-labile hemolysin. Its target for lysis has been regarded as membrane cholesterol, because it is inactivated by a trace amount of cholesterol [1–3]. Ghost membranes of erythrocytes or cholesterol dispersions treated with a large amount of perfringolysin O reveal many ring- and arc-shaped complexes on electron microscopy [4–6], while ghost membranes treated with a small amount (2 hemolytic units) do not. The binding of perfringolysin O to membrane cholesterol is, therefore, thought to be the first step in the cell lysis.

This paper describes the effects of the evulsion of membrane cholesterol on the susceptibility to perfringolysin O of erythrocytes.

Materials and Methods

Preparation of perfringolysin O. Perfringolysin O was partially purified from the culture fluid of *Clostridium perfringens* PB6K N5-L9 as described [7,8]. The hemolytic activity was assayed by method of Roth and Pillemer [9], against human erythrocytes instead of sheep ones, in 0.1 M Tris-HCl buffer (pH 7.2) containing 50 mM NaCl. One hemolytic unit (HU) is defined as the activity which lyses half of the cells in 3 ml of a 1% (v/v) cell suspension. The toxin preparation used in this paper had a specific activity of $(1–1.5) \cdot 10^5$ HU/mg of protein.

Treatment of erythrocytes with phosphatidylcholine. Phosphatidylcholine (PC) was purified from egg yolk [10] and showed a single spot on a

thin-layer chromatogram (Silica-gel plate 60F-254, Merck). Washed human erythrocytes were added to 30% (v/v) to PC suspensions of various concentrations prepared by sonication in 0.1 M Tris-HCl buffer (pH 7.2) containing 50 mM NaCl, and the mixtures were incubated under the indicated conditions. The preincubated cells were collected, washed with the same buffer, and suspended in the same buffer to 1% (v/v).

Treatment of erythrocytes by the preincubated plasma method. Human plasma kept at 37°C for 20–40 h (preincubated plasma [11,12]) has a free cholesterol level of 50–70% of that before the incubation, probably due to its esterification by phosphatidylcholine-cholesterol acyltransferase, whereas plasma incubated at 56°C shows no decrease. These values were reasonably close to those reported by Murphy [11] and Gottlieb [13]. Washed erythrocytes were added to preincubated plasma or plasma treated at 56°C as control, to a 10% hematocrit value and incubated at 37°C for the indicated times. The cells were collected, washed with 0.1 M Tris-HCl buffer (pH 7.2) containing 50 mM NaCl, and resuspended in the same buffer to 1% (v/v).

Preparation of unsealed and resealed ghost membranes, and inside-out vesicles. Human erythrocytes were lysed in cold 5 mM phosphate buffer (pH 8.0) and ghost membranes were harvested (unsealed ghosts). To prepare resealed ghost membranes, unsealed ghost membranes were incubated in 5 mM phosphate buffer (pH 8.0) containing saline at 37°C for 1 h. Inside-out vesicles were prepared as described [14]. The preparation was judged to contain more than 90% of the inside-out vesicles, because its acetylcholine esterase activity, a marker enzyme of the exoplasmic half of the cell, was 0.25 μmol/min per mg of protein, or 8.6% of the activity of the ghost membranes treated with Triton X-100.

Other methods. Osmotic fragility of the cells was determined by the following method [15]. To 3.9 ml of 10 mM phosphate-buffered (pH 7.0) solutions with varying NaCl concentrations was added 0.1 ml of a 10% (v/v) cell suspension, and the mixture was incubated at 37°C for 5 min. After centrifugation, the absorbance at 550 nm of the supernatant was measured, and the osmotic fragility of the cells was expressed as the NaCl concentration giving 50% hemolysis. The lipids in plasma and membranes were extracted with chloroform/methanol (1 : 1, v/v). Total and free cholesterol were determined with reagents for the cholesterol enzymatic color test (Boehringer). Protein concentration was determined by the method of Lowry et al. [16] or by the micro-biuret method as described [17] using bovine serum albumin (Fraction V) as standard.

Chemicals. Triton X-100 and bovine serum albumin (Fraction V) were products of Sigma Chemical Co. All other chemicals were of analytical reagent grade and purchased from commercial sources.

Results

Preincubated cells required larger amounts of perfringolysin O for hemolysis than normal cells, which suggests a decreased susceptibility of the cells to the toxin. The decrease was affected by the conditions of preincubation (Fig. 1a, b). The susceptibility decreased with increase of PC concentration, reaching 16% of the control level on treatment on 10 mg PC/ml for 90 min (Fig. 1a). Preincubation temperatures of 15°C and below had no effect on susceptibility, while an increase of temperature from 23°C caused a proportional decrease in susceptibility (Fig. 1b). The toxin has been reported to bind to normal cells without lysis on incubation at 37°C for 10 min [2]. However, cells preincubated with 1.35 mg PC/ml at 37°C for 1.5 and 6 h showed decreases in binding ability to 22 and 2%, respectively (Fig. 2).

The decreased binding ability and susceptibility to the toxin of the preincubated cells are suggested to be due to the evulsion of cholesterol from cell membranes. On preincubation of cells with 1.35 mg PC/ml at 37°C for 4 h, membrane cholesterol decreased to 70% (Fig. 2). The toxin-inhibitory activity of the ghost membranes was affected not only by their cholesterol contents but also by the conditions of preincubation of cells with PC before hypotonic lysis. In amounts containing 6 μg or more of cholesterol, ghost membranes prepared from cells preincubated at 22.5°C for 90 min inactivated all of the toxin added (1.8 HU), while those prepared from cells preincubated at 37°C gave only about 20% inactivation even at the cholesterol content of 45 μg (Fig. 3).

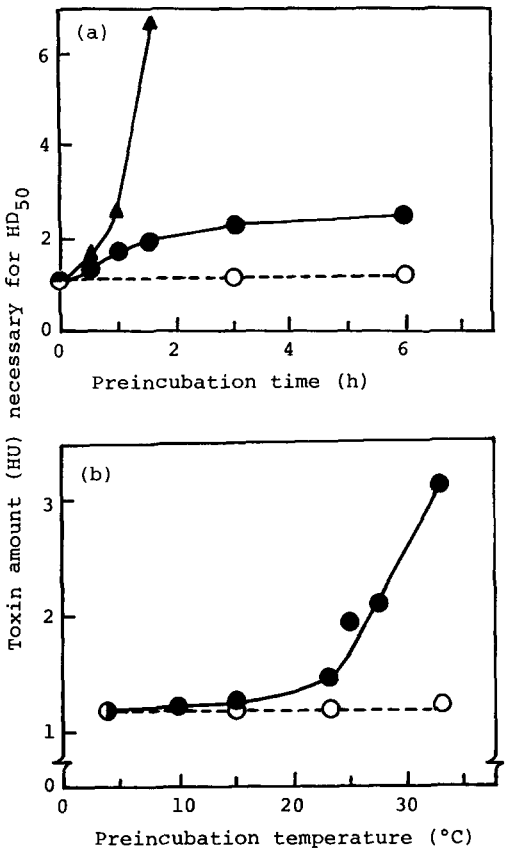

Fig. 1. Susceptibility to perfringolysin O of cells preincubated with PC. Susceptibility: the activity (HU) of the toxin necessary for lysis of half of the cells was measured under the assay conditions against preincubated cells instead of normal cells. (a) Effect of preincubation time of cells with 1.3 mg/ml (●) and 10.0 mg/ml (▲) of PC at 37°C, and with buffer only (○). (b) Effect of preincubation temperature of cells with 1.8 mg/ml of PC (●) and with buffer only (○) for 90 min.

In amounts containing 3 μg of cholesterol, inside-out membrane vesicles, unsealed and resealed ghost membranes inactivated 100, 65 and 48% of the toxin added (1.9 HU), respectively (Fig. 4). However, sonicated ghost membranes containing the same amount of cholesterol exhibited little change in inhibitory activity with preincubation conditions (Table I).

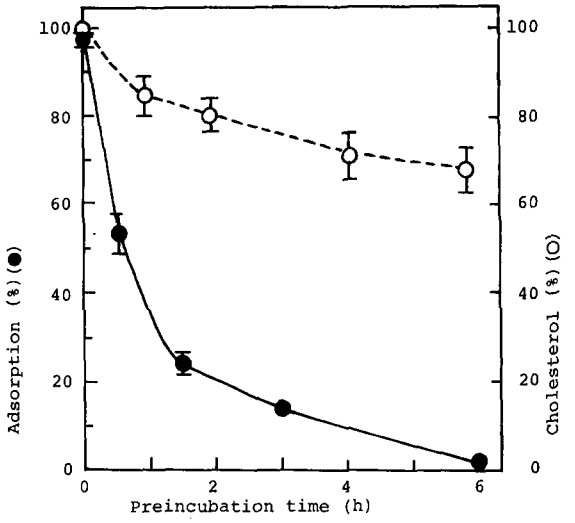

Fig. 2. Binding of perfringolysin O to cells preincubated with PC. Three ml of 1% (v/v) cell suspension was preincubated with 3 ml of 1.35 mg/ml of PC at 37°C for the indicated times. Cells were collected, washed with saline, and resuspended in 3 ml of 0.1 M Tris-HCl (pH 7.2) containing 50 mM NaCl. This preparation of preincubated cells was added to 3 ml of the toxin solution containing 1.3 HU. The mixture was incubated at 37°C for 10 min, and then centrifuged immediately to separate cells. At this time, lysis was not observed in any case. The activity in the supernatant, namely, that not adsorbed onto cell membranes, was measured under the assay conditions against normal cells. The adsorption (%) of toxin onto cell membranes was presented (●). An aliquot of the preincubated cells preparation was hypotonically lysed, and the cholesterol content of its ghost membranes was determined (○).

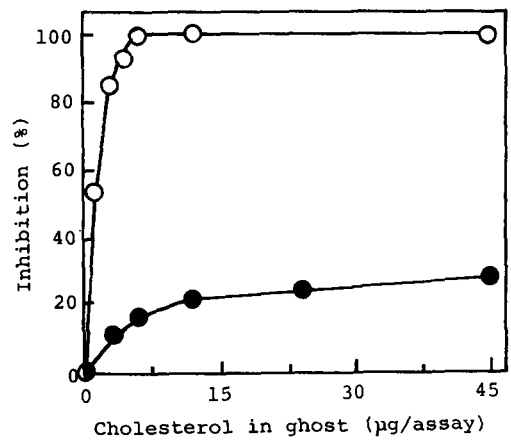

Fig. 3. Inhibition of perfringolysin O activity by ghost membranes prepared from preincubated cells. Cells were preincubated with 1.8 mg/ml of PC for 90 min at 22.5°C (○) or 37°C (●). After incubation, the cells were collected by centrifugation and hypotonically lysed. Cholesterol content of the ghost membranes was measured. The toxin (1.8 HU) was incubated with different amounts of the ghost membranes at 37°C for 10 min. Then normal cells (1%, 3 ml) were added to the mixture, and the remaining toxin activity was assayed.

TABLE I

RECOVERY OF THE INHIBITORY ACTIVITY OF THE GHOST MEMBRANES BY SONICATION

Cells preincubated with 1.0 mg/ml of PC at 37°C for the indicated times were hypotonically lysed. Cholesterol contents of the ghost membranes obtained was measured. Preparations of ghost membranes containing 0.6 μg of cholesterol and the sonicated preparations were each added to the toxin solution (2 HU), and the mixtures were incubated at 37°C for 10 min. After incubation, the remaining hemolytic activity in the mixture toward normal cells was measured under the assay conditions. n.d., not determined.

Preincubation time of cells with PC (h)	Inhibition (%) by ghost membranes; sonication (min)		
	0	3	5
None	75	82	95
1	65	82	100
2	20	55	98
4	9	n.d.	80

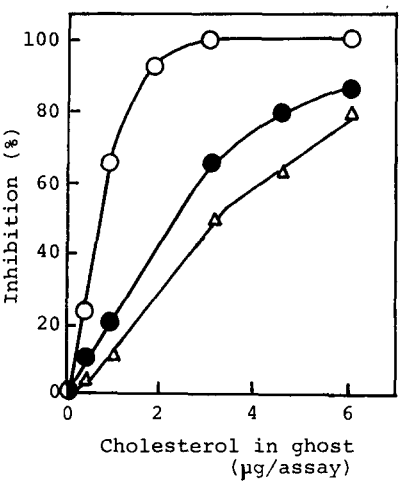

Fig. 4. Inhibition of perfringolysin O activity by inside-out membrane vesicles, and unsealed and resealed ghost membranes. Inside-out vesicles (○), unsealed (●) and resealed ghost membranes (△) were prepared as described [14] and their cholesterol contents were measured. The toxin (1.9 HU) was incubated with each ghost membrane containing the indicated amounts of cholesterol at 37°C for 10 min, then the hemolytic activity in the mixture was measured against normal cells.

TABLE II

EFFECT OF CHOLESTEROL EVULSION BY THE PREINCUBATED PLASMA METHOD ON THE SUSCEPTIBILITY OF CELLS TO HEMOLYTIC AGENTS

The susceptibility is shown as the activity (HU) of the toxin or concentration (μg) of the agents necessary for HD_{50}. LysoPC, lysophosphatidylcholine.

Preincubation time (h)	Cholesterol in ghost membranes (%)	Susceptibility of preincubated cells			
		Perfringolysin O (HU)	Saponins (μg)	Digitonin (μg)	LysoPC (μg)
None	100	1.0	16.5	2.1	4.4
1	93	1.8	19.8	2.5	3.7
3	87	2.5	–	2.7	3.4
6	80	3.5	21.3	3.2	2.9

TABLE III

DIFFERENCE IN EFFECTS OF CHOLESTEROL EVULSION METHODS ON ERYTHROCYTES

Cells were preincubated with 1.3 mg/ml of PC at 37°C for 2 h, or with preincubated plasma at 37°C for 4 h. The susceptibility to perfringolysin O is expressed as in Table II.

Cholesterol evulsion method	Cell shape [19]	Cholesterol in membranes (%)	Susceptibility to perfringolysin O (HU)	Osmotic fragility (NaCl, mM)
No treatment	Discocyte	100	1.0	61
Phosphatidylcholine	Spheroechinocyte I	80±2	3.0±0.2	93
Preincubated plasma	Spheroechinocyte II	79±5	2.7±0.2	61

20% of membrane cholesterol was evulsed from cells by incubation with preincubated plasma at 37°C for 4–6 h (Tables II and III). The susceptibility of these cells to the toxin decreased to one third, and their susceptibility to saponins (Merck) decreased to 77%. On the other hand, their susceptibility to lysophosphatidylcholine increased 1.5-fold (Table II).

The osmotic fragility of the cells was decreased by preincubation with PC, but not by treatment with preincubated plasma (Table III).

Discussion

The susceptibility of preincubated cells to perfringolysin O was strongly affected by preincubation time and temperature, and PC concentration (Fig. 1). The temperature dependency appeared at above 20°C, which may correspond to the phase transition of cell membranes. The decrease in the susceptibility of cells was detected by decrease in the binding ability of the toxin to cell membranes (Fig. 2) and by the increase in the amount of toxin required for HD_{50} (Fig. 1, Tables II and III). Ghost membranes prepared from preincubated cells also showed low inhibitory activity toward the toxin. The inhibitory activity, moreover, was not affected by the total amount of cholesterol in the membranes (Fig. 3). These results are suggested to be due to the disappearance of cholesterol from the intact cell membrane surface, because it is known that 10 or more molecules of the toxin bind irreversibly to cholesterol at a limited number of sites on the outer membrane surface, and thereby induce hemolysis [2,4]. On the other hand, the recovery of inhibitory activity on sonication of the ghost membranes (Table I) suggests that the toxin binds readily and nonspecifically to exposed cholesterol on the inner membrane. This suggestion is supported by the fact that the inhibition of resealed ghost membranes was lower than those of inside-out vesicles and unsealed membranes with the same cholesterol level (Fig. 4). Indeed, about 20–30% of cholesterol was evulsed from cells without lysis by treatment with PC or preincubated plasma (Fig. 2, Table II). We therefore propose that cholesterol interactive with the toxin can readily be evulsed by incubation with PC, while cholesterol which is firmly constituted in the membrane structure cannot bind to the toxin.

The susceptibility to saponins, whose target is regarded as membrane cholesterol [18], also decreased by cholesterol evulsion from cells, although the effect was less than that for the toxin. On the other hand, an increased susceptibility to lysophosphatidylcholine, whose target is not cholesterol, suggests that cell membranes are rendered more fluid by cholesterol evulsion.

The susceptibility to the toxin of erythrocytes of hypercholesterolemic rats, prepared by feeding with a high cholesterol diet, was about 2-fold that of untreated rats (unpublished data). This suggests that the susceptibility of the cells to the toxin is increased by increasing the amount of removable cholesterol in membranes.

References

1 Bernheimer, A.W. (1970) in Microbial Toxins (Ajl, S.J., Kadis, S. and Montie, T.C., eds.), vol. 1, pp. 200–202, Academic Press, New York and London
2 Hase, J., Mitsui, K. and Shonaka, E. (1975) Jap. J. Exp. Med. 45, 433–438
3 Hase, J., Mitsui, K. and Shonaka, E. (1976) Jap. J. Exp. Med. 46, 45–50
4 Mitsui, K., Sekiya, T., Nozawa, Y. and Hase, J. (1979) Biochim. Biophys. Acta 554, 68–75
5 Mitsui, K., Sekiya, T., Okamura, S., Nozawa, Y. and Hase, J. (1979) Biochim. Biophys. Acta 558, 307–313
6 Smyth, C.J., Freer, J.H. and Arbuthnott, J.P. (1975) Biochim. Biophys. Acta 382, 479–493
7 Mitsui, K., Mitsui, N. and Hase, J. (1973) Jap. J. Exp. Med. 43, 65–80
8 Mitsui, K., Mitsui, N. and Hase, J. (1973) Jap. J. Exp. Med. 43, 377–391
9 Roth, F.B. and Pillemer, L. (1955) J. Immunol. 75, 50–56
10 Hanahan, D.J., Turner, M.B. and Jayko, M.E. (1951) J. Biol. Chem. 192, 623–628
11 Murphy, J.R. (1962) J. Lab. Clin. Med. 60, 86–109
12 Murphy, J.R. (1962) J. Lab. Clin. Med. 60, 571–578
13 Gottlieb, M.H. (1976) Biochim. Biophys. Acta 433, 333–343
14 Steck, T.L. (1974) in Methods of Membrane Biology (Korn, E., ed), vol. 2, pp. 245–281, Academic Press, New York
15 Sato, T. (1973) Chem. Pharm. Bull. 21, 176–183
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Itzhaki, R.F. and Gill, D.M. (1964) Anal. Biochem. 9, 401–410
18 Seeman, P. (1974) Fed. Proc. 33, 2116–2124
19 Bessis, M. (1973) in Red Cell Shape (Bessis, M., Weed, R. and Leblond, P.F., eds), p. 1, Springer-Verlag, New York