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A modified θ-toxin produced by limited proteolysis and methylation: a probe for the functional study of membrane cholesterol

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A derivative of cytolytic θ-toxin from *Clostridium perfringens* was prepared by limited proteolytic digestion of the native toxin followed by methylation. Among the chloroform/methanol-extractable, lipid components of sheep and human erythrocytes, the proteinase-nicked and methylated derivative (MCθ) specifically binds to cholesterol. While MCθ retains binding affinity comparable to that of intact toxin, it causes no obvious membrane damage, resulting in no hemolysis at temperatures of 37°C or lower. Using MCθ, we demonstrated the possible existence of high- and low-affinity sites for θ-toxin on sheep erythrocytes at both 37°C and 10°C. The number of high-affinity sites on sheep erythrocytes was estimated to be approximately 3-times larger at 37°C than that at 10°C. In addition, high- and low-affinity sites were demonstrated in human erythrocytes and a lymphoma B cell line, BALL-1 cells. Both binding sites disappear upon simultaneous treatment of cells with sublytic doses of digitonin, suggesting that cholesterol is an essential component of both the high- and low-affinity sites and that the mode of cholesterol existence in plasma membranes is heterogeneous in these cells. Because of its high affinity for membrane cholesterol without causing any obvious membrane changes at physiological temperatures, MCθ may provide a probe for use in the functional study of membrane cholesterol.

Introduction

Although cholesterol is one of the major constituents of biological membranes and its importance for the structure and function of biological membranes is recognized [1,2], interesting questions concerning its organization in membranes along with its role in membrane function remain to be answered. Cholesterol functions as a receptor for polyene antibiotics [3], saponins [4], and thiol-activated cytolysins (hemolysins) such as θ-toxin (perfringolysin O) [5] and cereolysin [6]. Because of their specific interaction with cholesterol, these molecules have been used as probes to investigate the distribution of cholesterol in membranes [2,7]. However, these molecules have been known to disrupt membrane integrity after binding, resulting in redistribution of membrane cholesterol. Thus a probe that has a high affinity for cholesterol without causing disruption of membrane integrity is required to study the organization of cholesterol in membranes.

From this point of view, we have investigated the characteristics of θ-toxin and have prepared several modified toxins [8–11]. θ-Toxin is an exotoxin produced by *Clostridium perfringens* type A. It has been reported to damage a wide variety of cells, including erythrocytes [12], human fibroblasts [13], hepatocytes [10], and myocardial cells [14] after adsorption to membrane cholesterol. Some phenomena associated with the lytic process, such as stimulation of ion flux [15,16] and formation of arc- and ring-shaped structures of the toxins on red cell membranes, have been described [17,18]. Recently we obtained a proteinase-nicked derivative of θ-toxin. The nicked toxin (Cθ) hemolyzes

Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; TLC, thin-layer chromatography; RBC, red blood cell.

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cells at 37 °C but not at temperatures below 20 °C, even though it binds to membrane cholesterol at both temperatures [8,11]. Cθ was found to be a useful probe for studying the organization of membrane cholesterol at low temperatures because: (1) Cθ binds specifically to cholesterol but not to other membrane constituents; (2) it possesses a high affinity for membrane cholesterol ($K_d \approx 10^{-9}$ M); and (3) it causes no obvious membrane damage below 20 °C. Using Cθ as a probe we demonstrated the existence of two classes of cholesterol sites with high and low affinities for the toxin on sheep erythrocytes at 10 °C [11]. This finding implies that the mode of existence of cholesterol in sheep red cell membranes is heterogeneous, at least at 10 °C. To demonstrate the biological significance of cholesterol heterogeneity, the following problems should be clarified: (1) whether the mode of cholesterol existence is heterogeneous at physiological temperatures and (2) whether the heterogeneity is ubiquitous among many types of cells. To approach these problems we have prepared another modified toxin which binds to membrane cholesterol but causes no membrane damage at 37 °C or lower temperatures. In this report we characterize the modified toxin and demonstrate the heterogeneous mode of existence of membrane cholesterol in erythrocytes from several species and in cells of a lymphoma B cell line at physiological and/or lower temperatures.

Materials and Methods

Materials. Carrier-free $Na^{125}I$ and [^{14}C]formaldehyde were purchased from New England Nuclear. θ-Toxin and its proteolytic derivatives were prepared as described in our previous report [8]. Cθ and MCθ were iodinated by the solid-phase lactoperoxidase/glucose oxidase method as described previously [11]. The average molar ratio of incorporated ^{125}I to Cθ and MCθ were 1.3 and 0.7, respectively.

Cells. Erythrocytes from sheep blood stored in Alsever solution for 2–3 days and from fresh heparinized human and rat blood were washed three times with phosphate-buffered saline (PBS) (pH 7.0) immediately before use.

Lymphoma B cell line BALL-1 cells [19], kindly supplied by Dr. J. Fujisawa (Cancer Institute, Tokyo), were grown to $(4–8) \cdot 10^5$/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (HyClone, Logan, UT) and 50 μg/ml gentamycin under a 5% CO_2 atmosphere. The cells were then washed three times with Hanks' balanced salt solutions (without sodium bicarbonate, Cat. No. 450-1200, Gibco) containing 10 mM Hepes (pH 7.2) and 1 mg/ml bovine serum albumin (BSA, fatty-acid free, Sigma) and resuspended in the same buffer at a cell density of $8 \cdot 10^5$ cells/ml. The viability of cells was more than 97% when judged by Trypan-blue exclusion.

Determination of hemolytic activity of θ-toxin and its derivatives. The hemolysis assay reaction mixture was composed of 0.75 ml of sheep erythrocyte suspension (0.67% hematocrit) and 0.25 ml of toxin solution appropriately diluted in PBS containing 1 mg/ml BSA and 10 mM dithiothreitol. The degree of hemolysis after incubation for 30 min at the indicated temperatures was determined by centrifuging the samples and measuring the absorbance of the supernatant at 540 nm. One hemolytic unit was defined as the amount of toxin required to cause 50% hemolysis of sheep erythrocytes (1 ml 0.5% suspension) in 30 min at 37 °C.

Reductive methylation of toxins. Reductive methylation of toxins was carried out according to Means and Feeney [20] with the following modifications. Solutions (0.5–2.2 mg protein/ml) of θ-toxin and Cθ in 50 mM sodium borate-HCl (pH 8.6) were cooled in an ice bath. To 0.3 ml of the solution an equal volume of sodium borohydride ($NaBH_4$) freshly dissolved in 10 mM NaOH was added to give a final concentration of 0–30 mM. Then a total of 0.15 ml of formaldehyde (HCHO) in 0.2 M sodium borate-HCl (pH 9.0) was added in six aliquots at 3-min intervals and the reaction mixture was incubated for 20 min at 0 °C. The final amount of HCHO was kept at 0.53 equivalent of $NaBH_4$ ($NaBH_4$/HCHO, 1 : 2.13, mol/mol) in order that the reducing agent ($NaBH_4$) should be kept in slight excess. The reaction was stopped by the addition of 94 mM Tris-HCl (pH 8.8). The mixture was dialyzed against 20 mM sodium phosphate buffer (pH 7.0) to remove the excess reagents and stored at 4 °C.

The number of methyl groups incorporated into the proteins was determined using [^{14}C]HCHO (10 mCi/mmol). After the 20-min incubation of the above reaction mixture at 0 °C, aliquots (15 μl) were diluted to 1 ml with ice-cold 100 mM sodium borate buffer (pH 9.0) containing 10 mM $NaBH_4$ and 21.3 mM HCHO, and applied to nitrocellulose filters. The filters were washed four times with the same buffer, dried and the radioactivity retained on the filters was determined. Each 15-μl aliquot contained 50.9 pmol protein. The radioactivity of [^{14}C]HCHO retained on the filter in the absence of protein was less than 100 dpm (= 4.5 pmol).

Detection of direct binding of MCθ to lipid components. Lipids from human and sheep erythrocytes and standard lipid samples were prepared as described previously [11]. The concentrations of cholesterol in the extracts were 0.81 mg/ml and 0.65 mg/ml in sheep erythrocytes and human erythrocytes, respectively, as determined by the method of Richmond [21]. Lipids were applied to TLC plates and developed with two solvent systems successively [22]. For the detection of lipid components, the plate was cut after development and half of the plate was dried and soaked in a 3%

(w/v) cupric acetate/8% (v/v) phosphoric acid solution, and then heated at 140°C. The other half was soaked overnight at 4°C in 50 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl, 20 mg/ml BSA and 3 mg/ml gelatin. The chromatogram was then incubated with 10 μg/ml MCθ in the same buffer for 1 h at 37°C or room temperature, and washed with five successive change of 50 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl. The toxin bound to lipids was detected by immunostaining [11,23] after treatment of the plates with anti-θ-toxin antibody.

Electron microscopy. Sheep erythrocyte ghosts prepared according to Dodge et al. [24] were resuspended in 30 mM barbital buffer (pH 7.0) to make a 1.7 mg/ml protein suspension. The ghost suspension (30 μl) was incubated with 16 μg of θ-toxin, Cθ or MCθ in a 100 μl mixture. Aliquots of toxin-treated ghost membranes were negatively stained with 1% phosphotungstic acid (pH 6.5–7.2). Observations were made on a JEM-200CX electron microscope operating at 80 kV [18].

Binding of MCθ to erythrocytes and lymphoma cells. ^{125}I-MCθ was preincubated in PBS containing 1 mg/ml BSA for 5 min at 37°C. An aliquot of each toxin sample was then mixed with various amounts of unlabeled MCθ and incubated with erythrocytes (0.05–0.5% hematocrit) in a 0.1-ml reaction mixture containing PBS and 0.25 or 0.5 mg/ml BSA at the indicated temperatures. After incubation, the cells were sedimented by centrifugation and the radioactivities in the supernatant and the pellet were measured in a gamma counter to determine the percentage of ^{125}I-MCθ bound to the cells. Nonspecific binding was determined by adding an excess of unlabeled MCθ and was shown to be less than 3%.

The binding of the toxin to lymphoma B cells was carried out as described above except that the incubation was performed in Hepes-buffered Hanks' balanced salt solutions (pH 7.2) containing 0.75 mg/ml BSA. The viability of the lymphoma cells was not changed by the incubation with MCθ.

Results

Reductive methylation of θ-toxin and nicked θ-toxin

Limited proteolysis of θ-toxin (54 kDa) with subtilisin Carlsberg produces a nicked toxin (Cθ) which is a complex of 15-kDa N-terminal (C2) and 39-kDa C-terminal (C1) fragments [8]. Cθ is quite distinct from both native toxin and another nicked toxin (Tθ) produced by limited trypsin digestion [8] with respect to its temperature dependency of hemolytic activity. While θ-toxin and Tθ hemolyze cells over a wide temperature range of 4–37°C, Cθ causes almost no hemolysis at temperatures below 20°C (Fig. 1) while retaining nearly normal cholesterol binding activity at the low temperatures [8,11].

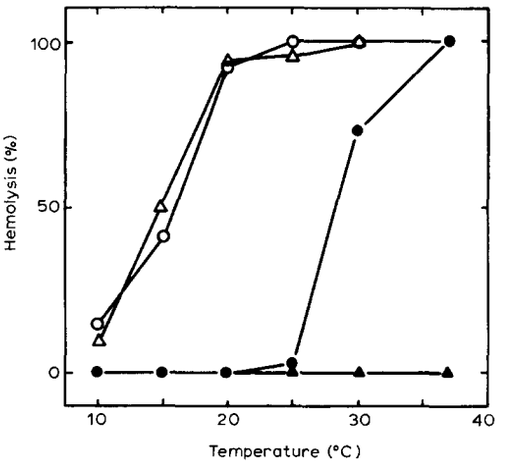

Fig. 1. Hemolytic activities of θ-toxin and its derivatives at various temperatures. Sheep erythrocytes (0.5% hematocrit) were incubated with 11.2 ng/ml of θ-toxin (○), Cθ (●), Tθ (△), or methylated Cθ (MCθ) (▲) for 30 min at the indicated temperatures and the degree of hemolysis was determined. MCθ was prepared in the presence of 30 mM $NaBH_4$ and 64 mM HCHO as described in Materials and Methods.

When Cθ was methylated with $NaBH_4$ and HCHO, the product was found to lack hemolytic activity not only at low temperatures, but also at higher, physiological temperatures (Fig. 1). The hemolytic activities of methylated θ-toxin (Mθ) and methylated Cθ (MCθ) are compared in Table I. When θ-toxin was methylated in the presence of up to 2.13 mM HCHO, the hemolytic activity was unchanged. Heavily methylated θ-toxin prepared in 64 mM HCHO still retained 14% of its hemolytic activity at 37°C. On the contrary, the hemolytic activity of MCθ was greatly reduced as the concentrations of HCHO and $NaBH_4$ were increased. When 64 mM HCHO was used to methylate Cθ, no hemolytic activity of MCθ was detected even at concentration of 100 μg/ml in the assay mixture. This corresponds to a 10^5-times higher concentration than the 1.0 ng/ml of Cθ that is required for 50% hemolysis under the same conditions. Since the reducing reagent ($NaBH_4$) alone had no effect on hemolytic activity, the decrease in activity is ascribed to the methylation of the toxins. The difference in hemolytic activity between Mθ and MCθ is not due to the efficiency of methylation, because under the same reaction conditions almost the same number of methyl groups was incorporated into each toxin (Table I).

A plot of hemolytic activity vs. methyl groups incorporated suggests that Cθ loses its activity due to preferential incorporation of 8–9 methyl groups into some specific α- and/or ε-amino groups of the toxin molecule (Fig. 2). The activity of θ-toxin, on the other hand, was not affected by the incorporation of up to 19 methyl groups per molecule.

TABLE I

Reductive methylation of θ-toxin and Cθ

θ-Toxin and Cθ were methylated with [^{14}C]HCHO and the amounts of [^{14}C]methyl groups incorporated into the proteins were determined in 15-μl aliquots (Materials and Methods). The remainder of the samples was used to determine hemolytic activity at 37°C. The activity (hemolytic units/mg protein) of each modified toxin was determined as described in Materials and Methods and expressed as a relative value (%) to that of unmodified toxin.

Reagents (mM)		Hemolytic activity of modified toxins (%)		[^{14}C]Methyl groups incorporated (mol/mol of protein)	
$NaBH_4$	HCHO	θ-toxin	Cθ	θ-toxin	Cθ
0	0	100	100		
30	0	100	100		
0.15	0.32		60		3.2
0.3	0.64	100	30	6.0	7.2
0.5	1.07		16		11.5
1	2.13	100	2.6	18.6	19.7
10	21.3	34	0.011	46.7	45.5
30	64	14	< 0.001		53.8

To examine whether the methyl groups are distributed differently in the Mθ and MCθ molecules, θ-toxin was methylated with various amounts of [^{14}C]HCHO and digested into C1 and C2 fragments with subtilisin Carlsberg. The distribution of radioactivity in the fragments was compared with that in the corresponding fragments of [^{14}C]methylated Cθ. The degree of methylation of each fragment did not vary significantly between the toxins (data not shown).

Binding of MCθ to membrane cholesterol

Since MCθ, but not Mθ, prepared in 30 mM $NaBH_4$ and 64 mM HCHO has no hemolytic activity at temperatures of 4–37°C, the MCθ preparation was used to examine the cholesterol binding characteristics.

The binding affinity of MCθ was compared with that of Cθ which has the same binding affinity to sheep erythrocytes as θ-toxin [11]. Both unlabeled MCθ and Cθ inhibited the binding of ^{125}I-Cθ to sheep erythrocytes with the same dose dependency (Fig. 3), indicating that the affinity of MCθ for erythrocytes is not signifi-

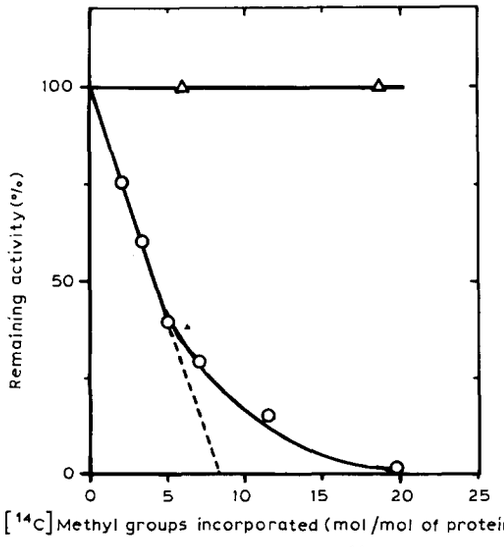

Fig. 2. Correlation between the number of [^{14}C]methyl groups incorporated into θ-toxin and Cθ and their hemolytic activities. θ-Toxin (△) and Cθ (○) were methylated with various amounts of [^{14}C]HCHO. The number of [^{14}C]methyl groups incorporated and the hemolytic activities were determined as described in Materials and Methods.

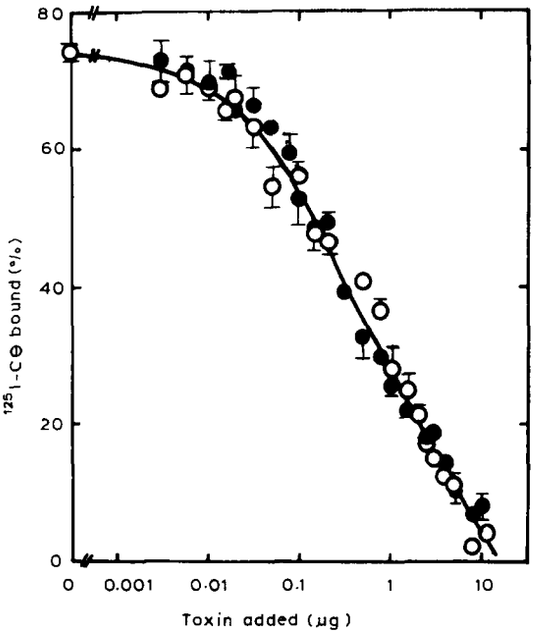

Fig. 3. Binding competition between ^{125}I-Cθ and unlabeled toxins. ^{125}I-Cθ was preincubated in PBS (pH 7.0) containing 1 mg/ml BSA for 5 min at 37°C. An aliquot of the toxin sample (0.77 ng) was then mixed with the indicated amounts of unlabeled Cθ (○) or MCθ (●) and incubated for 1 h at 10°C with 0.5% sheep erythrocyte suspensions in a 0.1-ml reaction. The cells were sedimented by centrifugation and the amounts of ^{125}I-Cθ bound to the cells were determined. The results are means ± S.E. of three experiments.

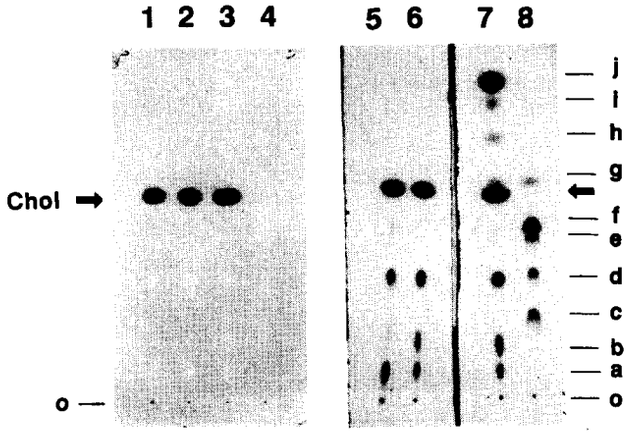

Fig. 4. Detection of lipid components that bind MCθ. Aliquots (2 μl) of the chloroform/methanol extracts of sheep erythrocytes (lanes 1, 5) or human erythrocytes (lanes 2, 6) and 2 μg each of standard neutral lipids (lanes 3, 7) or 1.6 μg each of standard acidic lipids (lanes 4, 8) were applied to a TLC plate and developed with two solvent systems. Half of the plate was then incubated with 10 μg/ml MCθ for 1 h at 37°C, and the lipid components that bind MCθ were detected by immunostaining (lanes 1–4) (Materials and Methods). Incubations at room temperature gave essentially the same results. The positions of lipid components were determined in lanes 5–8. The migration position of cholesterol (Chol) is shown by arrows. The migration positions of other standard lipids are shown as follows: *o*, origin; a, sphingomyelin; b, phosphatidylcholine; c, phosphatidylinositol; d, phosphatidylethanolamine plus phosphatidylserine; e, phosphatidic acid; f, cardiolipin; g, oleyl alcohol; h, oleic acid; i, triolein; j, cholesteryl oleate.

cantly different from that of Cθ. Competition experiments using ^{125}I-MCθ instead of ^{125}I-Cθ gave essentially the same results (data not shown).

θ-Toxin and Cθ bind specifically to cholesterol [11]. Fig. 4 demonstrates that MCθ also binds only to cholesterol among the chloroform/methanol-extractable components of sheep and human erythrocytes at temperatures of 37°C or lower. θ-Toxin and Cθ recognize only cholesterol and its analogues which have both a 3β-OH group and an aliphatic side chain [5,11]. MCθ retains the same cholesterol binding specificity as θ-toxin, as judged by the direct binding assay of the toxin to various cholesterol analogues including esterified cholesterol (Fig. 4, lane 3 and data not shown). Thus the methylation of Cθ amino groups does not affect its binding characteristics.

Toxin assembly on membranes

θ-Toxin and other thiol-activated cytolysins form ring- and arc-shaped structures on erythrocyte ghosts and on cholesterol dispersions [17,18]. Since formation of these structures has been suggested to be related to hemolytic activity, the formation of these structures by θ-toxin, Cθ and MCθ was investigated at various temperatures (Fig. 5). A large number of ring- and arc-shaped structures were visible on sheep erythrocyte ghost membranes treated with θ-toxin both at 37°C and 20°C (Figs. 5a, d). Cθ formed such structures at 37°C (Fig. 5b), but not at 20°C where no hemolysis occurs (Fig. 5e). No such structures were detected in specimens treated with MCθ either at 37°C or 20°C (Figs. 5c, f). Thus the formation of ring- and arc-shaped structures correlates well with hemolytic activity. Furthermore, the data suggest that MCθ does not disrupt membrane integrity at either 37°C or lower temperatures.

High- and low-affinity toxin binding sites on erythrocytes at 37°C

Since MCθ has a high affinity for membranes and does not disrupt membrane integrity at physiological temperatures, it can be used to characterize toxin binding sites on cell membranes under physiological conditions. The binding of MCθ to sheep and human erythrocytes was analyzed at 37°C and 10°C. Scatchard analysis of the binding of MCθ to sheep and human erythrocytes gave curvilinear plots at both 37°C and 10°C (Fig. 6), suggesting that at both temperatures, the erythrocyte membranes of these species contain at least two classes of binding sites. Analysis of toxin binding to rat erythrocytes gave similar results (data not shown). The binding of MCθ to both the high- and low-affinity sites was strikingly inhibited in the presence of sublytic doses of digitonin (4 μM) (data not shown), a compound that has been shown to bind specifically to cholesterol [25]. These results and those from Fig. 4 showing that MCθ binds only to cholesterol strongly suggest that cholesterol molecules are essential constituents of both the high- and low-affinity sites.

The dissociation constants and the number of high- and low-affinity binding sites were estimated by computer analysis of the plots (Table II), using a program

TABLE II

Binding affinity and number of binding sites of MCθ to erythrocytes and lymphoma B cells

Binding experiments were carried out with 0.06% (hematocrit) sheep red blood cells (RBC), 0.05% human RBC or $4 \cdot 10^5$ lymphoma B cells in 0.1 ml of reaction mixture at 10°C for 3 h, at 37°C for 40 min, or at 16°C for 3 h. The values are expressed as mean ± S.E. ($n = 3$ for RBC and $n = 4$ for lymphoma cells).

Cells	Incubation	High affinity		Low affinity	
		B [a] (pmol)	K_d [b] (nM)	B [a] (pmol)	K_d [b] (nM)
Sheep RBC	10°C	0.26 ± 0.01	2.7 ± 0.6	5.7 ± 1.0	300 ± 130
	37°C	0.83 ± 0.02	3.9 ± 0.4	3.8 ± 1.6	170 ± 20
Human RBC	10°C	0.64 ± 0.03	1.4 ± 0.4	14 ± 1	360 ± 150
	37°C	1.9 ± 0.2	4.7 ± 2.6	11 ± 6	400 ± 180
Lymphoma B cells	16°C	0.26 ± 0.06	2.3 ± 0.9	10 ± 1	210 ± 90

[a] Number of maximum binding sites.
[b] Dissociation constant.

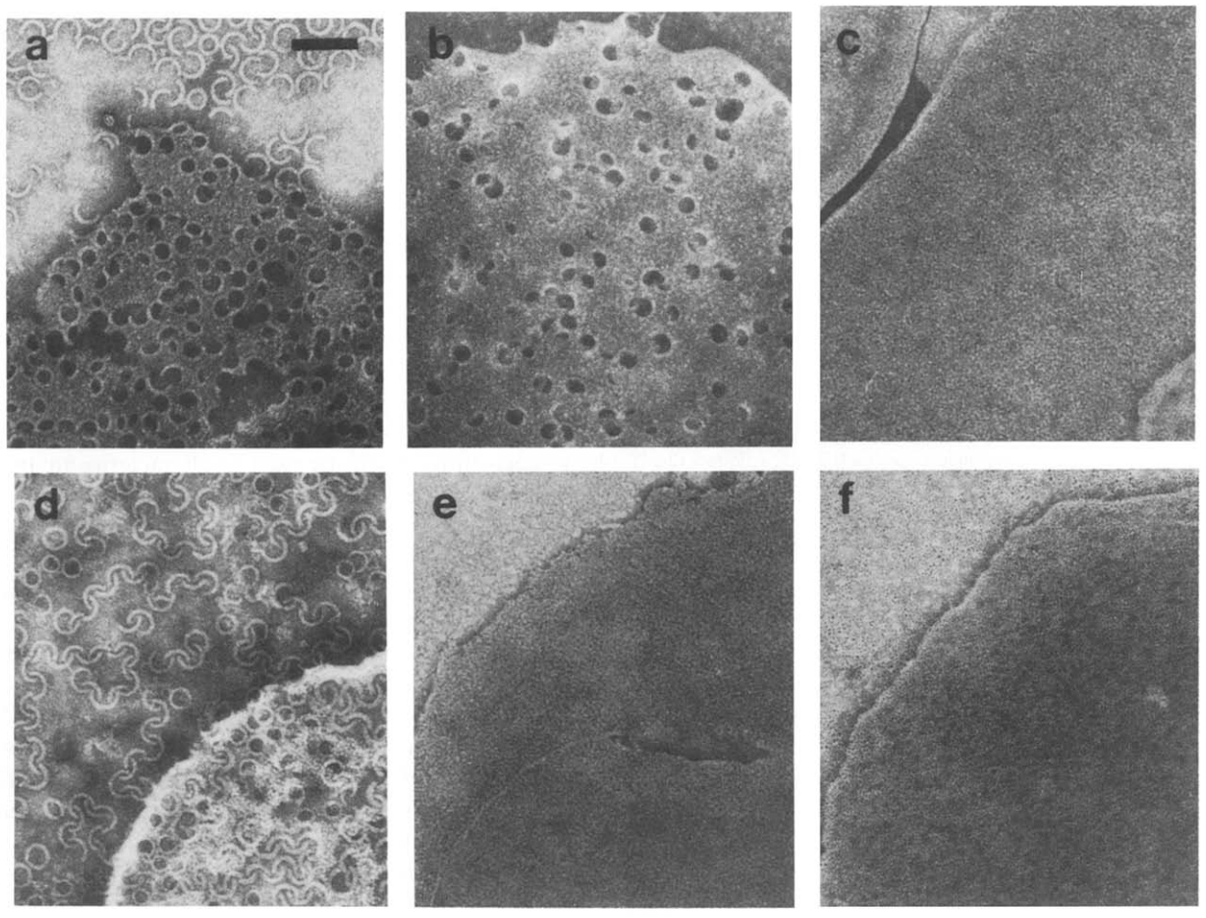

Fig. 5. Negatively stained specimens of erythrocyte ghost membranes treated with θ-toxin and its derivatives. Sheep erythrocyte ghosts were treated with θ-toxin (a, d), Cθ (b, e), or MCθ (c, f) at 37 °C for 10 min (a–c), or at 20 °C for 15 min (d–f). Bar represents 100 nm.

which fits a hyperbola to the Scatchard plot and gives two asymptotes of the hyperbola, when two types of binding sites with different affinities exist in the system [26]. In sheep erythrocytes the number of high-affinity

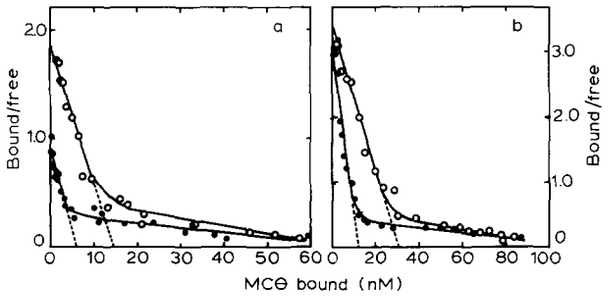

Fig. 6. Scatchard analysis of MCθ binding to sheep and human erythrocytes. Various amounts of MCθ containing 0.54 ng each of ^{125}I-MCθ as a tracer were incubated with sheep (a) and human (b) erythrocyte suspensions (0.06% and 0.05% hematocrit, respectively) in 0.1 ml of reaction mixture for 3 h at 10 °C (●) or 40 min at 37 °C (○). The amounts of free and bound toxin were determined as described in Materials and Methods. The data are representative of three experiments.

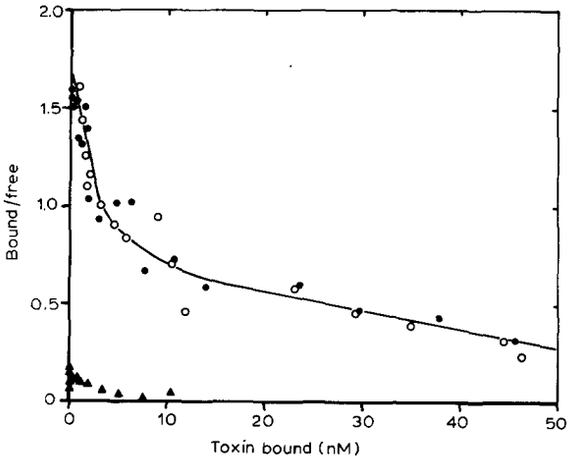

Fig. 7. Scatchard analysis of MCθ binding to lymphoma B cells. Various amounts of MCθ containing 0.40 ng each of ^{125}I-MCθ as a tracer were incubated with lymphoma B cells (BALL-1, $4 \cdot 10^5$ cells) in 0.1 ml of reaction mixture for 30 min (●, ▲) or 3 h (○) at 16 °C in the absence (●, ○) or presence (▲) of 4 μM digitonin. The amounts of free and bound toxin were determined as described in Materials and Methods. The data are representative of three experiments.

binding sites is approximately 3-times larger at 37°C than at 10°C, whereas the total number of binding sites does not vary significantly at these temperatures (Table II). Essentially the same results were obtained for human erythrocytes (Table II).

θ-Toxin binding sites on lymphoma B cells

To examine whether cells other than erythrocytes have heterogeneous toxin-binding sites, the binding of MCθ to lymphoma B cells (BALL-1) was examined (Fig. 7). To minimize endocytosis of the MCθ during incubation, the binding assays were performed at 16°C [27]. MCθ binding equilibrated within 30 min at 16°C, since 30-min and 3-h incubations gave similar results. As in erythrocytes, both high- and low-affinity toxin sites were demonstrated in lymphoma cells. The binding of MCθ to both the high- and low-affinity sites was inhibited by digitonin (Fig. 7), strongly suggesting that cholesterol is also an essential component of the high- and low-affinity sites in lymphoma B cells.

The data on the binding affinity and number of binding sites in lymphoma B cells are summarized in Table II. The high-affinity sites comprise 2.5% of the total apparent binding sites. The dissociation constants for MCθ were estimated to be 2.3 and 210 nM. These are of the same order as the corresponding dissociation constants obtained for sheep and human erythrocytes (Table II), suggesting that the characteristics of the high- and low-affinity binding sites are similar among these cells.

Discussion

Well-characterized cholesterol-binding proteins, such as sterol and squalene carrier protein [28–30], cholesterol oxidase [21,31] and cholesterol-7α-hydroxylase [32] are known. However, sterol and squalene carrier protein has been reported to have a broad specificity for biosynthetic precursors of cholesterol [28] and long chain fatty acids [30]. On the contrary, θ-toxin and other thiol-activated cytolysins recognize only cholesterol or analogues which have a 3β-OH group and aliphatic side chain [5,12]. Cholesterol oxidase and cholesterol-7α-hydroxylase specifically recognize cholesterol molecules and analogues containing a 3β-OH group [31,32], but their K_m values for cholesterol are approx. 10^{-5} M [21,32], indicating a remarkably lower affinity for cholesterol than θ-toxin. These observations suggest the specificity and binding affinity of θ-toxin to cholesterol is superior to these other cholesterol-recognizing proteins.

To take the advantage of the specificity and high affinity of θ-toxin for cholesterol, we have prepared θ-toxin derivatives which retain the binding characteristics of θ-toxin without its hemolytic activity. In this report we have described the preparation and characterization of a nicked and methylated derivative of θ-toxin, MCθ. We demonstrated first that MCθ possesses as high specificity and affinity ($K_d \approx 10^{-9}$ M) for membrane cholesterol as θ-toxin and that it causes neither hemolysis nor obvious membrane damage at physiological temperatures.

Secondly, we characterized toxin-binding sites using MCθ as a probe, and revealed the existence of high- and low-affinity binding sites at physiological temperatures. The existence of high- and low-affinity sites was demonstrated in various types of cells, including sheep and human erythrocytes (Fig. 6), rat erythrocytes, lymphoma B cells (Fig. 7), and mouse thymocytes (Ohno-Iwashita, Y., unpublished data). The fact that only cholesterol among all membrane lipids was detected as a binding constituent for MCθ (Fig. 4), and that digitonin greatly reduced the numbers of both high- and low-affinity sites (Fig. 7), strongly suggests that cholesterol constitutes both types of sites on these plasma membranes. The dissociation constants obtained for erythrocytes and lymphoma B cells were on the order of 10^{-9} M and 10^{-7} M for high- and low-affinity sites, respectively, in all cases, suggesting a common mode of existence of cholesterol in the membranes of these cells. In addition, the fact that this cholesterol heterogeneity was observed at physiological temperatures and in a wide variety of cells suggests the significance of the cholesterol heterogeneity in membrane organization. Qualitative heterogeneity of membrane cholesterol has also been recently suggested by measuring the fluorescence lifetime of a fluorescent sterol [33].

Interaction of cholesterol with other membrane components might cause such cholesterol heterogeneity. The existence of some cholesterol antibodies which react with liposomes containing 71% cholesterol but not with those containing 43% cholesterol [34] suggests that the cholesterol in cholesterol-rich microdomains of membranes might have a unique configuration or orientation. It has also been suggested that the phospholipid and protein composition of liposomes or membranes might affect the rate of exchange of cholesterol with external cholesterol pools [35–37] as well as the susceptibility of cholesterol to cholesterol oxidase [37,38]. The etiology of the high- and low-affinity cholesterol sites is now under investigation.

Cholesterol has been reported to be essentially for Sendai virus-mediated lysis [39] and membrane fusion [40] and for the regulation of unsaturated fatty acid uptake into *Mycoplasma capricolum* [41]. It would be quite interesting to investigate whether the high- and low-affinity sites are involved in these phenomena. Analyses using modified θ-toxin would make it possible to quantify heterogeneous cholesterol pools and to visualize their localization in membranes. Thus studies with modified θ-toxin would provide new insight into

the organization of cholesterol in and its relation to the function of biological membranes.

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