Interaction of Streptolysin O from *Streptococcus pyogenes* and Theta-Toxin from *Clostridium perfringens* with Human Fibroblasts

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The membrane-damaging properties on human diploid embryonic lung fibroblasts of streptolysin O (from *Streptococcus pyogenes*) and theta-toxin (from *Clostridium perfringens*) were compared. The results are consistent with the suggested mechanism for hemolysis by streptolysin O involving one fixation site and one lytic site of this cytotoxin. However, the membrane-damaging activity of the two toxins differed with respect to (i) relative cytolytic activity on human diploid lung fibroblasts compared with that on sheep erythrocytes, (ii) binding to the fibroblast membrane, (iii) activity at 0°C, (iv) membrane repair after more than 30 min, and (v) effect on influx of amino acids. It is concluded that the mechanism of membrane damage caused by theta-toxin differs from that of streptolysin O, since the former toxin was active at 0°C and did not bind to the cytoplasmic membrane. These results question the current concept that all thiol-activated, cholesterol-inactivated bacterial toxins are similar both structurally and functionally.

Streptolysin O (SLO) from *Streptococcus pyogenes* and theta-toxin from *Clostridium perfringens* are exoproteins which belong to the group of thiol (SH)-activated bacterial cytotoxins. These membrane-damaging proteins have similar biological properties; they are reversibly inactivated by oxygen, they give rise to cross-reacting antibodies, and they are lethal and cardiotoxic, but their exact role in the pathogenesis of infections caused by the bacteria producing them is not presently known (8, 29). The present investigation is concerned with the membrane-damaging (cytolytic) properties of SLO, considered to be the prototype of the group, in comparison with the less well-studied theta-toxin.

The SH-activated bacterial toxins lyse or alter the membrane permeability of mammalian cells that contain cholesterol in their plasma membranes, and lysis is inhibited by preincubation of the toxins with low amounts of cholesterol. These facts point to cholesterol as the membrane receptor for the SH-activated toxins (1, 7, 29). Indeed, it has been clearly established that SLO binds to cholesterol in various membranes (3, 26). Also, theta-toxin has been claimed to bind to cholesterol in erythrocyte membranes (17). Furthermore, both SLO and theta-toxin induce the formation of rings and arcs on erythrocyte membranes, presumably by interaction with membrane cholesterol (14, 29, 31), but the toxin concentrations needed to induce rings are relatively high. Thus, the significance of these

structures in the lytic process may be questioned (29).

At the present time, the SH-activated toxins are believed to cause hemolysis by binding to membrane cholesterol, causing its withdrawal from other membrane lipids (29). Furthermore, Alouf (1) demonstrated that SLO possesses two active sites, one responsible for fixation of SLO to the membrane cholesterol and the other responsible for the subsequent lytic step.

Duncan and Buckingham (13) studied the effects of sublytic doses of SLO on transport functions of HeLa cells. SLO was found to inhibit the membrane transport of certain amino acids but not that of nucleosides and glucose analogs. We have previously investigated the effects of SLO and theta-toxin on the plasma membranes of cultured human lung fibroblasts by using leakage of different-sized cytoplasmic markers as indicators of plasma membrane damage. Both toxins caused leakage only of markers with molecular weights less than 25,000 and thus induced relatively small "holes" in the fibroblast plasma membranes (34, 35).

In this investigation a closer comparison between the effects of SLO and theta-toxin on human lung fibroblasts was made since preliminary experiments indicated that theta-toxin was active at a low temperature, whereas SLO was not. The results indicate that SLO and theta-toxin act differently at the membrane level despite many similarities.

MATERIALS AND METHODS

Chemicals. Eagle minimal essential medium, newborn calf serum and trypsin were obtained from Flow Laboratories, Ltd., Irvine, Scotland; Hanks balanced salt solution (BSS) was obtained from the National Bacteriological Laboratory, Stockholm, Sweden; [5-³H]uridine (specific activity, >25 Ci/mmol), [1-¹⁴C]α-aminoisobutyric acid (AIB) (specific activity, >50 mCi/mmol), 2-[1,2-³H]deoxy-D-glucose (specific activity, 30 to 60 Ci/mmol), [methyl-³H]thymidine (specific activity, 20 Ci/mmol), and Aquasol universal cocktail were obtained from New England Nuclear Chemicals GmbH, Frankfurt, West Germany; sodium pyruvate, sodium borate, sodium chloride, boric acid, cetyltrimethylammonium bromide, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and tris(hydroxymethyl)aminomethane (Tris) were purchased from E. Merck, Darmstadt, W. Germany; Triton X-100 was from Rohm and Haas, Co., Philadelphia Pa.; and filipin (U-5956, reference 8393-DEG-11-18 crystalline complex, 66% pure) was kindly supplied by G. B. Whitfield, The Upjohn Co., Kalamazoo, Mich. All chemicals were of analytical grade.

Toxins. SLO from *S. pyogenes* strain C203S was purified by the method of Smyth and Fehrenbach (30). Theta-toxin was purified by the method of Smyth (28) from *C. perfringens* type A (ATCC 13124). Before use, the toxins were activated by 1 mM dithiothreitol. Theta-toxin had a specific activity of approximately 47,000 hemolytic units (HU) per mg of protein. The specific activity of SLO was 94 HU per mg of protein. Cholesterol (1 μg/ml) abolished the membrane-damaging effects of the toxin preparations on both erythrocytes and human fibroblasts.

Cell culture. The cells were cultivated as previously described in detail (32, 33). Human diploid embryonic lung fibroblasts (line MRC-5) were grown in Eagle minimal essential medium (15) supplemented with 10% newborn calf serum, 5 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells to be used for cytotoxicity tests were grown to confluency in polystyrene wells (30-mm diameter) for 5 to 7 days.

Measurements of increased membrane permeability (structural damage). Membrane damage was assessed by the leakage of three different-sized cytoplasmic markers. Cell monolayers were prelabeled with AIB (molecular weight, 103) by the method of Thelestam and Möllby (35) or with [5-³H]uridine to obtain either a labeled nucleotide (molecular weight, <1,000) or labeled ribonucleic acid (molecular weight, >200,000) (32).

Labeled cultures were rinsed three times with Hanks BSS before exposure to toxins. Unless otherwise stated, the toxins were diluted in Tris-buffered saline (TBS; 0.9% NaCl buffered with 0.02 M Tris-hydrochloride, pH 7.0), and the cells were treated under various conditions (see below). Released radioactivity was measured in 0.1-ml fractions added to 10 ml of Aquasol. The results were expressed as percentage of a maximal release obtained when the cell membranes were completely ruptured by treatment with a 0.06 M sodium borate buffer at pH 7.8 (32).

Measurement of membrane transport. Toxin-treated cells were carefully rinsed twice in Hanks BSS

(20°C) and then incubated with isotope solutions for 15 min at 37°C. AIB (1 μCi/ml) was diluted in Hanks BSS with 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4. 2-[1,2-³H]deoxy-D-glucose (1.5 μCi/ml) was diluted in TBS containing 10% serum. [3H]uridine (1 μCi/ml) was supplied in Eagle minimal essential medium with 10% serum. The isotope solutions were warmed to 37°C before addition to cell cultures. After incubation the solutions were withdrawn, and the cells were quickly washed twice with ice-cold Hanks BSS. The cells were ruptured in 1% Triton X-100 (1 ml/culture) and centrifuged, and the radioactivity in a 0.1-ml fraction of the supernatants was counted. The results were expressed as percentage of controls in which the toxin treatment was omitted. The accumulated radioactive solutes could also be extracted in sodium borate buffer (as above) or with perchloric acid with essentially the same results as with Triton X-100.

Binding of toxins to cells. Nucleotide-labeled cells were treated at 0°C for 15 min with various concentrations of toxins to allow for adsorption to the cells. These toxin solutions were then removed from the cell monolayers and transferred to another set of nucleotide-labeled cell cultures which were incubated for 30 min at 37°C. As controls, fresh toxin solutions, which had not been in contact with cells, were used for treatment of a parallel set of nucleotide-labeled cultures. The cultures initially incubated at 0°C were carefully rinsed twice with Hanks BSS; then TBS was added, and the temperature was raised to 37°C. The incubation was terminated after 30 min.

Measurement of thymidine incorporation into DNA. (i) **Scintillation.** Toxin-treated cells were rinsed twice with Hanks BSS and treated with [3H]-thymidine (0.5 μCi/ml) in Eagle minimal essential medium with 10% serum for 1 h. The solution was carefully removed by rinsing with ice-cold Hanks BSS three times. The cell material was transferred to test tubes in 1 ml of 0.5 M NaOH, and deoxyribonucleic acid was precipitated by adding 4 ml of 10% trichloroacetic acid. The tubes were allowed to stand in the cold for at least 30 min. The precipitates were collected on membrane filters (pore size, 0.22 μm; Millipore Corp.) which were washed with ice-cold 5% trichloroacetic acid three times before they were counted in 10 ml of Aquasol.

(ii) **Autoradiography.** Toxin-treated cells on cover slips were rinsed twice and incubated with [3H]thymidine in Eagle minimal essential medium as above. The cover slips were rinsed and fixed in ethanol-chloroform-acetic acid (6:3:1), rinsed in ethanol, air dried, and mounted on slides. These were dipped in Kodak Nuclear Track Emulsion, exposed in the dark at 4°C during 1 week, developed, and stained with Giemsa solution. The percentage of nuclei incorporating radioactivity was determined by counting 10 fields per culture.

RESULTS

Dose-response curves and induced changes in fibroblast morphology. Upon incubation of the human diploid fibroblasts with graded doses of the toxins for 30 min, SLO and

theta-toxin induced relatively small functional holes in the fibroblast membranes since the labeled ribonucleic acid in both cases was released only to a low degree. (The modern concept of biomembranes as dynamic, fluid structures seems to exclude the possibility of fixed holes present in the membrane. The word "hole" is used herein as a purely operational term to indicate a change in membrane permeability allowing leakage of cytoplasmic substances.) The leakage patterns (Fig. 1) were rather similar for both toxins; however, theta-toxin was about 15 times more membrane-damaging to fibroblasts than was SLO, compared with their respective effects on sheep erythrocytes.

Both toxins caused similar morphological changes, as determined by light microscopy. The nuclei became swollen with highly prominent nucleoli, and the cytoplasm was granulated. However, the toxins did not seem to affect any of the cytoskeletal structures since the cells remained well spread on the solid substrate, as is also the case after treatment of cells with membrane-damaging nonionic detergents (23).

Effects of divalent cations. The membrane-damaging activities of both SLO and theta-toxin were abolished in the presence of 10 mM Zn^{2+} and significantly reduced by 5 mM of the same ion (Table 1). Also, Ca^{2+} ions were inhibitory to both toxins, but Mg^{2+} ions had no significant effect.

Time course relationships. When prelabeled fibroblasts were treated with high doses of SLO and theta-toxin (15 and 2 HU/ml, respectively) for various time periods, roughly similar leakage patterns were obtained for both toxins (Fig. 2). Maximal AIB release occurred after 10 min of incubation, whereas the labeled nucleotide was released more slowly.

Release of nucleotide and labeled ribonucleic

acid was also monitored during prolonged toxin treatment of prelabeled cells (Fig. 3). SLO induced a continuously increasing release of ribonucleic acid amounting to 90% after 6 h. The release of ribonucleic acid with theta-toxin remained 50%, and this maximum value was reached in 2 h. Thus, SLO eventually caused larger functional holes in the membrane than did theta-toxin. However, in both cases the granulated cells remained attached and as well spread on the plastic surface at 6 h as after 30 min of toxin treatment.

Effects of temperature. At 0°C the membrane-damaging activity of high concentrations of SLO was strongly reduced, and that of low doses of SLO was abolished (Fig. 4). In contrast, low concentrations of theta-toxin were almost equally active at 0 and 20°C as at 37°C (Fig. 4), and the membrane-damaging effect occurred almost as rapidly at 0°C as at 37°C (data not shown).

Effects of toxins on membrane transport functions. Functional membrane damage (decreased cellular uptake of various solutes) by toxin treatment was investigated in relation to the induced structural membrane damage (nucleotide release) (Fig. 5).

Uptake of uridine was inhibited by both toxins in direct proportion to the induced structural membrane damage (Fig. 5A). In contrast, the uptake of 2-deoxy-D-glucose was inhibited by both toxins to about 40% at concentrations causing no significant nucleotide release (Fig. 5B). The uptake of AIB was affected in different ways by the toxins. Theta-toxin induced a strong inhibition of AIB accumulation at concentrations causing no nucleotide release. SLO, in contrast, inhibited the AIB uptake to less than 25% even at concentrations causing structural membrane damage (Fig. 5C).

FIG. 1. Release of labeled cytoplasmic markers from human diploid embryonic lung fibroblasts treated for 30 min at 37°C with SLO (A) and theta-toxin (B) at the concentrations indicated. Symbols: $\square$, AIB; $\circ$, nucleotide; Δ , ribonucleic acid.

Thus, it appears that the structural membrane damage induced by SLO and theta-toxin caused dysfunction of the uridine and 2-deoxy-D-glucose carriers. Theta-toxin at sublytic doses inhibited the system for AIB transport, whereas the AIB carrier was not much altered by the membrane perturbation induced by SLO.

Binding of toxins to cells. The binding of SLO and theta-toxin to fibroblasts was investi-

TABLE 1. Effects of divalent cations on the membrane-damaging activities of SLO and theta-toxin^a

Ion added	Concn (mM)	Nucleotide release (% of control)	
		SLO (10 HU/ml)	Theta-toxin (1 HU/ml)
None (controls)		100	100
Zn ²⁺	10	0	0
	5	17	64
	1	125	115
Ca ²⁺	10	10	49
	5	41	61
	1	31	53
Mg ²⁺	10	76	98
	5	95	88
	1	81	93

^a Nucleotide-labeled human diploid embryonic lung fibroblasts were incubated at 37°C for 30 min with SLO and theta-toxin diluted in TBS (controls) and in TBS with ZnCl₂, CaCl₂, or MgCl₂ at the concentrations indicated. The results were calculated as percentage of the maximal release as described in the text. Results are expressed as percentage of the nucleotide release in the absence of added divalent cations (TBS controls), for which the actual experimental values, i.e., percentage of maximal release, were 58% (SLO) and 85% (theta-toxin).

gated in an indirect manner as described above. The results presented in Fig. 6 indicate the following. (i) SLO bound to the cells at 0°C, since the solutions that had been in contact with fibroblasts had lost their membrane-damaging capacity as compared with fresh solutions. Upon incubation at 37°C in TBS, the bound SLO induced the expected membrane damage. (ii) Theta-toxin did not seem to adsorb to the fibroblasts during the incubation in the cold, since the solutions that had been in contact with cells at 0°C were as membrane damaging as the fresh solutions. The membrane-damaging activity in the transferred theta-toxin solutions was neutralized by cholesterol, indicating that the cytolytic activity remaining in these solutions was not due to some other membrane-damaging factor which might conceivably have been released from the fibroblasts during the first incubation. (iii) During the first incubation in the cold, theta-toxin and SLO induced some sort of damage foci, since an equal amount of labeled nucleotide was released during the subsequent incubation in TBS as found with the fresh toxin.

These findings were confirmed by the experiment described in Table 2. Most of the SLO was adsorbed to the cells during the cold treatments, whereas this was not the case with theta-toxin. This difference was also reflected in the behaviour of the cells incubated at 37°C with TBS after the 1-min toxin exposure. Considerably less radioactivity was released from the eighth culture than from the first in the case of SLO, whereas no significant difference was found with theta-toxin.

Binding experiments were also performed with the cationic detergent cetyltrimethylammonium bromide and the polyene antibiotic filipin, since both give rise to leakage patterns similar to those of SLO and theta-toxin (35).

FIG. 2. Release of labeled cytoplasmic markers from human diploid embryonic lung fibroblasts treated at 37°C for the time periods indicated with 15 HU of SLO per ml (A) and 2 HU of theta-toxin per ml (B). Symbols: □, AIB; ○, nucleotide; △, ribonucleic acid.

FIG. 3. Release of labeled cytoplasmic markers from human diploid embryonic lung fibroblasts treated at 37°C for the time periods indicated with 15 HU of SLO per ml (A) and 2 HU of theta-toxin per ml (B). Symbols: ○, nucleotide; △, ribonucleic acid.

FIG. 4. Release of labeled nucleotide from human diploid embryonic lung fibroblasts treated for 30 min at 0, 20, or 37°C with SLO (A) and theta-toxin (B) at the concentrations indicated. Symbols: □, 0°C; ●, 20°C; ○, 37°C.

Filipin is also known to bind to cholesterol in natural and artificial membranes (9, 11). Both substances were as active in the cold as at 37°C when incubated with fibroblasts for 30 min (data not shown). Both agents also appeared to adsorb to the fibroblast membranes (Fig. 7).

Survival of cells after toxin treatment. Cell survival after toxin treatment was monitored by measurements of incorporation of [³H]thymidine into deoxyribonucleic acid after 0, 24, and 48 h as described above.

The thymidine incorporation was drastically lowered immediately after treatment with more than 5 HU of SLO per ml and 0.5 HU of theta-toxin per ml (Fig. 8). After 48 h, the theta-toxin-treated cells seemed to have completely recovered their ability to incorporate thymidine into deoxyribonucleic acid, whereas the SLO-treated cells still showed a slightly poorer capacity for deoxyribonucleic acid synthesis.

Autoradiography of fibroblasts similarly exposed to toxin and thymidine indicated that the percentage of nuclei incorporating thymidine in

all cases was roughly the same as that in controls, but that the toxin-treated cells had fewer grains per nucleus. Thus, the lowered thymidine incorporation per culture, as measured by scintillation, was due to a generally lowered thymidine incorporation per cell nucleus, and not to complete loss of deoxyribonucleic acid synthesis in only a fraction of the cells.

In summary, a 30-min toxin treatment inducing about 60% release of nucleotide still did not lead to the death of the cells, provided the toxin solutions were replaced with normal growth medium plus serum.

DISCUSSION

In this study it is reported that SLO and theta-toxin acted similarly on cultured human fibroblasts as regards (i) morphological effects, (ii) cell survival after toxin exposure, (iii) response to divalent cations, (iv) character of initially induced functional holes, and (v) effects on uptake of uridine and 2-deoxy-D-glucose. However, it is also demonstrated that SLO and theta-toxin

FIG. 5. Uptake of [3 H]uridine (A), 2-[1,2- 3 H] deoxy-D-glucose (B), and 14 C-labeled AIB (C) by human diploid embryonic lung fibroblasts after treatment with various concentrations of SLO and theta-toxin for 30 min at 37°C. The uptake values were plotted against the percent of nucleotide release in parallel cell cultures treated similarly. Symbols: ●, 1 to 15 HU of SLO per ml; ○, 0.01 to 2 HU of theta-toxin per ml.

differed from each other with respect to (i) relative activities on fibroblasts as compared with erythrocytes, (ii) binding to cells before occurrence of membrane-damaging effects, (iii) dependency on temperature of membrane-damaging effect, (iv) size of the functional holes induced after prolonged (>30 min) incubation with the toxins, and (v) effect on AIB uptake after toxin treatment.

Compared with theta-toxin, SLO was more active on erythrocytes than on fibroblasts. This fact may be explained by the differential binding to the membrane of the two toxins since the fibroblast membrane is more complex than that

of erythrocytes. Masking of cholesterol sites by membrane glycoproteins could be of less importance for the action of theta-toxin than for that of SLO, since the toxin does not need to attach irreversibly (i.e., bind) to exert its action on the membrane. On the other hand, the fact that less than 0.1 HU of theta-toxin was needed to cause structural membrane damage might indicate that in the fibroblast membrane some secondary mechanism for membrane damage is activated by the primary hits of the toxin. The initial effect could be multiplied by release of some temperature-independent, detergent-like activity present in fibroblasts but absent from erythrocytes. Such a course of events has recently been proposed for membrane damage to 3T3-fibroblasts caused by melittin and staphylococcal delta-toxin (27).

It is interesting that fibroblasts treated for a limited time period with concentrations of toxin causing about 60% release of nucleotide were able to repair the induced damage and survive, as indicated by regained deoxyribonucleic acid synthesis (Fig. 8). The fact that SLO-treated cells survived almost as well as those exposed to theta-toxin, despite the differential binding, may be explained simply by a gradual inactivation of bound SLO due to components in the medium. This result agrees with the finding that fibroblasts in confluent monolayers have active membrane repair (38), and supports the idea that the toxicity of SLO and theta-toxin is restricted to the plasma membrane level. Preliminary experiments with alveolysin, the SH-activated cytolyisin from *Bacillus alvei* (2), similarly indicated that its action was restricted to the plasma membrane level (M. Thelestam, unpublished data).

Calcium and magnesium were shown by Oberley and Duncan (22) to inhibit SLO-induced hemolysis, but Van Epps and Andersen (37) claimed that Mg^{2+} was without initial inhibiting effect. Pneumolysin, another SH-activated toxin, was inhibited by Ca^{2+} and Zn^{2+} but not affected by Mg^{2+} (19). Our results are in agreement with the latter investigation and show that theta-toxin also interacted similarly with these cations (Table 1). The inhibiting effects of Zn^{2+} and Ca^{2+} may be due to a direct inhibitory action on the toxins, e.g., by reaction with free SH groups (5, 37). These ions are also known to have a stabilizing effect on the fibroblast plasma membrane (24, 39), which may render it less susceptible to any membrane-damaging activity.

Both toxins exhibited sigmoid dose-response curves, which together with the time course relationships indicate multi-hit kinetics in agreement with previous investigations on erythrocytes (4, 12, 18, 20). We found no difference between the cytolytic effects of SLO and theta-

toxin after short exposures, but prolonged incubation with toxins indicated that the functional holes induced by SLO were enlarged (Fig. 3). SLO, when firmly bound to the membrane (4), appears to exert a more continuous membrane-disorganizing effect, overcoming the cellular repair activity. Since theta-toxin was not adsorbed (or only transiently adsorbed), its action may be of a more transient character, and the induced holes may thus be more readily repaired. These findings are also in agreement with the lack of binding of theta-toxin to sheep erythrocytes observed earlier by Roth and Pillemer (25) and by us (unpublished results).

Experiments concerning effects on membrane transport functions are not so easily interpreted in the case of membrane-damaging toxins. Decreased uptake of a metabolite may be due to

true inhibition of the transport, by competition for the carrier, by inhibition of energy metabolism in the case of active transport, or simply by general perturbation of the microenvironment around the carrier. However, decreased isotope uptake might also be measured when the transport function is intact but the accumulated metabolite leaks out again through the functional holes induced in the membrane. Therefore, the results of our uptake experiments were always related to the leakage tests.

Our findings on uptake of uridine after SLO treatment are in agreement with those of Duncan and Buckingham (13), who studied the effects of SLO on HeLa cells, in that at sublytic SLO concentrations the uridine uptake was hardly affected. Inhibition of uptake of uridine by theta-toxin was also directly correlated to

FIG. 6. Binding of toxins to human diploid embryonic lung fibroblasts (as described in the text). Symbols: ○, Release of labeled nucleotide from cells treated for 30 min at 37°C with fresh solutions of SLO (A) and theta-toxin (B) at the concentrations indicated; ▲, release of labeled nucleotide from a parallel set of cells treated for 15 min at 0°C with the toxins; △, release of labeled nucleotide from cells treated initially at 0°C, then washed and further incubated for 30 min at 37°C in TBS; ●, release of labeled nucleotide from a third parallel set of cells treated with the toxin solutions previously exposed to one set of cells at 0°C, transferred from the exposed cell cultures, and incubated at 37°C for 30 min.

TABLE 2. Adsorption of SLO and theta-toxin to fibroblasts^a

Toxin	Concn (HU/ml)	Nucleotide release (%)									
		Toxin solution		TBS no.:							
		Fresh	Transferred	1	2	3	4	5	6	7	8
SLO	10	59	14	35	34	32	27	26	22	13	10
	5	35	12	18	13	12	11	10	9	8	6
Theta-toxin	0.5	51	56	32	37	28	28	29	36	24	28
	0.1	37	42	12	10	10	8	12	10	8	8

^a Nucleotide-labeled human diploid embryonic lung fibroblasts were incubated at 0°C with toxin solutions which were transferred over eight cultures and stayed in contact with each for 1 min. The final transferred toxin solutions (from culture no. 8) were then incubated with another set of nucleotide-labeled cells for 30 min at 37°C. Fresh toxin solutions were used as controls. The cultures treated initially at 0°C were then carefully rinsed with Hanks BSS at 20°C and incubated with TBS for 30 min at 37°C, and the nucleotide release was scored (right part of the table). All figures denote nucleotide release expressed as percentage of the maximal release after reduction of appropriate background counts per minute.

FIG. 7. Binding of cetyltrimethylammonium bromide and filipin to human diploid embryonic lung fibroblasts (as described in the text). Release of labeled nucleotide from cells treated with cetyltrimethylammonium bromide (A) and filipin (B). Incubations and symbols are as described in the legend to Fig. 6. At concentrations causing nucleotide release in the cold, the experimental values for the subsequent release in TBS were low, since much of the releasable material had already escaped the cells. These values have been corrected in the figure by addition of the counts released at 0°C.

FIG. 8. Incorporation of [^3H]thymidine into deoxyribonucleic acid of human diploid embryonic lung fibroblasts treated at 37°C for 30 min with SLO (A) and theta-toxin (B) at the concentrations indicated. Thymidine incorporation was measured (as described in the text) after 0 (●), 24 (Δ), and 48 h (□). The open circles (○) denote release of labeled nucleotide after 30 min of toxin treatment at 37°C.

induced structural damage. These results may be explained by either of the above-suggested courses of events.

As regards effect upon accumulation of AIB, theta-toxin and SLO acted differently (Fig. 5C). The inhibition of AIB uptake by low concentrations of theta-toxin indicates a selective attack on the AIB carrier (10). Whether this attack is a competition with or a disorganization of the carrier or surrounding functional molecules is not known at present.

SLO did not release any nucleotide label at 0°C, whereas low concentrations of theta-toxin caused structural membrane damage at this temperature. Also, alveolysin was active upon fibroblasts at 0°C (M. Thelstam, unpublished data). The only other bacterial cytolysins known to be active on fibroblasts in the cold are staph-

ylcoccal delta-toxin and the hemolysin from *Pseudomonas aeruginosa* (36, M. Thelstam, unpublished data), both believed to damage membranes by virtue of their surfactant properties (7, 21). The cationic detergent cetyltrimethylammonium bromide was studied for comparison and shown to be active at 0°C. Unlike theta-toxin, however, it was adsorbed to the membranes during incubation in the cold (Fig. 7A).

Interestingly, the polyene filipin was also able to induce fibroblast membrane damage in the cold, indicating that specific interaction with cholesterol can indeed also lead to structural damage in fairly rigid membranes. However, filipin and theta-toxin otherwise differ in several respects. Filipin is a polyene (molecular weight, 654), whereas theta-toxin is a protein (molecular weight, about 69,000). Furthermore, filipin

bound to the fibroblast membrane (Fig. 7B), in agreement with previous findings with other membranes (11, 16), whereas theta-toxin did not bind.

In conclusion, our data support the current concepts regarding action of SLO on membranes (29), and they do not contradict the idea of interaction with membranes via two sites as suggested by Alouf (1). However, the action of theta-toxin on fibroblasts differs from that of SLO. The latter has been regarded as the prototype of SH-activated toxins, although an anomalous hemolytic behavior of SLO compared with other SH-activated cytotoxins was pointed out already 30 years ago (6). Furthermore, our preliminary experiments with alveolysin (M. Thelestam, unpublished data) indicated a greater similarity to theta-toxin than to SLO. The similarities between the SH-activated cytotoxins will thus have to be reconsidered when their molecular effects on the cytoplasmic membrane have been further investigated.

Theta-toxin seems to act via a hit-and-run mechanism which is not typical for other cholesterol-interacting substances, such as filipin, or for true detergents, such as cetyltrimethylammonium bromide, which also operate in the cold. At this stage, it is not possible to suggest an alternative molecular mechanism for the membrane-damaging action of theta-toxin, but hopefully future studies on artificial membrane systems will cast light upon this problem.

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