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RESISTANCE TO STREPTOLYSIN O IN MAMMALIAN CELLS TREATED WITH OXYGENATED DERIVATIVES OF CHOLESTEROL

CHOLESTEROL CONTENT OF RESISTANT CELLS AND RECOVERY OF STREPTOLYSIN O SENSITIVITY

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Summary

Cultures of L cells and HeLa cells were made resistant to the cytolytic toxin, streptolysin O, by incubating them in the presence of 20α-hydroxycholesterol or 25-hydroxycholesterol. Such cells were also found to be more resistant to the cytotoxic effects of saponin and digitonin, agents known to interact with membrane cholesterol. Sterol synthesis in L cells that had been treated with either of the oxygenated derivatives of cholesterol was reduced by almost 90%, and the free cholesterol content of streptolysin O-resistant HeLa and L cells fell to approx. 50% of control cell levels. Significant recovery of sensitivity to streptolysin O occurred in about 6 h when refractory L cells were incubated in serum or cholesterol. Partial recovery was observed when the cultures were incubated for 24 h in mevalonate or lipid-depleted serum. The results provide further support for the role of membrane cholesterol in the cytotoxic action of streptolysin O on mammalian cells.

Introduction

Streptolysin O is a cytolytic toxin produced by *Streptococcus pyogenes*, an important human pathogen. Like other thiol-activated cytolysins, streptolysin O is thought to alter the function and integrity of cell membranes by interacting with cholesterol in the membrane bilayer [1—3]. This concept has been supported by the observation that L cells and HeLa cells that have been treated with certain oxygenated derivatives of cholesterol become increas-

ingly resistant to the lytic effects of streptolysin O [4,5]. The cholesterol derivatives, including 20α-hydroxycholesterol and 25-hydroxycholesterol have been shown to inhibit sterol synthesis in several types of cells and tissues by suppressing the activity of the regulatory enzyme in the cholesterol biosynthesis pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (reviewed in Ref. 6). In the absence of serum or other exogenous cholesterol sources, the cholesterol content of the cells is significantly reduced [6,7]. When cells treated in this way were exposed to streptolysin O, they were found to be up to 50-times more resistant to the cytolytic effects of the toxin than were control cells. Furthermore, most of the toxic activity could be recovered from the culture supernatants, suggesting that streptolysin O did not effectively bind to the membrane of the resistant cells [4]. This report describes experiments that relate the increased toxin resistance of hydroxycholesterol-treated cells to a decrease in cellular cholesterol concentration. In addition, the recovery of streptolysin O sensitivity following incubation of protected cells in serum, cholesterol, or mevalonate (the product of the 3-hydroxy-3-methylglutaryl-CoA reductase reaction) is demonstrated.

Materials and Methods

Cell cultures. Stock cultures of L cells and HeLa cells were grown as monolayers in Eagle's minimal essential medium containing Hank's salts, supplemented with 5% fetal calf serum and antibiotics as previously described [4]. Cells used in the experiments were cultured in 35 × 10 mm plastic dishes.

Streptolysin O. Streptolysin O was prepared by $(NH_4)_2SO_4$ precipitation of culture supernatants and gel filtration on Sephacryl S-200 as previously described [4]. The Sephacryl column fractions that contained the peak of toxin activity were lyophilized, resuspended in water containing 10 mM dithiothreitol, and passed through a G-25 Sephadex column equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl and 1.0 mM EDTA. The fractions containing peak activity were added to an activated SH-Sepharose 4B column equilibrated with the same Tris buffer at 4°C. The toxin remained in the SH-Sepharose overnight, then the column was washed with 5—10 vol. of Tris buffer. Streptolysin O was eluted by the addition of 20 mM dithiothreitol in Tris buffer. The toxin had a specific activity of approx. $7 \cdot 10^4$ hemolytic units (HU) per mg protein.

For titration of streptolysin O activity, the toxin was diluted in Hank's balanced salt solution or phosphate-buffered saline, then incubated for 30 min at 37°C with an equal volume of a 1.7% rabbit erythrocyte suspension. Lysis was measured by determining hemoglobin release colorimetrically at 540 nm. Complete hemolysis was determined by including 0.1 ml of 1% saponin in the suspension. 1 HU of toxin is defined as the greatest dilution of streptolysin O that produces 50% hemolysis under these conditions.

Exposure to oxygenated derivatives of cholesterol. Stock solutions of 20α-hydroxycholesterol (Sigma) or 25-hydroxycholesterol (Steraloids, Inc.) were prepared in absolute ethanol, then brought to a concentration of 200 μg/ml in 0.14 M NaCl containing 5% fatty acid-free bovine serum albumin. For each experiment the stock solutions were diluted into minimal essential

medium containing lipid-depleted fetal calf serum to a concentration of 0.5 μg 20α-hydroxycholesterol per ml or 0.25 μg 25-hydroxycholesterol per ml, then added to cell monolayers for various periods of time [4].

Fetal calf serum was lipid-depleted by extraction with butanol/isopropyl ether (40 : 60) for 1 h [8]. The aqueous phase was re-extracted and after separation by centrifugation, traces of solvent were removed under vacuum. The lipid-depleted serum was dialyzed overnight against phosphate-buffered saline, sterilized by filtration and brought to the original volume with sterile phosphate-buffered saline.

Toxicity assays. The cytotoxic effect of streptolysin O was measured by examining the ability of the cells to transport 2-deoxy-D-[^{3}H]glucose (8.26 Ci/mmol; New England Nuclear) after a 20 min exposure to the toxin. Previous studies have demonstrated that deoxyglucose uptake in streptolysin O-treated cells accurately reflects cell viability as determined by trypan blue staining [4,9]. The results are expressed as 'viable cells' or 'protection', the percentage of labeled deoxyglucose uptake in toxin-treated cultures relative to label uptake in cultures not treated with toxin.

Cholesterol synthesis. L cell cultures in 60 × 15 mm plastic dishes were exposed to the oxygenated cholesterol derivatives for 24 h. The cells were washed, then incubated for an additional 4 h in identical medium which also contained 1 μCi [^{14}C]acetate (56.1 mCi/mM; ICN) per ml. The monolayers were washed and the cells were removed by scrapping with a rubber policeman. After centrifugation, 1 μCi [^{3}H]cholesterol (44.0 Ci/mmol; New England Nuclear) was added as an internal standard, and the cells were extracted in 1 ml 95% ethanol containing 25 μl of a solution of 1% Triton X-100 and 20 mM sodium cholate [10]. The extraction mixture was sonicated for 10 s using the microtip of a Heat Systems sonifier, and centrifuged for 15 min at 3000 × *g*. The pellet was retained for protein determinations and the supernatant was evaporated under N_2, then resuspended in 200 μl $CHCl_3$. Portions of the extract, usually 25 μl, were spotted on silica gel-60 thin-layer chromatography plates (Merck) and run first in isopropyl ether/acetic acid (96 : 4), then in petroleum ether/diethyl ether/acetic acid (90 : 10 : 1) for the analysis of neutral lipids [11,12]. The plates were developed by exposure to iodine vapor, and the spot corresponding to cholesterol or desmosterol (L cells have been shown to synthesize desmosterol as their major sterol [13]) was scraped off and the amount of ^{14}C and ^{3}H radioactivity present was determined by scintillation counting. The data were expressed as picomoles of acetate incorporated into [^{14}C]cholesterol or [^{14}C]desmosterol per h.

Determination of cellular cholesterol. The cholesterol content of normal and streptolysin O-resistant HeLa cells was determined by an enzymatic method essentially as described by Gamble et al. [10]. The procedure is based on the fact that H_2O_2, generated when free cholesterol is oxidized by cholesterol oxidase, reacts with *p*-hydroxyphenylacetic acid in the presence of horseradish peroxidase to form a stable fluorescent product. Total cholesterol can be determined by including cholesterol ester hydrolase in the assay system.

Cultures in 60 × 15 mm plastic dishes were exposed to appropriate concentrations of the cholesterol derivatives for 0, 18, 24, or 36 h. After washing, duplicate cultures of each group were exposed to streptolysin O to determine

their sensitivity to the toxin. Three culture dishes from each group were scraped with a rubber policeman, the cells were centrifuged, resuspended and counted. The cells were then extracted in ethanol/Triton X-100/sodium cholate as described above. The free cholesterol content of the cells was determined by adding to 0.2 ml of extract, 1 ml of a solution containing 8 parts K_2HPO_4 (0.1 M, pH 7.4), 2 parts cholesterol oxidase (1 unit/ml; Boehringer Mannheim), 2 parts of a mixture of 1% Triton X-100 and 20 mM sodium cholate, and 3 parts *p*-hydroxyphenylacetic acid (4 mg/ml; Sigma). After incubation at 37°C for 30 min, 1 ml of K_2HPO_4 buffer was added, and fluorescence was measured in an Aminco-Bowman spectrophotofluorometer with an excitation wavelength of 325 nm and an emission wavelength of 415 nm. For total cholesterol determinations, the procedure was identical except that 2 parts cholesterol ester hydrolase (0.1 unit/ml; Boehringer Mannheim) were added to the reaction mixture, replacing 2 parts buffer. Standard curves were prepared with cholesterol and cholesterol oleate. The results are expressed as μg cholesterol/mg protein. Protein determinations were carried out on the pellet remaining after cholesterol extraction.

Recovery experiments. Duplicate L cell cultures were incubated for 24 h in 25-hydroxycholesterol. After washing with phosphate-buffered saline, one group of cultures was tested for protection against streptolysin O. The remaining cultures were incubated for various times in minimal essential medium alone, or minimal essential medium plus fetal calf serum, lipid-depleted fetal calf serum, mevalonic acid (Calbiochem), or cholesterol (Sigma). Cholesterol was first dissolved in absolute ethanol containing 5% fatty acid-free bovine serum albumin, then diluted in minimal essential medium.

Other reagents. Digitonin and saponin were obtained from Sigma.

Results

Effect of streptolysin O and other cholesterol-reactive substances on cells treated with oxygenated sterols

The cytotoxic effect of streptolysin O has been determined by measuring the uptake of 2-deoxy-D-[^{3}H]glucose into HeLa and L cells [4,5]. In order to show that under the experimental conditions described here deoxyglucose transport itself is not affected by the oxygenated cholesterol derivatives, HeLa cells were exposed to minimal essential medium plus lipid-depleted serum in the presence or absence of 0.25 μg of 25-hydroxycholesterol per ml for 24 or 36 h. The cells were then incubated in minimal essential medium containing 1 μCi/ml 2-deoxy-D-[^{3}H]glucose for 30 min and the amount of radioactive label taken up by the cells was determined. The results in Table I show that deoxyglucose transport in control and 25-hydroxycholesterol-treated cultures was virtually the same. Similar results have been obtained with L cells [4].

In our previous studies, L cells and HeLa cells were shown to become resistant to streptolysin O after incubation with 25-hydroxycholesterol or 20α-hydroxycholesterol in minimal essential medium for 24—48 h [4,5]. We subsequently found that the cells became even more resistant to streptolysin O when lipid-depleted fetal calf serum was added to the hydroxycholesterol/minimal essential medium solution (Fig. 1A). Unless otherwise noted, the

TABLE I

2-DEOXY-D-[^{3}H]GLUCOSE TRANSPORT IN CONTROL AND 25-HYDROXYCHOLESTEROL-TREATED HeLa CELLS

Values expressed as the mean ± S.E. for six determinations. 25-Hydroxycholesterol added at 0.25 μg/ml.

Time	Addition	2-Deoxy-D-[^{3}H]glucose uptake (cpm/mg protein)
0	control	26 282 ± 1799
24	control	31 152 ± 1918
	25-hydroxycholesterol	32 029 ± 802
36	control	28 140 ± 1179
	25-hydroxycholesterol	30 361 ± 1692

experiments described here were carried out in the presence of lipid-depleted fetal calf serum. The effect of other cholesterol-reacting compounds on the viability of hydroxycholesterol-treated cells was also examined. Treated L cells were more resistant to the cytotoxic effects of saponin and digitonin (Fig. 1B and C).

Cholesterol concentration of streptolysin O-resistant cells

To demonstrate that sterol synthesis was in fact inhibited under conditions where the cells became resistant to streptolysin O, L cells were incubated for 24 h in the presence of 20α-hydroxycholesterol or 25-hydroxycholesterol. The cells were washed and incubated for an additional 4 h in identical medium that contained 1 μCi [^{14}C]acetate per ml. The cultures were extracted as described in Materials and Methods, [^{3}H]cholesterol was added as an internal

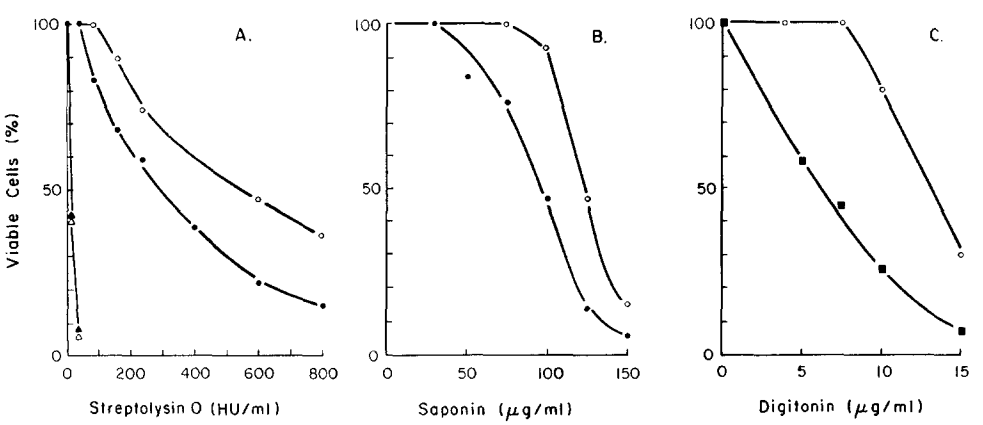

Fig. 1. Effect of streptolysin O, saponin or digitonin on L cells treated with 0.25 μg 25-hydroxycholesterol per ml for 24 h. (A) L cells were incubated in 25-hydroxycholesterol in minimal essential medium (●) or 25-hydroxycholesterol in minimal essential medium plus lipid-depleted fetal calf serum (○), then exposed to various concentrations of streptolysin O: minimal essential medium (▲) and minimal essential medium plus lipid-depleted fetal calf serum (△) were controls. (B and C) L cells were incubated in minimal essential medium plus lipid-depleted fetal calf serum and 25-hydroxycholesterol (○) or minimal essential medium plus lipid-depleted serum alone (●, ■), then exposed to various concentrations of saponin or digitonin for 20 min.

TABLE II

STEROL SYNTHESIS IN STREPTOLYSIN O-RESISTANT L CELLS

Addition	[^{14}C]Acetate → [^{14}C]Cholesterol (pmol/mg protein per h)	Protection * (% viable cells)
Control	277.00 (100%)	16
+ 20-α-hydroxycholesterol (0.50 μg/ml)	33.52 (12%)	86
+ 25-hydroxycholesterol (0.25 μg/ml)	32.25 (12%)	83

* Cells were treated with 160 HU streptolysin O.

standard, and the extracts were analyzed using thin-layer chromatography. The radioactivity associated with the spot corresponding to cholesterol (or desmosterol) in each sample was determined by scintillation counting. Duplicate cultures were treated with 160 HU of streptolysin O to assess their sensitivity to the toxin. The average values of two such experiments (Table II) demonstrate that sterol biosynthesis was inhibited by about 90%.

The sterol content of control and streptolysin O-resistant cultures was also examined. HeLa cell cultures were treated for 18, 24, or 36 h with the oxygenated sterols, and the amount of free cholesterol present in the cells was determined. Duplicate cultures were treated with 160 HU of streptolysin O at each time to test their sensitivity to toxin. The results of these experiments (Table III) show that after 18 h of treatment, the free cholesterol content of the cells had been reduced by about 20%, and that slightly more than half the cells remained viable after exposure to 160 HU of toxin. With increased incubation times, the free cholesterol content continued to fall, and there was a concomitant increase in resistance to streptolysin O. Virtually complete protection against this concentration of streptolysin O was seen when free cholesterol had been reduced to about half the level found in control cultures. The same pattern was observed when L cells were tested in similar experiments.

Recovery of sensitivity to streptolysin O

The ability of toxin-resistant cultures to recover their sensitivity to strepto-

TABLE III

CHOLESTEROL CONCENTRATION IN STREPTOLYSIN O-RESISTANT HeLa CELLS

Values of the free cholesterol concentrations are expressed as the mean ± S.E. for six determinations.

Time (h)	Addition	Free cholesterol concentration (μg/mg protein)	Protection * (% viable cells)
0	control	25.28 ± 4.34	4 ± 2
18	control	19.55 ± 1.35	
	25-hydroxycholesterol	15.78 ± 1.34 (81%)	57 ± 6
24	control	19.57 ± 1.66	
	25-hydroxycholesterol	12.87 ± 1.11 (66%)	72 ± 7
36	control	20.80 ± 1.26	
	25-hydroxycholesterol	11.47 ± 0.64 (55%)	92 ± 2

* Cells were treated with 160 HU of streptolysin O.

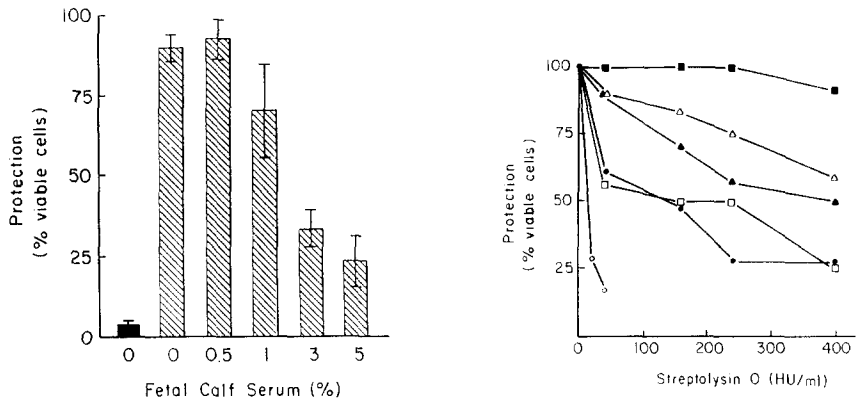

Fig. 2. Effect of fetal calf serum on recovery of sensitivity to streptolysin O. L cell cultures were incubated in minimal essential medium and lipid-depleted serum alone (solid area) or plus 25-hydroxycholesterol (shaded areas) for 24 h, then minimal essential medium containing various concentrations of fetal calf serum for an additional 24 h. The cultures were then tested for the degree of protection retained against 240 HU of streptolysin O.

Fig. 3. Recovery of streptolysin O sensitivity by 25-hydroxycholesterol-treated cells. L cells were incubated in 25-hydroxycholesterol for 24 h, and for an additional 24 h in the reagents listed. The cultures were then tested for sensitivity to streptolysin O. Control cultures (○) were not treated with 25-hydroxycholesterol. Cells were incubated in: minimal essential medium (■); 5% lipid-depleted fetal calf serum (△); 93 μM mevalonate (▲); 136 μM cholesterol (□); 5% fetal calf serum (●).

lysin O was also studied. L cell cultures that had been made toxin-resistant by incubation in 25-hydroxycholesterol for 24 h were washed and incubated for an additional 24 h period in minimal essential medium containing various concentrations of fetal calf serum. The cells were then exposed to 240 HU of streptolysin O. The results (Fig. 2) show that significant recovery to a toxin-sensitive condition occurred in the presence of 3 or 5% serum; these cultures, however, were still not as sensitive to streptolysin O as were control cells that had not been incubated in 25-hydroxycholesterol. In addition to serum, other substances were tested for their ability to stimulate recovery to toxin sensitivity. The addition of cholesterol (136 μM) to the protected cultures was as effective as fetal calf serum in bringing about recovery (Fig. 3). Mevanolate (93 μM), the product of the 3-hydroxy-3-methylglutaryl-CoA reductase reaction, and lipid-depleted fetal calf serum (5%) in minimal essential medium both stimulated partial recovery by 24 h, but they were less effective than whole serum or cholesterol. There was virtually no recovery in minimal essential medium alone. The possibility that protected cells could recover their sensitivity to streptolysin O in a shorter period of time was examined by incubating the cultures in various concentrations of fetal calf serum for 6 h. The cells became increasingly sensitive to the toxin with increasing serum concentrations, and substantial recovery was seen in cultures incubated with 20% fetal calf serum for 6 h (Fig. 4). Conversely, there was little recovery at this time when the cells were incubated in 20% fetal calf serum that had been lipid-depleted. Significant recovery was also observed in cultures that were incubated in minimal essential medium containing cholesterol for 6 h (data not shown).

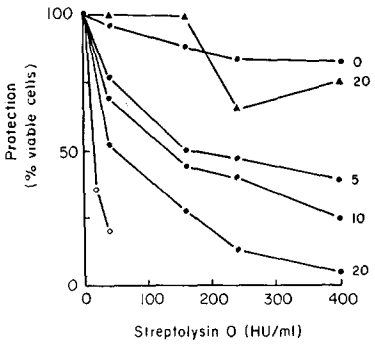

Fig. 4. Recovery of streptolysin O sensitivity in serum. L cells were incubated in 25-hydroxycholesterol for 24 h, incubated in various concentrations of fetal calf serum (●) for 6 h, then tested for sensitivity to streptolysin O. A duplicate set of cultures was incubated in 20% lipid-depleted fetal calf serum (▲). Control cultures (○) were not treated with 25-hydroxycholesterol.

Discussion

A variety of experiments have made use of mammalian erythrocytes to support the hypothesis that the binding site and target of streptolysin O and other thiol-activated bacterial toxins is membrane cholesterol. Our previous studies with oxygenated derivatives of cholesterol and the experiments described here provide additional evidence that streptolysin O action on more complex, nucleated mammalian cells is also related to cellular cholesterol. The oxygenated sterols inhibit cellular cholesterol synthesis by depressing the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, and if exogenous sources of cholesterol are not provided, the sterol content of the cells decreases, finally reaching a level that is about half that of the initial concentration (Ref. 6, and Table III). These events appear to account for the resistance of such cells to the action of streptolysin O and other cholesterol-reactive compounds. Sterol biosynthesis was found to be significantly inhibited in L cells exposed to the hydroxycholesterol compounds, and the toxic effects of streptolysin O were accordingly reduced (Table II). In addition, the cholesterol content of streptolysin O-resistant cells was indeed found to be reduced (Table III), and the extent of the reduction generally corresponded to the degree of toxin protection.

Several comments about the altered cholesterol content of the cells can be made. First, we found that when HeLa cells were grown in 5% serum, two-thirds of the cellular cholesterol was present as free cholesterol, but after 24 h of growth in lipid-depleted serum, greater than 90% of the total cholesterol was free, i.e., unesterified (data not shown). Second, in the absence of exogenous cholesterol, the free sterol content of cells treated with 25-hydroxycholesterol (Table III) or 20α-hydroxycholesterol was diminished, and after 36 h incubation, the free (and total) cholesterol concentration was about 50% of that found in control cells. The treated cells were highly resistant to streptolysin O at that point. Studies with human fibroblasts have shown that nearly all of the free cholesterol in the cell is found in the plasma membrane [4], and essentially all of the sterol present in cell membranes is in the free

form [15]. Based on inactivation studies, streptolysin O is thought to react only with free cholesterol (and certain related sterols possessing a 3β-OH group and aliphatic side chain), but not with cholesterol esters [3,16]. It seems reasonable, therefore, that the reduced streptolysin O sensitivity of cells treated with oxygenated derivatives of cholesterol is due to the reduction of free (membrane) cholesterol.

One observation deserving further comment here is that considerable protection against streptolysin O was observed in HeLa cells in which the free cholesterol content had been reduced by only about 20% (Table III). It appears that the production of cytotoxic effects by this particular concentration of toxin is quite sensitive to rather small reductions in membrane cholesterol. This may be related to the fact that lysis of cells (erythrocytes) by streptolysin O is a multi-hit process [3], and the toxin concentration used in this experiment may fall in a critical region of the dose-response curve where even a small reduction of free cholesterol in the cell precludes many of them receiving a threshold level of toxin sufficient to produce lysis. At this cholesterol concentration (80% of control), the cells would presumably be almost completely protected against low toxin doses, but more sensitive to higher doses. A related phenomenon was observed in a study on the effects of the closely related toxin, tetanolysin, on liposomes. Tetanolysin produced maximum release of trapped glucose when the liposomes contained 50 mol% cholesterol, but no release occurred below 40 mol% cholesterol [17].

The possibility that alterations in the membrane (other than decreased cholesterol) secondary to the inhibition of sterol synthesis account for the increased resistance to streptolysin O cannot definitely be ruled out, but it seems less likely. We previously found that the binding of toxin to protected cells was significantly reduced [4], and in this study, cells that were resistant to streptolysin O were also more resistant to saponin and digitonin, agents that are also known to react with membrane cholesterol. In addition, the nature of the substances that allow toxin-resistant cells to return to a sensitive state, and the speed with which this occurs, further support the concept that protection against streptolysin O is directly related to reduced membrane cholesterol. The addition of serum to the incubation medium at concentrations of at least 5% markedly stimulated the recovery of the cells. This is probably the result of the uptake of serum cholesterol, present in low density lipoprotein, via receptor-mediated endocytosis [18]. Significant recovery occurred even within 6 h after the addition of 20% fetal calf serum, and this effect was abolished by the removal of lipid from the serum (Fig. 4). The direct addition of cholesterol was also very effective in promoting recovery of the cells. The mechanism was not explored here, but exogenous cholesterol may be taken directly into the membrane [19], thus providing suitable binding/target sites for streptolysin O action. Recovery in mevalonate or lipid-depleted serum appears to require longer incubation periods. This is not unexpected, since under these conditions, it would be necessary for the cells to synthesize the sterol in order to replace it in the membrane.

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