PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 233–237 (1981)

Cholesterol Inhibition of Streptolysin O Toxicity for Myocardial Cells in Tissue Culture (41266)

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Abstract. Cholesterol has been shown to inhibit the hemolytic activity and the immunogenicity of streptolysin O. It has been found that in tissue culture, cholesterol also protects spontaneously contracting rat myocardial cells from the cytotoxic injury caused by streptolysin O.

Cholesterol inhibits two of the recognized biologic activities of streptolysin O, its hemolytic (1) and its immunogenic properties (2). In addition, cholesterol has been shown to block the lethal effect of intravenously injected streptolysin O in mice (1) and the toxic effect on isolated guinea pig atria (2). Although it has been suggested that this lipid compound may also block yet another biologic property of this streptococcal extracellular product, its direct cytotoxic effect on myocardial cells (3), to our knowledge this has not been documented. In these semiquantitative studies we have obtained evidence indicating that cholesterol neutralizes streptolysin O cytotoxicity for isolated spontaneously contracting myocardial cells in tissue culture.

Materials and Methods. *Myocardial cell preparation.* For each experiment, the hearts of 4-day-old Sprague–Dawley rats were sterilely excised and placed in cold Hanks' balanced salt solution (HBSS) containing penicillin, streptomycin, and gentamicin. The hearts were cut into very small pieces and treated with type I collagenase (Sigma Chemical Co., St. Louis, Mo.) in a concentration of 360 units/ml of HBSS; the pieces were then placed in a flask with gentle stirring at 37° for 10 min. The initial supernatant (which largely consists of epithelial cells) was discarded and fresh collagenase was added; slow stirring was continued for an additional 30–40 min.

Medium. Cold Eagle's minimum essential medium with Earl's salts (MEM) (Gibco, Grand Island, N.Y.) containing L-glutamine, penicillin, streptomycin, and gentamicin, with 10% heat-inactivated fetal calf serum (complete MEM), was added to the cells. The cells were centrifuged, washed, and resuspended in the cold complete MEM.

Chambers. Sykes-Moore tissue culture chambers were filled with the cell suspension and incubated at 37° for 3 hr, then inverted and incubated for 48 hr. The medium was changed, and at 72 hr the chambers were ready for use in experiments. Experiments to evaluate the effect on the viability of myocardial cells were performed (i) with tissue culture medium, (ii) with tissue culture medium plus streptolysin O, (iii) with tissue culture medium plus cholesterol, and (iv) with tissue culture medium plus streptolysin O which had been preincubated with cholesterol. All materials were warmed to 37° and adjusted to pH 7.2, prior to infusion into the chambers. Once the myocardial cell cultures had been established, fetal calf serum was eliminated from the medium used in cytotoxicity experiments to ensure that the cholesterol or similar lipids present in fetal calf serum did not influence streptolysin O cytotoxicity during the experiments. Chamber controls using established myocardial cell cultures and MEM without fetal calf serum showed no change in cell viability during a 48-hr period when com-

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0037-9727/81/100233-05$01.00/0

pared with myocardial cells incubated with complete MEM.

Streptolysin O. Streptolysin O was prepared by adding phosphate-buffered saline (PBS) at pH 6.6 to reduced streptolysin O (Difco Laboratories, Detroit, Mich.) with a resulting concentration of 25 minimum hemolytic doses/ml (7–9). The streptolysin O was then dialyzed for 18 hr against two 4-liter changes of cold buffer and, finally, for 3 hr against 1 liter of cold MEM. For the cytotoxicity experiments, appropriate dilutions were made in MEM (without fetal calf serum).

In vitro streptolysin O assay. In vitro semiquantitative streptolysin O assays were performed before and after dialysis. To 1.0 ml of the streptolysin O dilution in MEM, 0.5 ml of 3% washed ovine erythrocytes (RBC) was added, and the mixture was incubated for 30 min at 37°. Following slow centrifugation, hemolysis was evaluated visually (4+ = complete hemolysis; 0 = no detectable hemolysis). The highest dilution of streptolysin O in MEM resulting in complete hemolysis was determined before and after dialysis. Streptolysin O activity was usually reduced by approximately 50–75% of the original strength after dialysis.

Cholesterol. Ash-free cholesterol (99% pure, Difco Laboratories) suspensions at a final concentration of 500 μg/ml were prepared by dissolving cholesterol in methanol and then pouring the methanol solution into boiling HBSS (resulting in evaporation of the methanol). Appropriate dilutions of the cholesterol suspension for use in cytotoxicity experiments were made in MEM (without fetal calf serum).

Inhibition ratio of streptolysin O to cholesterol. To determine the appropriate inhibition ratio of streptolysin O to cholesterol for these *in vitro* experiments, 0.5 ml of dialyzed undiluted streptolysin O was incubated for 15 min at 37° with 0.5 ml of the cholesterol suspension being tested. Then, 0.5 ml of 3% RBC was added and the mixture was incubated at 37° for an additional 30 min. Hemolysis was evaluated semiquantitatively (0–4+).

After the cells were established in culture, spontaneously and regularly contracting myocardial cells were observed by phase microscopy. Photomicrographs of the same cells were taken at zero time and 5 min, 15 min, 30 min, 1 hr, 2 hr, and 18 hr after infusion of the test sample. A Reichert Zetopan phase microscope (Reichert, Vienna), equipped with a warming stage and a Wild photoautomat MPS 55 camera (Wild Heerbrugg Instruments, Inc., Heerbrugg, Switzerland) with high-contrast copy film (Kodak, Rochester, New York), was used. Experiments were repeated several times to assure reproducibility.

Results and Discussion. Concentrations of streptolysin O of 10–20 minimum hemolytic doses/ml rapidly and uniformly killed the myocardial cells (Fig. 1). Regular rhythmic contraction ceased and fibrillation began within 30 sec of exposure to the toxin. Small bleb formations, similar to those described by Thompson *et al.* (4), occurred on the cell membrane within 2 min. These blebs enlarged to larger balloon-shaped formations within 15 min. The intracellular organelles appeared disorganized. The cells never recovered, although the bleb and balloon formations became reduced in number and smaller in size after 2 hr of incubation.

More dilute concentrations of streptolysin O (2.5 minimum hemolytic doses/ml) were also lethal, but the onset of the toxic effect did not become visually evident until 5 min after exposure to the toxin and killing was less rapid. Bleb formation was noted at 15 min, but progression to large balloon forms was rarely observed. With dilute concentrations of streptolysin O (0.5 hemolytic doses/ml) beating continued for 2 hr, although the rhythm was slow and appeared irregular. Neither bleb nor balloon formation was observed. Myocardial contractions ceased after 18 hr of exposure to this low contraction of streptolysin O, but the cells remained grossly intact with none of the typical intracellular changes noted.

In high concentrations (500 μg/ml or greater), cholesterol alone was lethal for the myocardial cells (not shown); large intercellular fat-filled vacuoles formed and myocardial contractions ceased. Choles-

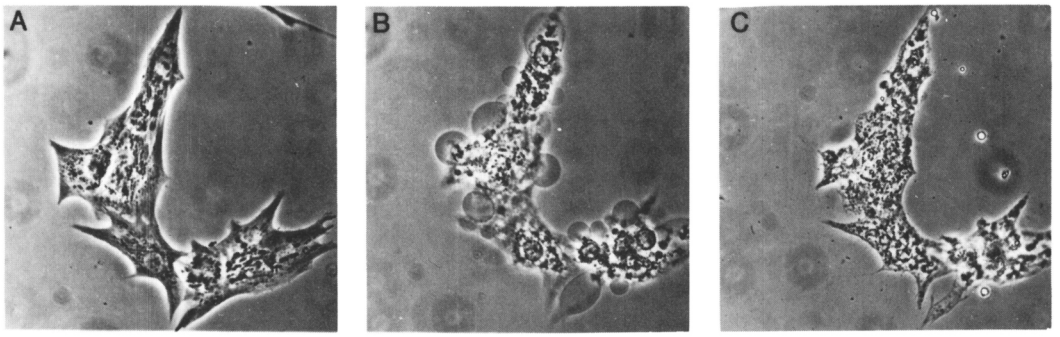

FIG. 1. Effects of streptolysin O on myocardial cells. ×380. (A) Before addition of streptolysin O, this group of myocardial cells was contracting regularly at a rate of 120/min. (B) Five minutes after the addition of 12.5 minimum hemolytic doses/ml, all contractions ceased and large membrane blebs were observed. (C) After 18 hr the cellular contents were disorganized and the blebs had disappeared.

terol concentrations of 125 μg/ml or less of cholesterol also resulted in accumulation of fat vacuoles in cells, but with no discernable adverse effect on the cells (Fig. 2) other than, in a few instances, transient reduction in rate of contraction lasting a few hours.

When dialyzed streptolysin O (minimum concentration of 10 minimum hemolytic doses/ml) was incubated with cholesterol (at a ratio of 1.0 hemolytic dose of streptolysin 0 to 1.0 μg cholesterol) for 15 min at 37°, no cytotoxic effect was observed (Fig. 3). At 18 hr the myocardial cells continued regular contractions and no changes were

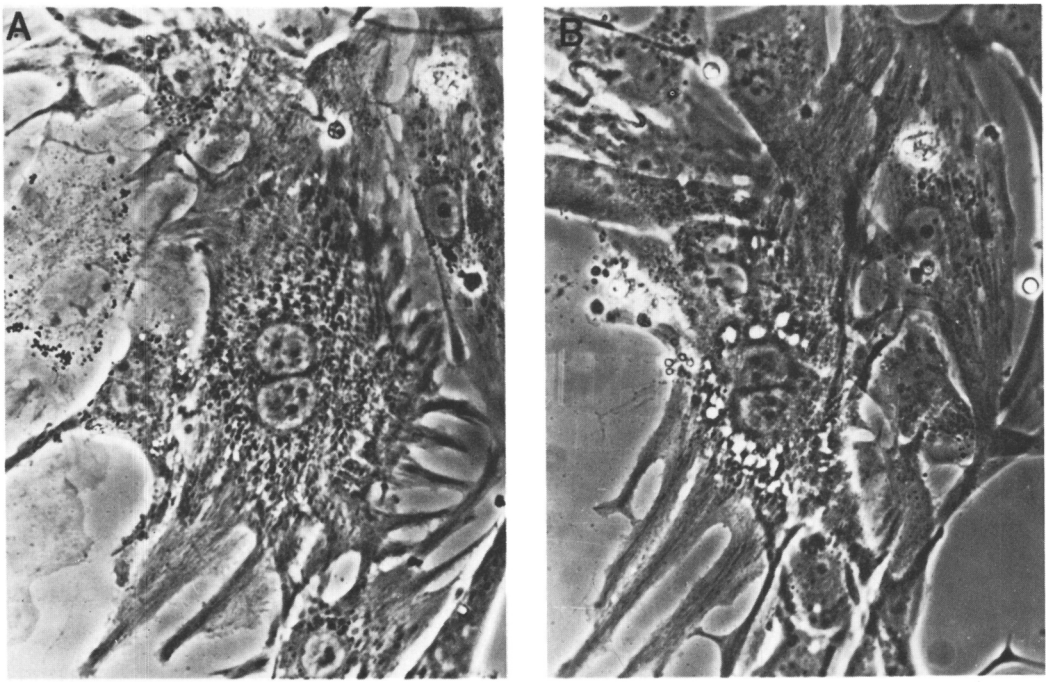

FIG. 2. Effects of cholesterol on myocardial cells. ×380. (A) Before addition of cholesterol this binucleated myocardial cell was contracting at a rate of 88/min. (B) Cholesterol (125 μg/ml) was added and 18 hr later the rate was 60/min.

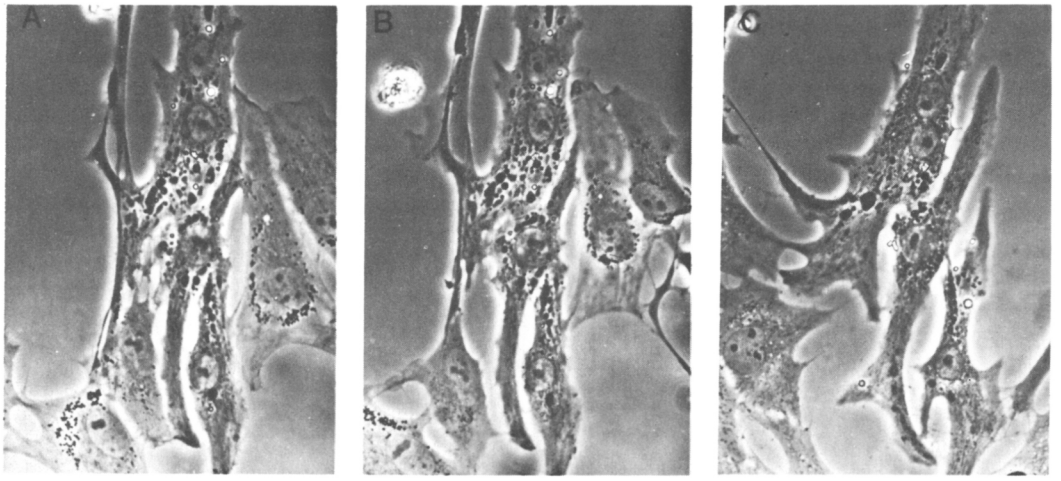

FIG. 3. Effects of inhibition of streptolysin O cytotoxicity by cholesterol. ×380. (A) Before addition of the streptolysin O–cholesterol mixture, the rate of contraction was 100/min. (B) A mixture of 12.5 minimum hemolytic doses of streptolysin O and 12.5 μg cholesterol was added to the 1.0 ml Sykes-Moore chamber and 2 hr later the rate was 80/min. (C) After 18 hr the cells were still contracting at a regular rate. [*Note.* the surrounding epithelial cells have migrated on the coverslip. Myocardial cells usually remain in place, but send out myopodia to contact other cells (10).]

noted in the intracellular organelles. This suggests that other streptococcal extracellular products that could be present in the streptolysin O preparation (i.e., products unaffected by cholesterol) had no cytotoxic effect. When the ratio was increased to 1.0 hemolytic dose streptolysin O:0.1 μg cholesterol the changes were variable. In some experiments the cells continued to contract, and in others they did not, although no cellular damage was visually evident. At a ratio of 1.0 hemolytic dose streptolysin O/0.01 μg cholesterol the myocardial cells consistently showed characteristic evidence of streptolysin O damage. A more exact endpoint could be determined for inhibition of hemoysis; 1.0 hemolytic doses of dialyzed streptolysin O were totally inhibited by 0.08 μg of cholesterol.

The mechanism(s) of inhibition of streptolysin O toxicity for myocardial cells has not been elucidated. All eukaryotic cells have cholesterol present in their cell membranes, and it is likely that the streptolysin O cytotoxicity is blocked by competitive binding by the exogenous cholesterol, as is the case with streptolysin O-induced hemolysis of erythrocytes (1). Duncan and Buckingham found that L-cell fibroblast cultures treated with oxygenated derivatives of cholesterol (which inhibit cholesterol biosynthesis) had an increased resistance to streptolysin O toxicity (5). They proposed that a reduction in the number of streptolysin O binding sites due to the decrease in membrane cholesterol was responsible for the resistance of the fibroblasts to the toxin. Thompson *et al.* (4) found that the cytotoxicity of streptolysin O is also eliminated by heating it to 56° for 30 sec or combining it with rabbit antiserum.

It has been suggested that streptolysin O may play a role in the myocardial injury associated with rheumatic fever (5, 6). Our data indicate that this toxin's lethality for myocardial cells can be blocked by cholesterol, but the relevance of this observation to the development of rheumatic carditis remains undefined.

This work was supported by a grant from the National Institutes of Health (AI 08724) and in part by the Dwan Family Fund. Dr. Wannamaker is a Career Investigator of the American Heart Association.

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Received February 23, 1981. P.S.E.B.M. 1981, Vol. 168.