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Cytotoxic Effects In Vitro of Highly Purified Streptolysin O on Mouse Macrophages Cultured in a Serum-Free Medium

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ABSTRACT

FAUVE, ROBERT M. (Institut Pasteur, Garches (Hauts de Seine), France), JOSEPH E. ALOUF, ALBERT DELAUNAY, AND MARCEL RAYNAUD. Cytotoxic effects in vitro of highly purified streptolysin O on mouse macrophages cultured in a serum-free medium. J. Bacteriol. 92:1150–1153. 1966.—After the addition of highly purified streptolysin O to mouse macrophages cultured in medium 199 containing albumin, the following cellular alterations were observed. The disappearance of the stellate shape of the cells and the formation of bleb-like vesicles extruding from the cytoplasmic membrane occurred within a few minutes. Later, massive degranulation and formation of cytoplasmic vesicles were followed by cell death.

Streptolysin O (SLO) is known as a potent extracellular hemolytic protein produced by most strains of *Streptococcus pyogenes*. It has been shown that this toxin, which is lethal for mammals, displays cytolytic and cytotoxic properties towards polymorphonuclear leukocytes (14, 20), Ehrlich tumor cells (11), and rabbit alveolar macrophages (14). It has been emphasized that this cytolytic action is related to lysosomal disruption (6, 15, 18, 19) and mitochondrial swelling (15).

Most of the previous experiments were performed with partially purified SLO. The toxic effects observed are most certainly induced by streptolysin itself, as shown indirectly by the use of oxidized SLO preparation or SLO after addition of cholesterol. However, the presence of many streptococcal enzymes in such preparations (12) does not rule out a possible interfering action of these substances with SLO. On the other hand, the mammalian cells used in experiments remained coated with serum globulins, since it is well known that even repeated washings cannot remove all the proteins strongly bound to cellular membrane. Thus, it seemed advisable to use, on the one hand, a SLO preparation devoid of other detectable streptococcal enzymes and, on the other hand, serum-free cells. In vitro cultured mouse macrophages appeared to be a suitable material, since these cells no longer contain detectable amounts of serum globulins after 3 days of incubation (R. M. Fauve, *unpublished data*). Moreover, the large size of such cells, and the easy observation of their cytoplasmic organelles, after in vitro culture, make them a very convenient experimental model.

In this paper, we report the study of the morphological alterations of mouse macrophages after the addition of reduced SLO.

MATERIALS AND METHODS

Streptolysin O. Highly purified SLO (5,200-fold purified) was obtained, after fractionation of crude culture supernatant fluid of *S. pyogenes* A78 strain, grown on the medium described previously (2). During the first steps, purification procedure was similar to the one previously reported (1), but it was highly improved, mainly by subsequent gel filtration on Sephadex G 50 and G 100 (J. Alouf and M. Raynaud, *to be published*). Specific activity of the purified toxin was 36,000 combining units (LhT) per milligram of nitrogen. One LhT is equivalent to 35 hemolytic units (HD_{50}, where 1 HD_{50} is 5×10^{-3} μg of protein) in the experimental conditions of titration described elsewhere (3). The preparation obtained was homogenous in the ultracentrifuge. Immunodiffusion tests, performed according to Oakley and Fulthorpe technique (16), in which a high concentration of antigen was allowed to diffuse against horse antiserum (3) or human γ globulins (Centre National de Transfusion Sanguine, Paris) showed a main diffusion band and a weaker one.

A suitable volume of the stock solution was diluted

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as needed in medium 199 to a final titer of 5,000 HD_{50} per milliliter. Toxin activation was carried out before dilution in presence of 0.02 M cysteine at room temperature for 10 min. Diluted toxin solution was then kept ice-cooled until use. Since medium 199 partially inhibited hemolytic activity of SLO, the titer refers to SLO diluted in this medium and not in phosphate-buffered saline as usual. Under these condition, the LD_{50} for N.C.S. mice after intravenous injection was in the range of 150 HD_{50} (0.75 μg of protein).

Culture of mouse macrophages. Collection and culture of macrophages were carried out according to the technique reported previously (8). Briefly, the peritoneal cavities of 25-g N.C.S. (9) mice were washed with 5 ml of medium 199, containing 2.5% bovine serum albumin (Poviet Amsterdam, The Netherlands) and 5 units/ml of heparin (Choay, Paris, France). From the pool of ice-cooled peritoneal washings, 2.5 ml of the medium, containing 5×10^5 to 7×10^5 macrophages per milliliter was placed in Leighton tubes flushed with an air (95%) and CO^2 (5%) mixture. After 2 hr at 37 C, the liquid phase was discarded and tubes were filled again with the same medium without heparin. After 3 days at 37 C, the cellular population consisted essentially of macrophages.

Incubation of macrophages with SLO. Before the addition of SLO to 3-day-old cultures, the liquid phase was removed, and the macrophages monolayers were washed twice in the tubes with plain medium 199. A 2-ml amount of this medium containing 860 HD_{50} of SLO was introduced in the tubes, which were then incubated in a water bath at 25 $\pm$ 0.5 C. After different periods of incubation, the liquid was removed; the cells were washed once with saline and then were fixed with 1% OsO_4 solution (17), as described previously (10). After fixation, morphological studies were carried out with a Leitz ortholux phase microscope (Heine condensor and P.V. apo, oil 90/1.15 lens). Pictures were taken with a Leica microattachment and Kodak panatomic X film.

RESULTS

Striking morphological modifications of macrophages were observed within a few minutes after the addition of SLO. A normal macrophage (Fig. 1) shows a kidney-shaped nucleus surrounded with cytoplasmic organelles, such as osmiophilic granules (G), lipid droplets (LD) and mitochondria (M). The cytoplasm, which is highly osmiophilic, is well spread and shows a diffuse cytoplasmic membrane.

After 1 min (Fig. 2), the typical stellate shape of healthy macrophages is lost in some cells. In a few cells, more pronounced alterations of cell surface appeared, as one can see from the very large bleb-like vesicles (V) which very likely represent cytoplasmic membrane alteration.

After 5 min (Fig. 3), most of the cells were highly damaged and showed various alterations of the cell surface, as well as of cytoplasmic organelles. On cell surface, many bleb-like protrusions were observed, as mentioned before. Inside the cell, numerous punched cytoplasmic holes were seen in the centrosphere area, contrasting with the surrounding osmiophilic granules. A few non-osmiophilic foci (RG) were also observed.

After 15 min (Fig. 4), almost all cells were severely injured. Many large intracytoplasmic vesicles (CV) appeared. The number of osmiophilic granules was greatly reduced. The mitochondria, which are so easily recognized in normal macrophages (Fig. 1), were no longer observed. Only the nucleus looked apparently unaffected at this time, except in a few cells where it appeared less dense and smaller.

Horse anti-SLO serum, as expected, protected cells from SLO injury, when the toxin was previously incubated for 15 min with the required amount of antiserum for the neutralization of its hemolytic activity.

Also, 3-day-old macrophage culture supernatant fluid exhibited a highly protective action against both cytotoxic and hemolytic effects of SLO. Although this inhibitory property has not been investigated, it is likely that cholesterol, which is known to be synthesized by macrophages (7), may be involved in this phenomenon, since this substance is a potent inhibitor of SLO (13).

DISCUSSION

The observations reported here on the very potent cytotoxic action in vitro of highly purified SLO on mouse macrophages cultured in a serum-free medium are consistent with the experiments reported in other investigations with partially purified preparations of that toxin. It is now evident that SLO damages many kinds of mammalian cells, especially blood cells (4, 5). Motion-picture studies of toxin action on rabbit polymorphonuclear leukocytes has been carried out by Hirsch et al. (14). These authors have shown that the initial morphological alteration is a rapid and extensive destruction of cytoplasmic granules, before the appearance of membrane alterations. Weissman et al. (18, 19) have shown in vitro that SLO disrupts isolated rabbit peritoneal polymorphonuclear leukocyte granules as well as lysosome-rich granular fractions of various mammalian cells (6, 15, 18, 19). In our experiments on cultured mouse macrophages, different cellular events occurred. Large cytoplasmic bleb-like protrusions, equivalent sometimes to half the length of the cell, appeared first, followed later by cytoplasmic granule disruption. We did not observe at any time the long filamentous threads extruding from cytoplasm, that Hirsch et al. (14) and Zucker-Franklin (20) reported. Our experimental

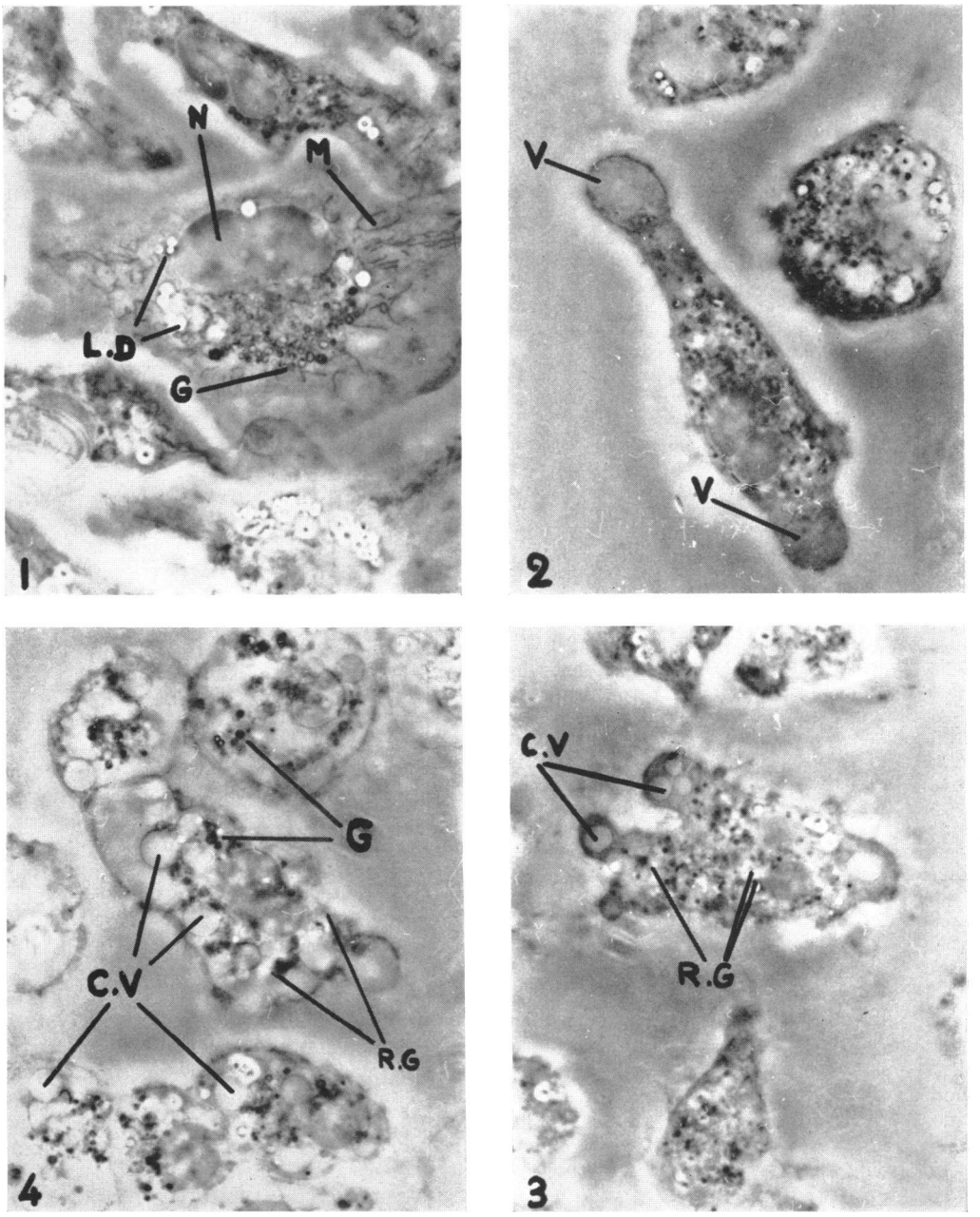

FIG. 1. *Mouse macrophages after 3 days in culture. Note nucleus (N), lipid droplets (L D), osmiophilic granules (G), mitochondria (M).* × *1,200.*

FIG. 2. *Mouse macrophages after 1 min contact with streptolysin O. Note bleb-like vesicles (V).* × *1,200.*

FIG. 3. *Mouse macrophages after 5 min contact with streptolysin O. Note cytoplasmic vesicles (C V), destroyed granules (R G).* × *1,200.*

FIG. 4. *Mouse macrophages after 15 min of contact with streptolysin O. Note the decrease of osmiophilic granules (G), the cytoplasmic vesicles (C V), and destroyed granules (R G).* × *1,200.*

conditions may explain such differences, since we have used cultured mouse macrophages instead of rabbit polymorphonuclear leukocytes. Besides the possible different behavior of rabbit polymorphonuclear leukocytes and mouse macrophages, it is known that cells, freshly removed from a previously irritated peritoneal cavity, remain coated with serum proteins even after repeated washings. Therefore, it is not impossible that such proteins can protect the membrane against the lytic action of SLO. In our experiments, where macrophages are not coated with such proteins, SLO may act directly on the cellular membrane and may induce the formation of the observed bleb-like vesicles. We have seldom noticed nuclear alterations of mice macrophages.

It is noteworthy that a marked variation in sensitivity to SLO was recorded in all experiments we carried out with a relatively high concentration of toxin. Even after 15 min of contact followed by extensive cell damage, a few cells were unaffected. This striking difference in susceptibility of an apparently homogenous cell population, also reported by others (5, 20), is still unexplained. The results obtained with the highly purified SLO preparation strongly support the concept that the cytotoxic effects reflect the biological activity of a single substance. The rapidity with which activity and integrity of many kinds of cells are impaired suggest that the primary biochemical lesion is on cytoplasmic and organelle membranes.

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