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Decreased E-Rosette Formation following Streptolysin-O Treatment[1] (39556)

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Streptolysin-*O* (SO) is known to be cytotoxic to a number of cell types (1-5). Cell lysis and death at high SO doses (1, 2) and altered cell function at lower doses (3-5) have been reported. Andersen and Cone (5) have shown that lymphocyte blast transformation, as measured by [^{3}H]thymidine uptake, can be blocked by pretreating lymphocytes with sublytic doses of SO. Since SO has the potential of altering immunologic responses important in streptococcal infections, we further investigated the action of SO on human lymphocytes using the E (erythrocyte) and EAC (erythrocyte, antibody, complement) cell rosette techniques.

Materials and methods. Streptolysin-O. SO was produced by Todd-Hewitt broth cultures of Richards strain, group A type 3 *Streptococcus pyogenes*, and isolated using cryoprecipitation and Sephadex G100 chromatography as described by Van Epps and Andersen (6). A hemolytic unit (HU) was defined as the minimal amount of SO that lyses an equal volume of a 2% sheep red blood cell (SRBC) suspension.

Lymphocyte isolation. Using the Ficoll-Hypaque density gradient technique as described by English and Andersen (7), lymphocyte-rich fractions were isolated from whole normal human blood. The cells were washed three times with saline, counted in a hemocytometer, and adjusted to a concentration of 10^7 cells per ml.

E-Rosettes. SRBCs were washed three times in phosphate-buffered saline (PBS), pH 7.0, and suspended to give a final SRBC concentration of 2% in 10% fetal calf serum in minimal essential media. Equal volumes of SRBCs and lymphocytes (untreated or treated as described below) were incubated for 30 min at room temperature. The cells were centrifuged at 200*g* for 5 min and incubated overnight at 4° without disturbing the cell button. After gentle suspension of the cells, 1 drop of 0.5% toluidine blue-O was added. The cells were placed in a hemocytometer, kept at room temperature for 5 min, and examined by light microscopy. Two hundred lymphocytes were examined for each test, and those with three or more attached SRBCs were counted as rosettes. The percentage of lymphocytes forming rosettes was determined.

EAC-Rosettes. An equal volume of rabbit anti-SRBC diluted 1:300 was added to SRBCs that had been washed four times with PBS. After incubation at 4° for 1 hr, the cells were washed twice with PBS to remove free hemoglobin. These cells can be stored for 1 week in the refrigerator. Before use, 9.6 ml of a 5% solution of the EA cells were mixed with 0.4 ml of fresh normal human serum and incubated for 1 hr at 37°. These EAC cells were washed with PBS till the free hemoglobin was gone, and then adjusted to a 2% suspension with PBS. Equal volumes of EAC cells and treated or untreated lymphocytes were incubated at room temperature for 30 min while tumbling end-over-end at 30 rpm. Incubation was continued at 4° for 30 min with tumbling. Prior to counting rosettes, the preparation was mixed vigorously on a vortex mixer. The counting procedure was the same as that described for the E-rosettes.

Treatment protocol. Isolated human lymphocytes were used in all experiments. The cells were treated with hemolytically active SO or with SO inactivated by treatment with anti-SO serum, cholesterol, heat (56°C, 30 min), or oxygen. In some cases, the lymphocytes were treated first with SO, then with cholesterol or anti-SO serum to determine the reversibility of the SO effect. Treatment periods were always 30 min and incubation temperature was 37°. Following treatment,

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the lymphocytes were washed twice with PBS before the rosette assays were performed. All experiments were done in duplicate and every experiment was repeated at least once.

Results. When low levels of hemolytically active SO (SO ≤ 2 HU) were incubated with isolated lymphocytes, the percentage of T-cells, as determined by the E-rosette technique, consistently decreased (Fig. 1). This effect was dose related, and very few E-rosettes could be found when 2 HU of SO was used. Cholesterol, which inhibits the hemolytic activity of SO, was found to block the effect of SO on lymphocytes, but could not reverse the effect after SO had been incubated with the lymphocytes (Table I). The decrease in E-rosette levels was not due to lysis of lymphocytes by SO since there were no differences in the number of lymphocytes before and after SO treatment when 2 HU or less was used. There was no significant change in the percentage of EAC-rosettes following SO treatment.

Table II demonstrates that the effect of SO on E-rosette formation could not be reversed by the anti-SO serum after the SO had been incubated first with the lymphocytes. The SO effect could be blocked, however, when the SO was preincubated with the anti-SO serum. Other factors which will inactivate the hemolytic ability of SO, namely, heat (56°, 30 min) and oxygen were effective in blocking the action of SO on the formation of E-rosettes.

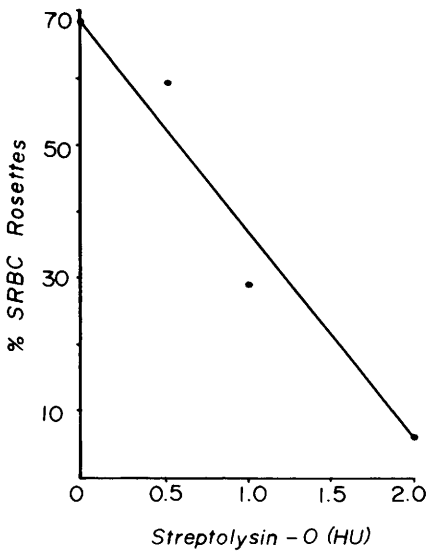

FIG. 1. The effect of varying concentrations of streptolysin-*O* on E-rosette (SRBC-rosette) formation.

Discussion. Small amounts of SO were capable of markedly reducing the number of E-rosettes found in isolated human blood lymphocyte preparations. The ability of agents which can block the hemolytic action of SO to interfere with the E-rosette blocking of SO suggests that the mechanism causing hemolysis is related to the one which blocks E-rosette formation. The effect of SO on E-rosette formation was not due to lysis of lymphocytes since lysis fails to occur at the levels of SO used in these experiments. A previous study of lymphocyte viability using the Evans blue dye exclusion method has shown that 1 HU of SO will not alter cell viability (5). No change was observed in the number of EAC-rosettes following SO treatment.

The effect of SO on E-rosettes was not due to the direct action of SO on the SRBCs, since SO was removed by washing the lymphocytes prior to the assay. Possible action of small amounts of residual SO on the SRBC-rosette was discounted by data shown in Table II. The SO effect on E-rosette formation could not be reversed by adding anti-SO serum following SO treatment. Since the anti-SO serum was added prior to the SRBCs, the antiserum would have neutralized any residual SO activity and protected the SRBCs from the effect of SO. In spite of this, the percentage of E-rosettes following SO treatment was markedly reduced compared to normal.

The mechanism of SO action on E-lymphocytes is unknown. Since other studies have indicated that its primary action is on cell membranes, it seems likely that SO is altering the SRBC receptor site of T-lymphocytes. The failure of cholesterol and anti-SO serum to reverse the effect suggests that membrane structure has been irreversibly altered.

An earlier study from this laboratory (5) has shown that T-lymphocyte blast transformation by PHA can be blocked by pretreatment of lymphocytes with SO. This action of SO may be due to the same or a similar membrane effect that has been postulated

TABLE I. EFFECTS OF STREPTOLYSIN-*O* AND CHOLESTEROL ON E- AND EAC-ROSETTES.

Lymphocyte treatment	E-Rosettes (%)		EAC-Rosettes (%)	
	Duplicates	Mean	Duplicates	Mean
None	67,70	68	15,15	15
SO (1 HU)	27,30	28	13,13	13
Cholesterol[a]	62,64	63	N.D.[b]	
SO (1 HU) → cholesterol[c]	33,36	34	N.D.	
SO (1 HU)/cholesterol[d]	62,66	64	N.D.	

[a] Lymphocytes treated with a saturated solution of cholesterol for 30 min at 37°.
[b] N.D., not done.
[c] Lymphocytes treated first with SO (1 HU) for 30 min at 37°, then incubated with an equal volume of a saturated solution of cholesterol for 30 min at 37°.
[d] SO (1 HU) mixed with a saturated solution of cholesterol prior to treatment of lymphocytes for 30 min at 37°.

TABLE II. EFFECTS OF ANTI-STREPTOLYSIN-*O* SERUM ON THE STREPTOLYSIN-*O* SUPPRESSION OF E-ROSETTES.

Lymphocyte treatment	E-Rosettes (%)	
	Duplicates	Mean
None	60,76	68
SO (2 HU)	10,14	12
SO (2 HU) → ASO[a]	16,17	16
ASO	64,68	66
SO (2 HU)/ASO[b]	67,73	70

[a] Lymphocytes treated first with SO (2 HU) for 30 min at 37°, then incubated for 30 min at 37° with an equal volume of human serum containing anti-SO activity (333 Todd units).
[b] SO (2 HU) mixed with human anti-SO serum (333 Todd units) prior to treatment of lymphocytes for 30 min at 37°.

for the E-rosette inhibition. These observations suggest that SO may act *in vivo* to alter the T-lymphocyte function of the host.

Summary. Doses of SO which did not cause lymphocyte lysis or reduced viability (≤2 HU), decreased E-rosette formation. SO, treated with agents which block hemolytic activity (heat, oxygen, cholesterol, and anti-SO serum), did not affect E-rosette formation. Cholesterol and anti-SO serum were not able to reverse the E-rosette-inhibiting action of SO. EAC-rosettes were not affected by SO.

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