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Streptolysin O Inhibition of Neutrophil Chemotaxis and Mobility: Nonimmune Phenomenon with Species Specificity

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The effects of streptolysin O (SO) (1 to 4 hemolytic units) on the mobility of neutrophilic leukocytes from humans, baboons, sheep, and rabbits were compared. After SO treatment, chemotaxis and random mobility of human neutrophils were markedly suppressed, baboon and sheep neutrophils were partially suppressed, and rabbit neutrophils were unaffected and demonstrated normal chemotaxis and mobility. The amounts of SO used in the mobility studies caused no leukocyte lysis or trypan blue uptake by human, baboon, or sheep cells, and minimal lysis or trypan blue uptake by rabbit cells. The possible involvement of immune mediators in the observed inhibition of human neutrophils was considered and excluded by the following studies. White blood cells from humans with humoral or cellular immune deficiencies responded in a manner similar to normal human cells; supernatant solutions from SO-treated human white blood cells did not contain a chemotactic suppressor; preincubation of SO with cholesterol (an inhibitor of SO hemolytic activity) caused loss of the chemotactic suppressive effect of the toxin on human leukocytes; and leukocytes from rabbits preimmunized with SO remained refractory to chemotactic suppression.

Streptolysin O (SO), the oxygen-labile hemolysin of beta-hemolytic streptococci, is capable of degranulating and lysing white blood cells (WBC) (1, 2, 4) when the cells are treated with high doses of the toxin. Andersen and Van Epps (1) have shown that treatment of human WBC with low doses of SO, which were incapable of lysing the WBC or of causing any observable morphological changes, resulted in suppression of neutrophilic chemotaxis and mobility. The present study is the result of further investigations of this effect and demonstrates species specificity and the absence of immune mediators as effectors in SO chemotactic suppression.

MATERIALS AND METHODS

Preparation of leukocytes. WBC preparations were obtained from the peripheral blood of humans, baboons, sheep, and rabbits. Coagulation was prevented by heparin (10 U/ml), except in the rabbit, where the excessive amounts of heparin needed to prevent coagulation were avoided by glass bead defibrination. Defibrination of human blood with glass beads gave results similar to those where heparin was used. Red blood cells (RBC) were then sedimented for 30 min with Plasmagel (Roger Bellon Laboratories, Neuilly, France; 1 ml per 5 cm^3) or with a one-third volume of pigskin gelatin (United Chemical and Organic Products, Calumet City, Ill.). In the rabbit and the sheep, pigskin gelatin proved to be a more efficient means of erythrocyte sedimentation. The supernatant solutions containing a high percentage of WBC were then decanted and centrifuged at 1,500 rpm (270 × *g*) for 10 min. The cells were washed twice with Hanks balanced salt solution (HBSS) and adjusted to the white cell count needed in each experiment. The human cell preparations contained approximately 65% polymorphonuclear leukocytes, 34% mononuclear cells, and a red cell-to-white cell ratio of approximately 1.4 to 1. In the rabbit, the red cell-to-white cell ratio was 1:2 and the WBC preparation consisted of approximately 47% polymorphonuclear cells and 50% mononuclear cells.

Trypan blue staining. The viability of leukocytes was measured by the trypan blue dye exclusion method. WBC preparations were mixed with trypan blue to a final dye concentration of 0.2%. The cell preparations were observed in a hemocytometer, and the percentage of cells containing nuclei staining with trypan blue was determined.

Chemotaxis. Chemotaxis was measured by a modification of the Boyden (3) technique using a miniaturized system of chemotaxis chambers developed in this laboratory and manufactured by the Mark-it Engineering Corp. (Chicago, Ill.). The chambers consisted of an upper and a lower compartment separated by a 3-μm Millipore membrane filter. The upper compartment had a 0.3-ml capacity, and the lower

compartment had a 0.25-ml capacity. The lower compartment was filled with a chemotactic attractant by injection through a small hole which was subsequently sealed with silicone grease. The upper compartment was open to the outside and was filled from the top with a WBC preparation containing 10^7 cells per ml, followed by incubation for 2 h at 37 C. The cells were then fixed on the filters with formaldehyde, stained with hematoxylin (3), and mounted in an inverted position with Permount and a cover slip so that the underside (side facing the chemotactic factor) faced up. The WBC migrating completely through the membrane could be seen clearly and counted. Ninety-eight percent of the cells migrating through the membrane could be easily identified as polymorphonuclear cells. The average number of cells per high-power field (HPF) was calculated from counts of 10 randomly selected HPFs. The method of chemotaxis was slightly modified when rabbit cells were used. A 3-h incubation period and an 8-μm Millipore membrane was substituted (3, 8). This modification gave maximal chemotaxis in the rabbit system. Base-line migration in these systems without chemotactic factors was less than 1 cell per HPF.

Chemotactic factors. In these experiments, the chemotactic factor consisted of a mixture of HBSS saturated with casein and 10% autologous serum for all species except the rabbit, where 20% serum was added. This mixture proved to be more potent than casein alone or immune complexes plus serum. Twenty percent serum in the rabbit experiments gave maximal chemotaxis. Results in the experiments with humans were similar when either 10 or 20% autologous serum was used.

Random mobility. A modified version of the technique of McCall et al. (9) was used to assess the effect of SO on WBC random mobility. WBC were prepared as described previously in a concentration that would give a 10% packed cell volume. This solution was then drawn into a capillary tube (1.5 by 90 mm) and flame-sealed at one end. The capillary tubes, centrifuged at 270 × *g* for 10 min to pellet the cells, were subsequently cleaved at the cell interface. The cell-containing portion was placed in a Sykes-Moore chamber and filled with HBSS. After an incubation period of 3 h at 37 C, the developing cell flares were observed and photographed. Studies by McCall et al. (9) have shown that 85% of the cells present in the zone of migration after 3 h of incubation are neutrophils. Our studies have also shown the cells to be primarily neutrophils.

Streptolysin O treatment of WBC. WBC (10^7/ml) were treated with an equal volume of a solution containing 1 to 4 hemolytic units (HU) per ml of active SO. The mixture was incubated for 30 min at 37 C. The cells were then centrifuged, washed, and resuspended in HBSS to the initial concentration. The control consisted of cells treated with a volume of phosphate-buffered saline diluted to the same extent as the SO with HBSS.

The SO used in treatment was obtained by Sephadex G-100 gel filtration chromatography of a 70% ammonium sulfate precipitation of a *Streptococcus pyogenes* (Richard strain group A) culture supernatant (10). Prior to gel filtration, 2-mercaptoethanol was added to obtain maximal activation of the toxin, but it was separated from the toxin as a result of the chromatographic procedure. The SO was then titered and diluted to 1 to 4 HU/ml with HBSS.

RESULTS

Chemotaxis of human peripheral leukocytes after streptolysin O treatment. Human WBC preparations containing 10^7 WBC/ml were treated with an equal volume of a SO solution containing 1 HU/ml. The chemotaxis of SO-treated cells was markedly suppressed when compared with controls (Table 1). A negative control using only HBSS in the lower chamber showed no migration of leukocytes. SO treatment (1 HU) had no effect on visible cell morphology, counts, or viability. The degree of anti-SO titer showed no correlation with the inhibitory effect of SO. Individuals with both high and low titers of anti-SO demonstrated similar degrees of chemotactic suppression.

Effects of cholesterol on the chemotactic activity of streptolysin O. To further associate the observed chemotactic inhibition and SO, the effect of cholesterol, a known inhibitor of the SO hemolytic activity, was investigated with respect to SO chemotactic inhibition. A solution of HBSS saturated with cholesterol was prepared. A 0.1-ml of volume of this solution was added to 1 ml of an SO solution containing 1 1 HU of active toxin. A 1:10 dilution of the cholesterol-saturated HBSS was capable of in-

TABLE 1. *Effect of streptolysin O on the chemotactic response of polymorphonuclear leukocytes from three healthy human donors*

Treatment of white blood cells	Chemotactic factor in lower chamber	Migration of neutrophils through membrane		
		Avg per chamber (HPF)[a]	Overall avg	Positive control (%)
Donor DV				
None ..	−	0, 0, 1	0	0
None ..	+	60, 51, 43	51	100
SO[b]	+	8, 5, 6	6	12
Donor NM				
None ..	−	0, 1, 0	0	0
None ..	+	344, 357, 365	355	100
SO[b]	+	13, 2, 4	6	2
Donor TB				
None ..	−	0, 1, 0	0	0
None ..	+	190, 154, 143	162	100
SO[b]	+	7, 16	12	7

[a] High-power field.
[b] One hemolytic unit of SO.

hibiting 4 HU of SO. Normal human WBC were treated for 30 min with either 1 HU of active SO, cholesterol-inhibited SO, a 1:10 dilution of cholesterol-saturated HBSS, or with HBSS alone (Table 2). Cholesterol treatment of the SO preparation blocked both the hemolytic activity and the chemotactic inhibitory activity of SO, thus supporting the role of SO as a chemotactic suppressor and attributing its action to its biological activity.

Chemotactic response of human cells with respect to time. It is possible that the inhibition of chemotaxis by SO was a function of the time allowed for cell migration. The presence of SO may have caused accelerated or decelerated migration, resulting in a shift in peak migration. To explore this possibility, a series of chemotaxis chambers were incubated for various times prior to fixing and staining the membranes. The suppressive effect of SO on chemotaxis was consistent throughout the 5-h incubation period (Fig. 1). It should be noted that, although suppression was consistent, the degree of suppression did vary.

Effect of streptolysin O on rabbit peripheral leukocyte chemotaxis. Rabbit WBC (10^7 cells per ml) were treated with SO (1 HU) in the usual manner and tested to ascertain the effect of SO on chemotaxis in this species. Anti-SO titers ranged from 0 to 1:16. Table 3 shows representative results of chemotaxis experiments on rabbit cells treated with 1 HU of SO as compared with a positive control. Treatment with larger amounts of SO (2 to 4 HU) sometimes resulted in a slight loss of WBC. In most cases, this loss was less than 10%, although with 4 HU reduction was as high as 40%. No substantial chemotactic inhibition was observed with 1 or 2 HU of SO, but higher doses of SO (4 HU) did produce some inhibition (<40%). This suppression could be entirely accounted for by cell loss. In the rabbit system some minor procedural changes were made (described in Materials and Methods) because of restricted conditions necessary to obtain optimal chemotaxis. However, when human neutrophils were subjected to the same modifications used in the rabbit system, chemotactic suppression occurred as before.

TABLE 2. *Inhibition of the chemotactic suppressive effect of streptolysin O by cholesterol treatment of the toxin*

Cell treatment	Migration of polymorphonuclear leukocytes through membranes		
	Avg per chamber (HPF)[a]	Overall avg	Positive control (%)
None	106, 111, 97	105	100
SO[b]	1, 1	1	1
Cholesterol[c]	82, 74, 62	73	100
SO plus cholesterol	82, 64, 84	77	105

[a] High-power field.
[b] One hemolytic unit.
[c] Used as a control for the cholesterol experiment and considered to be 100% migration.

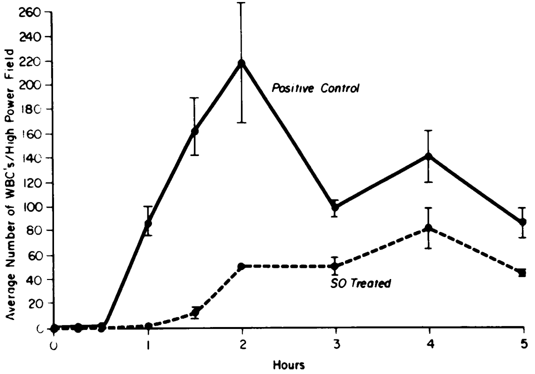

FIG. 1. *Chemotactic response of human control and streptolysin O-treated leukocytes with respect to time. The time variable represents the duration of incubation during the chemotaxis test and not the duration of SO treatment.*

TABLE 3. *Effect of streptolysin O treatment on rabbit leukocyte chemotaxis*

WBC treatment	Chemotactic factor in lower compartment	Neutrophils migrating through membrane		
		Avg/chamber (HPF)[a]	Overall avg	Positive control (%)
Rabbit no. 1				
None	−	0, 0	0	0
None	+	40, 42	41	100
SO, 1 HU	+	64, 43	54	132
Rabbit no. 2				
None	−	0, 0	0	0
None	+	19, 18	19	100
SO, 1 HU	+	17, 19, 15	17	90

[a] High-power field.

Chemotactic response of rabbit cells with respect to time. In an attempt to investigate the effect of SO with respect to duration of incubation, we treated rabbit WBC suspensions with 2 HU of SO or the equivalent control solution. Chemotaxis experiments were run in the usual fashion, with the exception that cell migration was stopped at varying times. The Millipore membranes were fixed and stained

after 1.5, 3.0, 4.5, and 5.5 h of incubation. SO treatment of rabbit WBC did not impair chemotactic capacity over a wide range of migration periods (Fig. 2).

Effect of streptolysin O on the random mobility of human and rabbit leukocytes. The effect of SO on the random mobility of human and rabbit leukocytes was compared. Rabbit cells with a 10% packed volume were treated with 2 HU of SO for 30 min at 37 C. The preparations were then used in a capillary tube random migration study. After 3 h of incubation, photographs were taken. A similar experiment was carried out on human cells and used for comparison. The effects of SO (1 HU on human cells, 2 HU on rabbit cells) on the random mobility of both human and rabbit cells can be seen in Fig. 3. SO was capable of suppressing the random migration of human peripheral leukocytes but had no effect on rabbit leukocyte mobility even when twice the concentration was used.

Sensitivity of rabbit and human leukocytes to streptolysin O toxicity. Two WBC preparations, one human and one rabbit, were subjected to various concentrations of SO to determine their susceptibility to SO lysis. The SO treatment utilized toxin which had 16 HU of activity in the absence of reducing agent. This was diluted with HBSS to yield the concentrations used for each part of the experiment. Cell preparations were treated for 30 min at 37 C with an equal volume of SO diluted to the appropriate strength. After treatment, WBC counts and trypan blue staining of leukocytes were determined for each sample (Table 4). The number of human WBC remaining after treatment with 4 HU of SO was essentially the same as the control. Trypan blue dye exclusion tests revealed that the cells were unaffected until a concentration of 8 HU of SO was attained. At this point, 23% of the cells demonstrated dye uptake. Rabbit cells responded differently to the toxin. Cell counts were diminished by 45% when 4 HU of SO were used in treatment. Trypan blue staining also took place at this point. Sixteen hemolytic units of SO reduced the WBC to 5% of the original count. It is

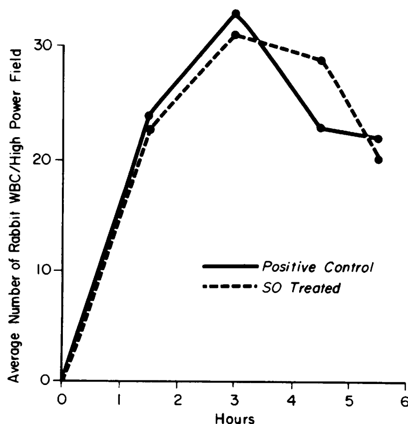

FIG. 2. *Chemotactic response of control and streptolysin O-treated rabbit leukocytes with respect to time. The time variable represents the duration of incubation during the chemotaxis test and not the duration of SO treatment.*

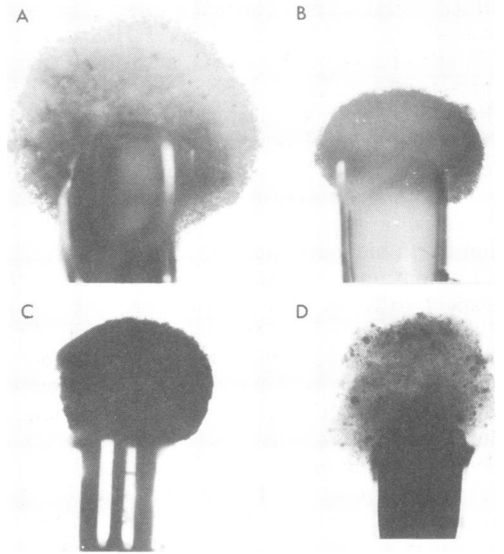

FIG. 3. *Effect of streptolysin O on the random mobility of human and rabbit peripheral leukocytes. (A) Human leukocyte positive control; (B) human leukocytes treated with SO (1 HU); (C) rabbit leukocyte positive control; (D) rabbit leukocytes treated with SO (2 HU). Differences in the density of the cell flares in the rabbit study are due to unlysed red blood cells in the control.*

TABLE 4. *Sensitivity of human and rabbit WBC preparations to streptolysin O toxicity*

SO (HU)	Human WBC		Rabbit WBC	
	WBC/ml $\times 10^6$	Staining with trypan blue (%)	WBC/ml $\times 10^6$	Staining with trypan blue (%)
0.0	4.0	0	4.0	0
0.5	4.6	0	4.6	0
1.0	4.8	0	4.0	0
2.0	4.4	0	3.8	0
4.0	4.2	0	2.2	6
8.0	2.6	23	2.0	9
16.0	2.8	34	0.2	23

apparent that rabbit WBC are more susceptible than human WBC to the lytic action of the SO preparation, although not to the chemotactic inhibitory activity.

Effect of streptolysin O on chemotaxis of various species. Further studies were performed on baboons and sheep to determine whether SO inhibition of neutrophilic chemotaxis was unique to human leukocytes. In both cases, no WBC lysis was observed after treatment with 4 HU of toxin. Chemotaxis experiments and cell preparation were carried out as before by using pigskin gelatin to sediment sheep RBC and Plasmagel to sediment baboon RBC. The anti-SO titer of the sheep was 1:1, whereas that of the baboon ranged from 1:16 to 1:32. In both cases, casein and 10% serum were used as the chemotactic factor. The data are shown in Table 5. Average results from rabbits and humans are also included. The rabbit showed an extremely low degree of susceptibility to chemotactic inhibition, whereas the sheep and baboons were intermediate in their susceptibility.

In addition to chemotaxis experiments, RBC from all of the species were tested for variations in SO hemolysis. The lysis of a 2% solution of RBC from each source showed no species distinctions when tested with a standard preparation of SO by the microtiter technique.

Effect of the supernatant solution from streptolysin O-treated WBC on leukocyte chemotaxis. To further study the mechanism of SO chemotactic suppression, we tested the supernatant solution from treated cells for its capacity to inhibit chemotaxis. If SO treatment resulted in release of a factor which subsequently caused inhibition, it should have been present in this solution. To test this hypothesis, two 1-ml volumes of a human WBC preparation were treated, one with an equal volume of SO (1 HU per ml) and one with HBSS. An incubation time of 30 min at 37 C was allowed. The supernatant solutions were then decanted. The cells were resuspended in HBSS and used in a chemotaxis experiment to confirm that SO inhibition had occurred. Two 1-ml samples of untreated WBC were centrifuged, and the resulting cell pellet was suspended in the supernatant solution from either the control or SO-treated cells. These cells were allowed to incubate for 30 min at 37 C, followed by centrifugation, resuspension in HBSS, and testing for chemotactic suppression. It was found that neither of the supernatant solutions contained any chemotactic inhibitory activity, suggesting that a mediator capable of inhibiting chemotaxis was not released after SO treatment of leukocytes.

TABLE 5. *Effect of streptolysin O on chemotaxis in various species*

Species of animal	Inhibition after SO treatment (%)		
	1 HU	2 HU	4 HU
Human	81 (12)[a]	52 (1)	95 (5)
Rabbit[b]	17 (8)	6 (1)	20 (2)
Sheep			84 (1)
Baboon	35 (3)	67 (2)	61 (1)

[a] Numbers in parenthesis represent the number tested.
[b] Values are uncorrected for cell loss.

Effect of streptolysin O on immune-deficient individuals. The possiblity remained that SO inhibition might be due to an immune mechanism arising from previous exposure to SO. To evaluate this possibility, immunologically deficient individuals were tested for SO chemotactic inhibition. Cells were obtained from fetal cord blood, patients with Bruton-type agammaglobulinemia, and a patient lacking a demonstrable delayed hypersensitivity response. Chemotaxis experiments were performed as before. Casein and serum were used as a chemotactic attractant. Fetal cord blood was collected in a heparinized tube from the umbilical cord immediately after birth. The humoral antibody-deficient patients were diagnosed as having Bruton-type agammaglobulinemia (anti-SO 1:8). The patient lacking delayed hypersensitivity was diagnosed as having multiple endocrine deficiencies and had been studied extensively and shown to produce no skin test delayed hypersensitivity or migration inhibitory factor. It was found that SO was capable of inhibiting the chemotactic capacity of neutrophils from all of these patients (Brutons agammaglobulinemia, 85% inhibited; absent delayed hypersensitivity, 98% inhibited; and human fetal cord blood, 68% inhibited), thus lending support to a nonimmune mechanism of chemotactic suppression.

Effect of SO on chemotaxis of immunized rabbits. To further investigate the role of immunological factors in SO chemotactic inhibition, rabbits were immunized with SO and used in a chemotaxis experiment. One rabbit was immunized by intravenous injection of SO and subcutaneous injection of SO mixed with Freund adjuvant. The peak anti-SO titer in this rabbit was 1:128, and the preimmunization titer was 1:16. The second rabbit was injected intramuscularly with three 1-ml samples of SO totaling approximately 100 HU. The injections were made over a period of 9 days. The anti-SO

titer 6 days later was 1:256. The leukocytes from both immune rabbits failed to show any chemotactic inhibition after SO treatment. These results add support to a nonimmune mechanism of SO chemotactic suppression and confirm species specificity.

DISCUSSION

This study lends support to the observation that SO will suppress the chemotactic activity of human leukocytes. In our previous report (1), it was shown that pretreatment of the toxin with heat, anti-SO serum, or air exposure prevented this phenomenon. We now have demonstrated that cholesterol, a known inhibitor of the hemolytic activity of SO, is also capable of reversing this effect. Although the SO preparations used in these studies contained a few other contaminating proteins (1), the ability of heat, oxygen, cholesterol, and anti-SO to block the chemotaxis-inhibiting ability of the preparation provides strong evidence that SO is the active constituent.

Species differences with respect to SO susceptibility were observed. When rabbit WBC were treated with SO in concentrations fourfold greater than that used to inhibit human cells, its effect on chemotaxis was minimal. Baboon and sheep WBC were both intermediate in their susceptibility to SO chemotactic suppression. When human and rabbit WBC were treated with varying amounts of SO, rabbit WBC were more readily damaged by higher concentrations of the toxin, as measured by trypan blue dye exclusion and cell lysis. It is interesting that, unlike human WBC, the chemotactic capacity and random mobility of rabbit WBC were unimpaired by SO treatment until the concentration of toxin was high enough to cause cell lysis. The loss of rabbit leukocytes after SO treatment of up to 3 HU amounted to no more than 20%. The observed chemotactic suppression did not exceed this amount and could be accounted for totally by cell loss.

The WBC preparation used in chemotaxis and mobility studies was not a pure polymorphonuclear leukocyte preparation and did contain some lymphocytes. The possibility than an immune mediator was released from these lymphocytes due to previous SO sensitization was diminished by several experiments. The supernatant solutions from SO-treated WBC when used in treatment of normal cells demonstrated no chemotactic suppressive activity. Although factors such as inadequate concentration or lability of the mediators could account for the negative results, the normal performance of leukocytes from immune deficiency states would make this seem unlikely. In addition, specific inhibition of the hemolytic activity of SO with cholesterol blocked the suppressive effect of the toxin. If immune mediators were involved, inhibition of the biological activity of SO should not inhibit all of its antigenic potential and the subsequent release of mediators. Finally, the possibility of previous exposure to SO as a factor in chemotactic suppression was reduced even further by the observation that WBC from SO-immunized rabbits were refractory to SO suppression.

It is possible that the SO chemotactic inhibition observed here may have been due to nonimmune inhibitory factors released from neutrophils upon exposure to the toxin. Such factors have been shown to be liberated from neutrophils after phagocytosis or exposure to acid pH (5). Since neutral pH conditions were maintained in our experiments, acidic conditions could not have been a factor. SO can cause degranulation of neutrophils (11), as does phagocytosis. This could result in the release of intracellular chemotactic inhibitors, but failure of the supernatants from SO-treated cells to inhibit untreated cells argues against this possibility. It thus appears that the neutrophil-immobilizing effect of SO is a species-dependent, nonimmunological phenomenon. Theoretically, the evidenced chemotactic suppression of human WBC by low doses of toxin and the lack of a low-dose suppressive effect on rabbit cells may be factors in the increased pathogenicity of group A streptococci in humans.

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