DISSOCIATION OF HEMOLYTIC AND LYMPHOCYTE-TRANSFORMING ACTIVITIES OF STREPTOLYSIN S PREPARATIONS*,‡

BY ANGELO TARANTA, M.D., GIROLAMO CUPPARI, M.D., AND FRANCO QUAGLIATA, M.D.

(From the Irvington House Institute and the Department of Medicine, New York University School of Medicine, New York 10016)

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Streptolysin S, a hemolytic toxin produced by beta hemolytic streptococci, has been reported to elicit the transformation[1] of human peripheral blood lymphocytes cultured in vitro into large, blast-like cells, and to stimulate mitosis (1). This effect appeared to differ from the transformation elicited by antigens in the lymphocytes of specifically sensitized subjects because the per cent of transformation was higher, and because transformation was obtained in the lymphocytes from all the normal subjects tested. Moreover, the preparation used, streptolysin S, had been reported not to behave as an antigen in naturally occurring streptococcal infections in man or in immunization experiments in animals (2, 3). It was concluded that the action of streptolysin S on cultures of human peripheral blood lymphocytes is nonspecific in the immunologic sense and qualitatively similar to that of phytohemagglutinin (PHA) (4), though quantitatively less marked. Preparations of the other hemolytic streptococcal toxin, streptolysin O, behaved like any other specific antigen (1). Streptolysin S is of interest because it is one of the most potent hemolytic agents known (5), and because it has been implicated in the pathogenesis of rheumatic fever (for reviews see references 6–8).

The great majority of strains of Group A beta hemolytic streptococci produce streptolysin S, but a few do not (9). One of these strains unable to produce streptolysin S, the C203U strain originally described as derived by mutation from a streptolysin S–producing strain (C203S), has recently been reported to produce a potent lymphocyte-transforming factor under conditions customarily adopted for the production of streptolysin S (10). This finding suggested the general

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[1] Other terms used in the literature are "activation," "stimulation," and "blast transformation."

hypothesis [I] that the hemolytic and the transforming activities of streptolysin S preparations may be independent of each other, and the specific hypotheses that they may be attributes of two different molecules [I*a*], or of two different active sites of the same molecule [I*b*].

We report here a series of experiments bearing on these hypotheses. Experiments based on the characteristic sensitivity of the hemolytic activity of streptolysin S to heat (11), to chymotrypsin (12), and to certain inhibitors (13, 14) supported the general hypothesis [I]. Support for the first specific hypothesis [I*a*] was derived from fractionation experiments.

Materials and Methods

Bacterial Strains.—Strain C203S of Group A streptococcus, used previously for streptolysin S production (12), and strain 010962 of *Diplococcus pneumoniae*, type I, were obtained from Dr. Alan W. Bernheimer. Strains of *Streptococcus viridans* and of Staphylococcus aureus (nonhemolytic) were isolated in this laboratory from healthy carriers.

Streptolysin S Preparations.—These preparations were obtained according to a method derived from that of Bernheimer (12) with minor modifications, including that of Ginsburg and Bentwich (15). Briefly, streptococci of the C203S strain were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and the overnight growth was inoculated 1:10 in 5 liter batches of the same broth. The streptococci were collected by centrifugation in the cold as soon as they approached the stationary phase of growth, usually 6–7 hr after inoculation. They were then washed twice and incubated in 70 ml of a medium (hereafter referred to as "suspension medium") containing phosphate, maltose (Fisher Scientific Co., Fairlawn, N.J.), L-cysteine base (Mann Research Laboratories, New York, N.Y.), and the residuum of pancreatic RNAse digestion of yeast RNA ("RNA core"—Worthington Biochemical Corp., Freehold, N.J.). Incubation was carried out for 2 hr at 37°C, at the end of which the streptococci were centrifuged out and the supernatant was passed through Millipore filters of 0.22 μ pore size. The preparations obtained were then dialyzed and lyophilized, or preserved in the frozen state, or subjected to fractionation experiments as described below. Various lots of streptolysin S preparation (also referred to in the literature as "C203S product" [10, 12] or RNA-hemolysin [16, 17]) had hemolytic activities ranging from 9,600 to 27,000 50% hemolytic units (HU_{50})/ml before dialysis, and from 15,000 to 27,000 HU_{50}/mg after dialysis and lyophilization. Most of the experiments reported here were done with a dialyzed and lyophilized lot containing 20,000 HU_{50}/mg. The term "streptolysin S preparation" is preferred to that of "streptolysin S" which may be misleadingly construed to imply purity of such preparations.

Determination of the Hemolytic Activity of Streptolysin S Preparations.—This procedure was carried out as described by Bernheimer (12) with a minor modification (the 0.35% human red cell suspension was standardized on the basis of the optical density at 541 mμ after lysis, and the determination of per cent hemolysis was also determined by the optical density at 541 mμ in a Beckman DU spectrophotometer). These determinations were done on the same days in which lymphocyte culture experiments or chromatographic fractionations of streptolysin S preparations were done. Since the sensitivity of washed erythrocytes to streptolysin S slowly increases even when the suspension is stored at 3–5°C (9), the red cell suspension was freshly prepared each day. The hemolytic activity was expressed in HU_{50}. The latter was defined as that amount of streptolysin S which lyses 50% of 1 ml of a 0.35% red cell suspension (12). Dilutions were made in pH 7 phosphate-buffered saline (1/15 M sodium phosphate, 1/12.9 M sodium chloride).

In critical experiments in which it appeared important to rule out the presence of minute amounts of streptolysin S hemolytic activity, lower than can be detected with the above standard test, a sensitized test was adopted, as suggested by Ginsburg et al. (18), using as diluent a hypotonic phosphate-buffered saline and substituting a 0.1% suspension of red cells for the standard 0.35% suspension.

Determination of the Transforming Activity of Streptolysin S Preparations.—Two different methods were used.

The *first method*, which was used in all the experiments, was derived from that of Bach and Hirschhorn (19). For each experiment, 400 ml of blood were collected in a plastic bag containing 2250 units of heparin sodium USP in 30 ml of sterile, pyrogen-free 0.9% sodium chloride solution buffered with sodium phosphate USP (Fenwall Laboratories, Morton Grove, Ill.), to which were added 40 ml of 6% dextran 70 (approximate molecular weight 70,000) in sterile, pyrogen-free, 0.9% sodium chloride solution (Travenol Laboratories, Morton Grove, Ill.). The blood was left to sediment in the plastic bag kept in an upright position at 37°C for approximately 1½ hr. The supernatant leukocyte-rich plasma was transferred into a flat-sided bottle which was kept lying on one of its wide sides for 1 hr at 37°C to expose the blood to a larger surface of glass, to which the neutrophils adhere, and thus to reduce the percentage of neutrophils. The plasma was then centrifuged at 130 relative centrifugal force (RCF) for 10 min and the sedimented cells were washed twice in 400 ml of Eagle's minimum essential medium modified for spinner culters (MEM-S, Grand Island Biological Co., Grand Island, N. Y.). The cells were resuspended in a medium containing 77% MEM-S, 20% fetal calf serum, 1% 200 mM solution of L-gluta-amine, and 2% of a solution containing 5000 units of penicillin and 5000 units of streptomycin/ml (all obtained from Grand Island Biological Co., Grand Island, N. Y.; this medium will be referred to as "enriched MEM-S"). The concentration of cells was adjusted to 10^6/ml. 4 ml aliquots of the cell suspension were transferred into flat-bottom, screw-cap, 30 ml bottles. Streptolysin S preparations were added in 0.1 ml amounts per bottle with exception of the chromatographic fractions, which were added in 0.2 ml amounts. In each experiment, positive controls were provided by duplicate or triplicate bottles to which 0.1 ml aliquots of a PHA preparation (Burroughs Wellcome, Wellcome Research Laboratories, Beckenham, England) were added, and negative controls were provided by triplicate or quadruplicate bottles to which neither PHA nor streptolysin S preparations were added. The caps were tightly closed, the contents mixed, and the bottles incubated for 5 days, with the exception of the ones containing PHA, which were incubated for 3 days. At the end of the incubation period, the cells were harvested by centrifugation for 10 min at 130 RCF and smeared on microscope slides, one slide for each bottle. The slides were air-dried and stained with Wright's stain. They were given random coded numbers and then read "blindly," i.e., without knowing whether PHA or streptolysin S preparation or neither had been added to the corresponding cultures, nor which were the sets of replicates. The percentage of transformation was determined by examining 1000 cells per slide. Cells in mitosis were counted as transformed cells. The per cent of transformation was recorded as such, i.e., without subtracting the per cent transformation observed in the control cultures with no additives. In such control cultures the transformation averaged 1.1%.

In a small series of experiments in which a single dose of heated and of unheated streptolysin S preparation were tested, only 30 ml of blood were used for each experiment, and the first steps of the above procedure were consequently modified as follows. The blood was collected in a plastic syringe and poured into glass tubes containing 200 units of heparin and 3 ml of 6% dextran in saline. It was then left to sediment in the tubes, inclined at a 45° angle for approximately 1 hr at 37°C. The rest of the procedure was as described above.

The *second method*, which was used only in the experiments on chromatographic fractions of streptolysin S preparations, was derived from those of Dutton and Eady (20) and of Caron

et al. (21) as modified by Valentine and Lawrence.[2] For each experiment, 400 ml of blood were collected and left to sediment as described above. However, to reduce the percentage of neutrophils, the supernatant leukocyte-rich plasma was poured into stoppered 100 ml glass syringes loosely packed with glass wool (previously sterilized by dry heat at 160°C for 4 hr), and incubated for ½ hr at 37°C. The stopper was then removed and the plasma collected. The syringes were then washed with MEM-S in an amount double that of the plasma recovered. The washings were added to the plasma, and the mixture was centrifuged at 260 RCF for 10 min. The sedimented cells were resuspended in 10 ml of enriched MEM-S. The concentration of the cells was adjusted to 10^6 cells/ml and the cell suspension was transferred in 4 ml aliquots into screw-cap glass tubes, 16 × 125 mm. The solutions to be assayed for transforming activity were added in 0.2 ml amounts. The tubes were flushed with 5% CO_2 in air. The caps were screwed tightly and the tubes incubated at 37°C for 3 days (for tubes containing PHA, i.e., positive controls) or for 5 days (for tubes containing chromatographic fractions of streptolysin S preparations, and for tubes containing no additives, i.e., negative controls).

24 hr before harvesting the cells, 0.1 ml aliquots of a solution of thymidine-2-^{14}C (specific activity 53.8 μc/mM, New England Nuclear Corp., Boston, Mass.) diluted to a concentration of 1 μc/ml in MEM-S medium, were added to each tube. The cells were harvested by centrifugation at 580 RCF for 15 min, and washed twice in normal saline. The supernatant was discarded and the tubes containing the sedimented cells were put in an ice water bath. 5 ml of ice cold 5% trichloroacetic acid (TCA) were added to each tube. The tubes were centrifuged at 4°C at 1250 RCF for 20 min, and the sediments were washed twice in 5 ml aliquots of 5% ice cold TCA and twice in 5 ml aliquots of ice cold methanol. The washed precipitates were left to dry overnight at room temperature.

The dry precipitates were suspended in 1 ml aliquots of hydroxide of hyamine 10-x (Packard Instrument Co., Inc., Downers Grove, Ill.) and heated at 56°C for ½ hr. The precipitates, resuspended in hyamine, were transferred into scintillation vials containing 5 ml of Liquifluor (New England Nuclear Corp., Boston, Mass.) in toluene. The tubes which had contained the precipitates were rinsed twice with 2.5 ml aliquots of Liquifluor and the washings were added to the scintillation vials. Radioactivity in the resulting suspension was assayed in a Packard scintillation spectrometer and expressed in counts per minute.

Lymphocyte Donors.—The majority of the experiments were carried out with 400 ml aliquots of blood, which were obtained from professional blood donors at a commercial blood bank. 21 different donors were used in these experiments. In experiments with a single dose of heated and unheated streptolysin S preparations, we used 30 ml aliquots of blood obtained from 7 healthy volunteers and from 17 ambulatory or hospitalized patients with inactive or active rheumatic fever, rheumatoid arthritis, or scleroderma.

Streptolysin S Inhibitors.—Vegetable lecithin was purchased from Nutritional Biochemical Corp., Cleveland, Ohio; egg L-alpha-lecithin from General Biochemicals, Chagrin Falls, Ohio; L-alpha-cephalin from Calbiochem, Los Angeles, Calif.; and trypan blue from Baltimore Biological Laboratory, Baltimore, Md.

Enzymes.—Chymotrypsin and trypsin were purchased from Worthington Biochemical Corporation, Freehold, N.J.; and papain, a suspension of a twice crystallized preparation, was purchased from Nutritional Biochemical Corp.

Chromatography.—This was carried out on DEAE-Sephadex A-25 (purchased from Pharmacia Fine Chemicals, Piscataway, N.J.) according to standard methods in jacketed, refrigerated columns at 4°C. Aliquots of 1 to 4 ml were collected from the columns by means of an automatic refrigerated fraction collector. Estimates of protein and nucleic acid were made by determining the optical density at 280 and 260 mμ respectively in a Beckman DU spectro-

[2] Valentine, F., and S. H. Lawrence. Manuscript in preparation.

photometer. Hemolytic activity was determined in each fraction, while transforming activity was determined in pools of two or three consecutive fractions.

RESULTS

Transforming Activity of Streptolysin S Preparations.—Streptolysin S preparations regularly induced high percentages of transformation when added to cultures of human peripheral blood lymphocytes from apparently healthy subjects. Lymphocytes obtained from the peripheral blood of 21 professional blood donors were tested. The percentages of transformation obtained with 50 HU_{50} of streptolysin S preparation ranged from 38 to 91 with an average of 65.8 (standard deviation 16.5). These results are in essential agreement with the previous report (1). By comparison, the percentage of transformation with PHA ranged from 60 to 100%, with an average of 94.2 (standard deviation 8.6), and the per cent transformation obtained in the negative controls, without additives, ranged from 0 to 6.5%, with an average of 1.1 (standard deviation 1.6).

To test the specificity of the transforming activity of streptolysin S preparations, uninoculated Todd-Hewitt broth and "suspension medium" were added in 0.001 to 0.1 ml amounts to replicate lymphocyte cultures. The transformation obtained did not exceed that found in the negative controls (without any additives).

As additional controls, one strain each of *Streptococcus viridans* (alpha streptococcus), *Staphylococcus aureus*, and *Diplococcus pneumoniae* type I was grown in Todd-Hewitt broth. The bacteria were collected, washed, and incubated exactly as for the production of streptolysin S by Group A beta hemolytic streptococci of the C203S strain. No transformation higher than that observed in the negative controls was obtained with 0.001 to 0.1 ml of any of these preparations. These results indicate that the transforming activity of streptolysin S preparations is due neither to constituents of the media used for their preparation, nor to nonspecific products of bacterial metabolism shared with these other Gram-positive organisms and obtainable in the experimental conditions described.

Heat Inactivation of Streptolysin S Preparations.—In general accordance with previous reports on the marked heat lability of streptolysin S preparations (11), heating at 56°C for 45 min reduced the hemolytic activity of a 500 HU_{50}/ml streptolysin S preparation to amounts undetectable by the standard method of hemolysin titration (12). One such heat-inactivated preparation was tested in five-fold dilutions for its ability to induce lymphocyte transformation. Corresponding amounts of a nonheated aliquot of the same batch of streptolysin S preparation were tested concurrently. Two S-shaped dose-response curves were obtained (Fig. 1). They indicated that the streptolysin S preparation heated at 56°C for 45 min was still capable of inducing lymphocyte transformation, though in percentages lower than those obtained with the unheated preparation,

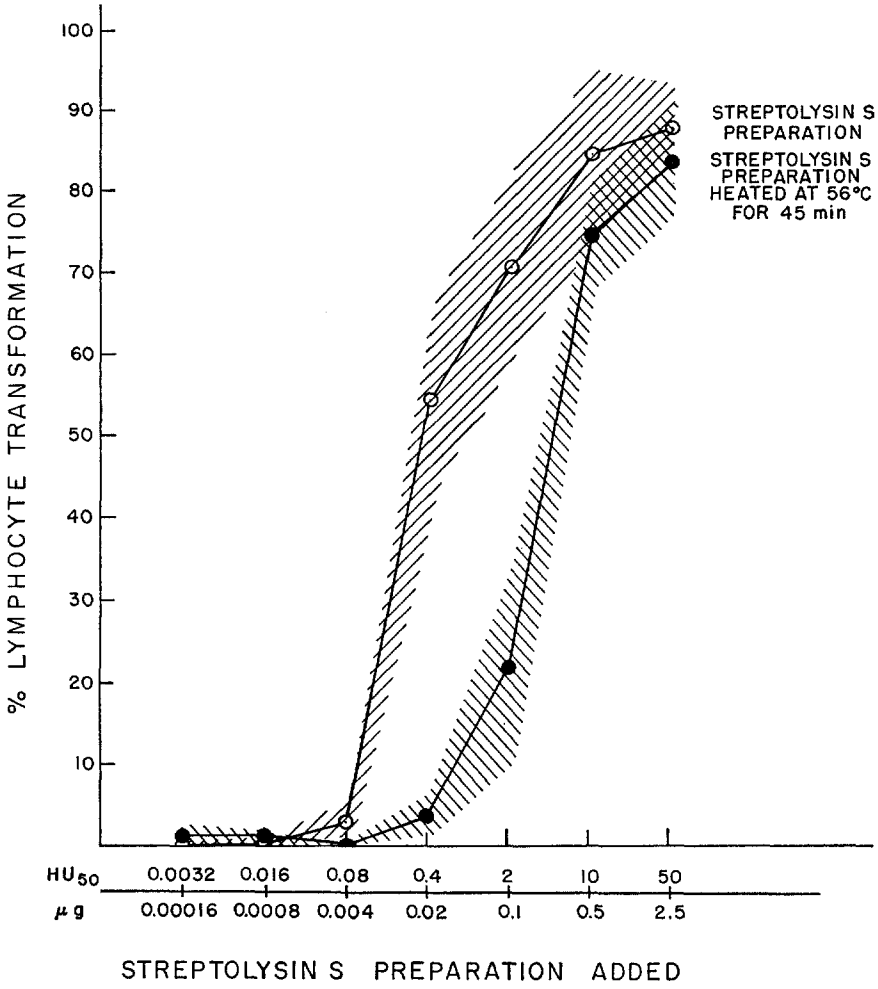

FIG. 1. Per cent lymphocyte transformation obtained in cultures of human peripheral blood lymphocytes to which various amounts of streptolysin S preparation and of streptolysin S preparation heated at 56°C for 45 min were added. In this and in the following figures the open and closed circles indicate the means of replicates and the shaded areas enveloping the solid lines indicate the values of the standard deviations.

the difference ranging from 1% to 52% depending on the amount of preparations used. The curves also indicate that similar percentages of transformation could be obtained with amounts of heated preparation 5–20 times greater than of unheated preparation, depending on the percentage of transformation chosen for the comparison.

Since it was considered critical to exclude the persistence after heating of even

minute amounts of streptolysin S hemolytic activity, lower than could be detected by the standard method of hemolysin titration, in later experiments a more sensitive test was adopted, with a hypotonic diluent and a lower concen-

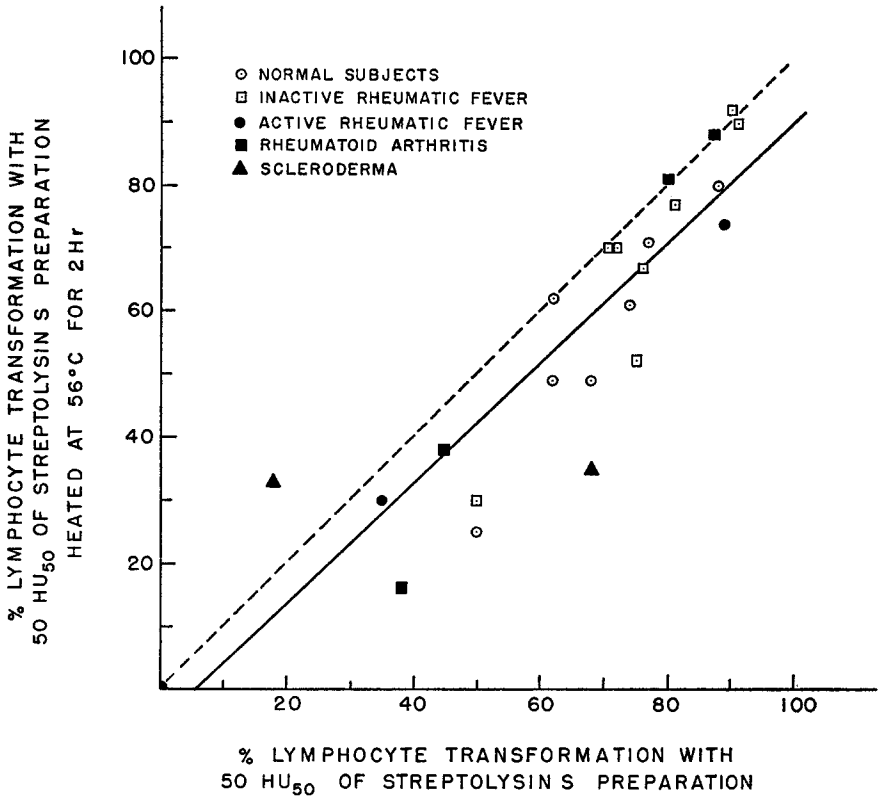

FIG. 2. Correlation between per cent lymphocyte transformation obtained with heated and unheated streptolysin S preparation in normal subjects and in patients with some rheumatic or connective tissue diseases. The solid line, fitted by the method of least squares, has a slope of 0.96. The broken line is an ideal line with a slope of 1, passing through the intersection of the X and Y axis. The mean of the transformations obtained with the heated preparation was 55.8%, while the mean of the transformations obtained with the unheated preparation was 64.5% (difference 8.7%). The two sets of percentages of lymphocyte transformation, obtained with the heated and the unheated preparation respectively, had a correlation coefficient of 0.90 ($P < 0.001$).

tration of red cells. By the use of this "sensitized" test, small amounts of hemolytic activity were indeed detected in streptolysin S preparations with a preheating titer of 500 HU_{50}/ml, after heating for 45 min at 56°C. Therefore the preceding experiments were repeated with streptolysin S preparations heated at 56°C for 2 hr, by which time the hemolytic activity had become undetectable

by either the standard or the sensitized test. The results were essentially similar to those just described.

One of the important features of the transforming activity of streptolysin S preparations that has been reported is that the lymphocytes of some patients, notably those with early active rheumatic fever, fail to transform (1). It was reasoned that, if the heat-inactivated and the native streptolysin S preparations shared the same transforming factor, the heat-inactivated preparation also should have the characteristic of inducing less lymphocyte transformation in some patients, notably those with active rheumatic fever. Therefore a series of normal volunteers, patients with previous rheumatic fever (inactive rheumatic fever) and patients with active rheumatic or connective tissue diseases were tested with aliquots of streptolysin S preparation containing 50 HU_{50} and with the same amount of the same preparation heated for 2 hr at 56°C, and therefore devoid of any detectable hemolytic activity. The results, summarized in Fig. 2, indicate a significant overall correlation between the per cent transformations obtained with the heated and those obtained with the unheated preparation ($r = 0.90$; $P < 0.001$). The percentage of transformation was generally lower with the heated than with the unheated preparation, both in the normal subjects and in the patients. A detailed analysis of the clinical correlation of these tests in a larger patient population will be presented separately.[3]

Experiments with Streptolysin S Inhibitors.—Vegetable lecithin, egg L-alpha-lecithin, L-alpha-cephalin, and trypan blue were tested in preliminary experiments to find the minimum dose which would inhibit completely the hemolytic activity of a 500 HU_{50}/ml streptolysin S preparation when incubated with it for 30 min at 37°C. Trypan blue was found to be the most potent inhibitor on a weight basis under the conditions used, followed by vegetable lecithin, giving complete inhibition in concentrations of 3 μg and 5 mg/ml respectively. At these concentrations, no hemolytic activity was detected either with the standard or the sensitized method of assay. Each of these two inhibitors was therefore used to abolish the hemolytic activity of a 500 HU_{50}/ml solution of streptolysin S preparation. The transforming activity of such treated preparations, tested in 0.1 ml amounts remained essentially unchanged, as compared to the untreated preparation.

To obtain more quantitative information on the relative transforming activities of streptolysin S preparations inhibited in their hemolytic activity, a further experiment was done in which fivefold dilutions of streptolysin S preparation inactivated by trypan blue were compared with equal amounts of the same batch of streptolysin S preparation incubated at 37°C for 30 min without trypan blue. The results, summarized in Fig. 3, indicate no loss of transforming activity of the trypan blue inactivated streptolysin S preparation over the range of con-

[3] Cuppari, G., F. Quagliata, A. Ieri, and A. Taranta. Manuscript in preparation.

centrations used. On the contrary, the trypan blue treated preparation resulted in slightly higher percentages of transformation, although the control cultures with trypan blue alone had no lymphocyte transformation higher than the negative controls (without additives).

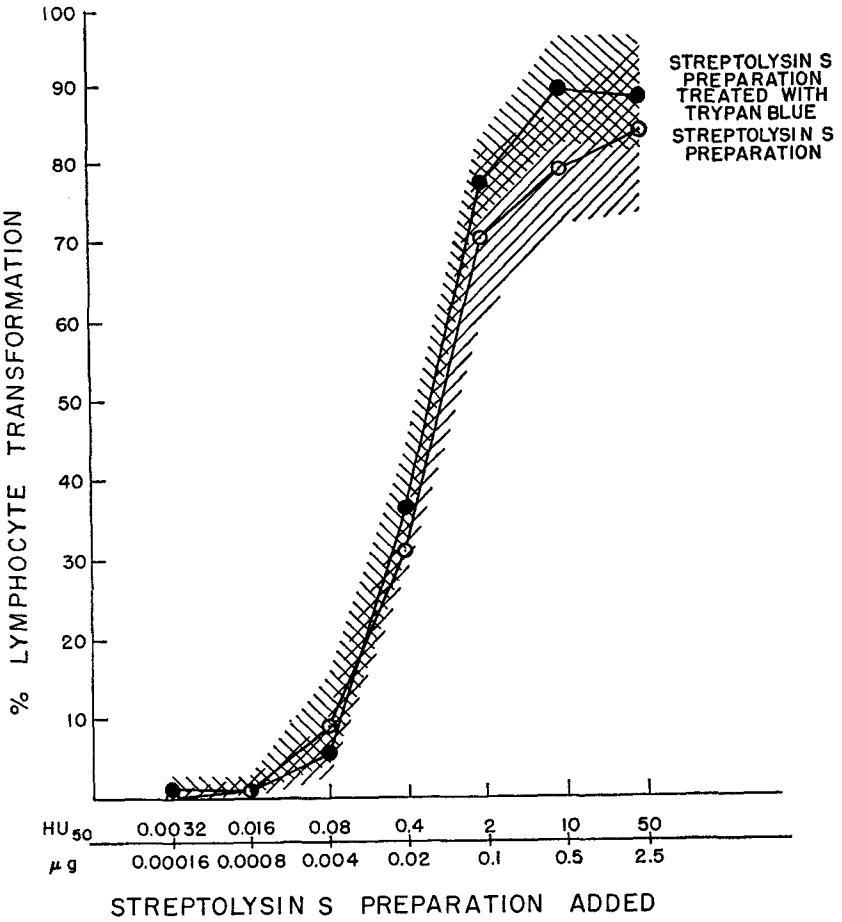

FIG. 3. Per cent lymphocyte transformation obtained with various amounts of streptolysin S preparation and of the same preparation treated with trypan blue. Both preparations were preincubated at 37°C for 30 min in solutions initially containing 500 HU_{50}/ml. Trypan blue was used in a concentration of 3 μg/ml.

Experiments with Enzymes.—Trypsin, chymotrypsin, and papain (the latter in 0.1% cysteine) were tested in preliminary experiments for their ability to reduce or eliminate the hemolytic activity of a 500 HU_{50}/ml solution of streptolysin S preparation in phosphate-buffered saline incubated at 37°C for 30 min.

In general agreement with previous findings (12), chymotrypsin and, to a slightly lesser extent, papain decreased drastically the hemolytic activity of streptolysin S preparations, while trypsin decreased it only slightly. Therefore chymotrypsin was chosen to inactivate streptolysin S; in addition, trypsin was used to determine whether a protein or a polypeptide different from streptolysin

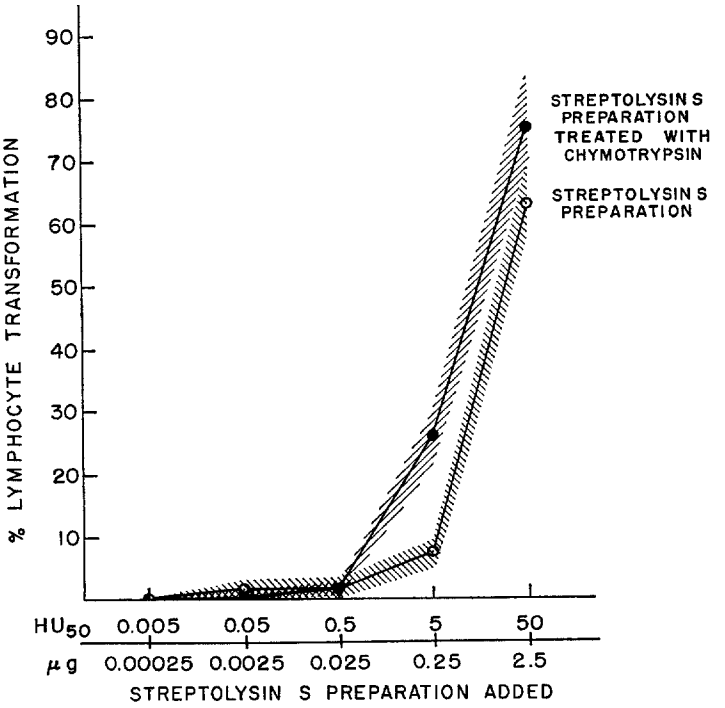

FIG. 4. Per cent lymphocyte transformation obtained with various amounts of streptolysin S preparation and of the same preparation treated with chymotrypsin. Both preparations were preincubated at 37°C for 30 min in solutions initially containing 500 HU_{50}/ml. Chymotrypsin was used in a concentration of 5 mg/ml.

S and sensitive to trypsin might be responsible for the lymphocyte transformation.

The transforming activity of 500 HU_{50}/ml solutions of streptolysin S preparation preincubated with 5 mg/ml of trypsin or of chymotrypsin and tested in 0.1 ml amounts was unchanged or increased as compared to control preparations incubated without enzymes, although the hemolytic activity of the chymotrypsin-treated preparation was undetectable by either the standard or the sensitized method of assay.

To obtain more definitive information on the relative transforming activity of

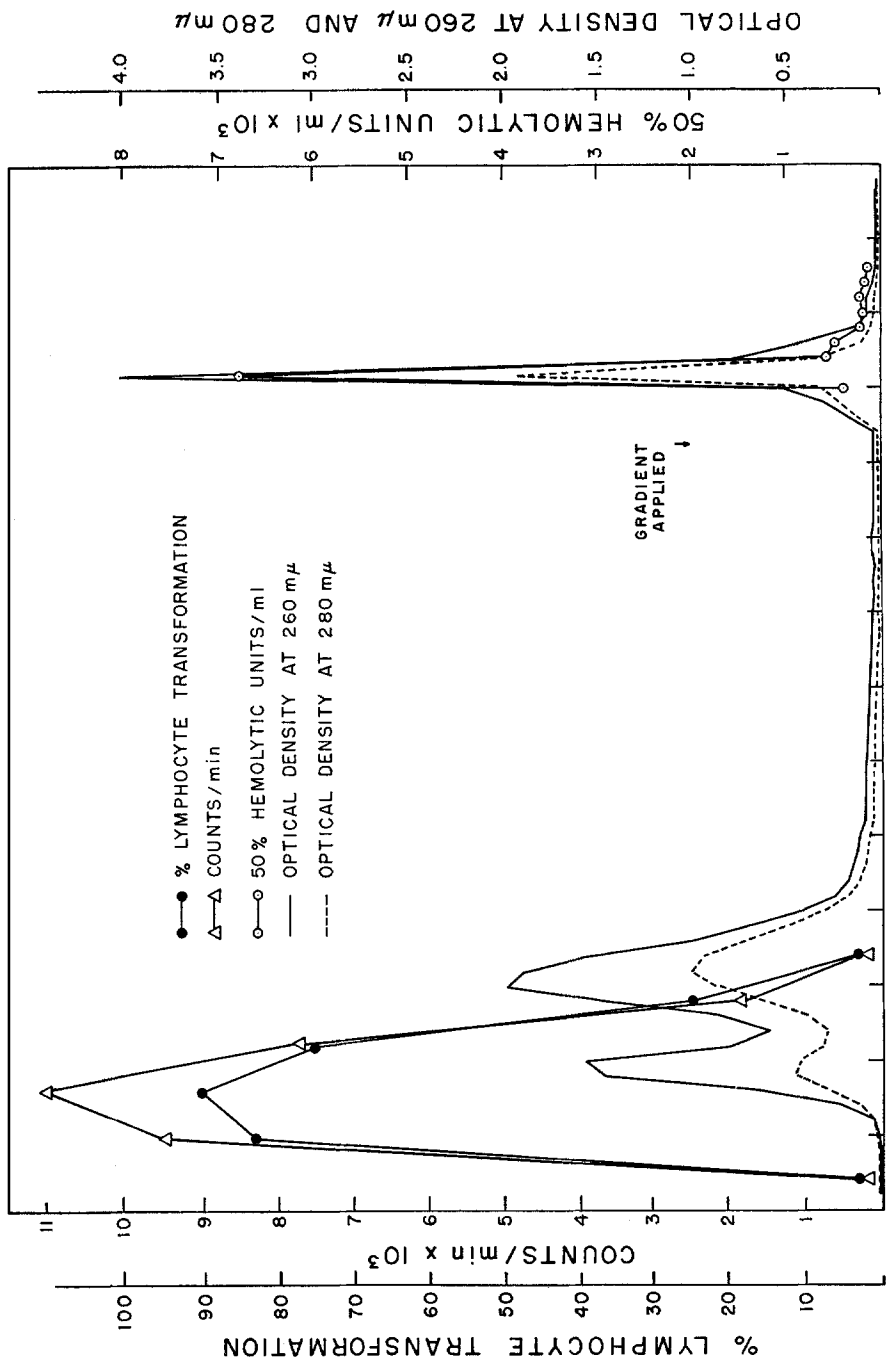

FIG. 5. Fractionation of streptolysin S preparation on a column of DEAE-Sephadex A-25. The recovery of the hemolytic activity was 60%. Quantitative determination of the recovery of transforming activity was not attempted. For reasons of clarity in the graphic presentation, notation of the hemolytic activity was omitted when less than 10 units/ml, and notation of the transforming activity was omitted when less than 3% (by morphology) or less than 0.15×10^3 (by ^{14}C-thymidine uptake). Negative controls (without additives) were less than 3% and less than 0.15×10^3; positive controls (with PHA) were 98% and 18.3×10^3.

streptolysin S preparations which had lost hemolytic activity upon digestion with chymotrypsin, two further experiments were done, with two different lymphocyte donors, but otherwise similar. Serial dilutions of a chymotrypsin-treated streptolysin S preparation were tested for their transforming activity and compared with equal amounts of the same batch of streptolysin S preparation incubated for 30 min at 37°C, but without chymotrypsin. One of these experiments (Fig. 4) yielded somewhat higher transformations with the chymotrypsin-treated preparation. The other experiment yielded essentially similar results with either preparation. Therefore, treatment with chymotrypsin does not decrease the transforming activity of streptolysin S preparations; whether a slight increase may result remains to be determined.

Chromatographic Fractionation of Streptolysin S Preparations on DEAE-Sephadex Columns.—Five fractionation experiments were carried out with small variations in technique, each yielding essentially similar results. Fig. 5 illustrates the results of a typical experiment, obtained with 1.7 ml of undialyzed streptolysin S preparation applied to a 0.9 × 15 cm column of DEAE-Sephadex A-25. Starting buffer was the suspension medium minus RNA core and maltose. A gradient of continuously increasing concentrations of sodium chloride in the same buffer was then applied. The gradient was obtained by connecting two cylindrical and equal reservoirs, A and B, with small bore tubing. Reservoir A was filled with the starting buffer; reservoir B was filled with the starting buffer saturated with sodium chloride. The buffer in reservoir A was kept in continuous agitation by a magnetic stirrer and was fed directly to the column.

Three optical density peaks were obtained, two with the starting buffer and one with the salt gradient. Hemolytic activity was associated with the last optical density peak only. Transforming activity was recovered only in the fractions immediately preceding and overlapping the first optical density peak, whether the transformation was measured morphologically (per cent transformation) or by incorporation of ^{14}C thymidine (counts per minute). Neither activity was contaminated with the other. The hemolytic activity recovered in the eluates had the characteristic sensitivities of streptolysin S: tested in 500 HU_{50}/ml solution it was completely inhibited by heating at 56°C for 2 hr, or by incubation with 3 μg/ml of trypan blue or 5 mg/ml of chymotrypsin at 37°C for 30 min. The appearance of the cells transformed by the chromatographic fractions could not be distinguished from that of cells transformed by unfractionated streptolysin S preparations.

Similar experiments with dialyzed and lyophilized streptolysin S preparations resulted in similar separation of the two activities. With the dialyzed preparations, the first two optical density peaks were markedly reduced while the third was unchanged.

The possibility was considered that the transforming activity of the early fractions might be due to substances originally present in the DEAE-Sephadex

rather than in the streptolysin S preparation. To test this possibility, "sham eluates" were obtained from a similarly prepared column that had been charged with 1.7 ml of complete suspension medium (including RNA core and maltose) rather than of streptolysin S preparation. Elution was carried out as for the streptolysin S fractionation experiments. These sham eluates proved to have no transforming activity.

The possibility was also considered that the lack of transforming activity in the hemolytic peak might be due to an inhibiting effect on transformation of the sodium chloride solution or of any substance that the sodium chloride solution might elute from the DEAE-Sephadex column. Therefore sham eluates obtained at the zone in the chromatogram of the suspension medium which corresponded to the hemolytic peak of the chromatogram of the streptolysin S preparation (tubes 55–57) were tested for their ability to inhibit transformation. Stimulus for transformation was provided by a pool of the early fractions (tubes 4–6), containing transforming activity. No inhibitory activity was found.

Finally, the hypothesis was considered that streptolysin S, although incapable of inducing transformation by itself, might be capable of enhancing transformation of lymphocytes stimulated by the transforming factor. Therefore, aliquots containing 50 HU_{50} of the fraction of streptolysin S preparation recovered at the summit of the hemolytic peak were added to a series of dilutions of a pool of early fractions, endowed with transforming activity. No stimulatory activity of streptolysin S was detected.

DISCUSSION

Many antigenic substances stimulate blastogenesis and mitotic activity in cultures of peripheral blood lymphocytes (reviewed in reference 22). The transformation thus obtained is limited to lymphocytes from previously sensitized donors. On the other hand, PHA (reviewed in reference 4), pokeweed extract (23, 24), staphylococcal filtrate (25), and antilymphocyte serum (26) ("nonspecific mitogens") are capable of inducing transformation in the lymphocytes of all normal subjects, and the per cent transformation usually exceeds that obtainable with specific antigens. Streptolysin S has been reported to be another nonspecific mitogen (1).

The present experiments confirm the observation that streptolysin S preparations induce transformation in the lymphocytes of normal subjects and that the per cent transformation is higher than that usually observed with antigens, though lower than that obtainable with PHA. They also show that the transformation obtained is indeed due to a product of beta-hemolytic streptococci, since the uninoculated media used for the preparation of streptolysin S had no transforming activity, and that this product has at least some degree of species specificity, since neither *Streptococcus viridans* nor *Diplococcus pneumoniae* nor *Staphylococcus aureus* proved capable of producing a lymphocyte transforming

factor when cultured and incubated as for the production of streptolysin S. In addition, the experiments here reported indicate that the lymphocyte transformation obtainable with streptolysin S preparations is not due to streptolysin S, but rather to another, nonhemolytic streptococcal product or products present in streptolysin S preparations but not previously recognized. Pending its further characterization, isolation, and possible identification with one or more previously known streptococcal products, it may be referred to as streptococcal mitogen in analogy with related nomenclature ("nonspecific mitogens," "pokeweed mitogen").

The possibility that the transforming activity of streptolysin S preparations might not be due to streptolysin S was first suggested by the observation that the C203U strain, a Group A streptococcus mutant derived from a streptolysin S producing strain (C203S) but itself unable to produce streptolysin S, had retained the capacity to produce a lymphocyte mitogen when cultured and incubated as for the production of streptolysin S (10). The resulting product (C203U product) was less active on a weight basis than the streptolysin S preparation obtained from the parent strain (C203S) but was capable of inducing, like the streptolysin S preparation, high percentages of lymphocyte transformation. The hypothesis that the transforming activity of streptolysin S preparations is not dependent on their hemolytic activity has now been supported by the observation that the transforming activity is less heat labile than the hemolytic activity and that, unlike the hemolytic activity, it is totally resistant to trypan blue, lecithin, and chymotrypsin. Although some of these experiments are open to criticism (for instance, lymphocytes may metabolize, absorb, or otherwise inactivate the inhibitors, and thus reinstitute in culture the hemolytic activity of streptolysin S) they constitute in their totality strong evidence that the hemolytic activity of streptolysin S preparations can be abolished in a number of ways with partial or complete preservation of their transforming activity. Moreover, preliminary observations indicate that streptolysin S preparations from some streptococcal strains of groups other than A have scanty or no transforming activity, despite a high hemolytic activity, and that the omission of RNA core from the suspension medium results in preparations with transforming activity but no hemolytic activity.[4] Finally, clear-cut separation of the two activities was achieved by chromatographic fractionation of streptolysin S preparations, with recovery of fractions endowed with transforming but not with hemolytic activity, and of fractions endowed with hemolytic but not with transforming activity. Although the recovery of the hemolytic activity was incomplete and the recovery of the transforming activity was not quantitatively determined, these results, in conjunction with those just considered, indicate that the hemolytic and transforming activities of streptolysin S preparations

[4] Taranta, A., and G. Cuppari. Unpublished observations.

are the attributes of two different streptococcal products, the first being streptolysin S and the latter streptococcal mitogen.

The dissociation of these two activities may have implications and applications of interest. Some previously described biologic activities of streptolysin S preparations may need to be reexamined with purified preparations of streptolysin S and of streptococcal mitogen to ascertain to which one they are due, and other possible biologic properties of the streptococcal mitogen will need to be explored. Other nonspecific mitogens have immunosuppressant properties (27, 28) and one of them, antilymphocyte serum, is being used to prolong the survival of organ transplants in man (29). It will be interesting to ascertain whether the streptococcal mitogen also may depress the immune response. If it does, the relation of the streptococcal mitogen to two apparently distinct streptococcal products with immunosuppressant activity (30, 31) will need to be determined. Another property of several lymphocyte transforming agents is the stimulation of interferon production (32–34), which is sought for prophylaxis and therapy of viral diseases (35), and the streptococcal mitogen deserves investigation in this respect.

The role that the streptococcal mitogen may play in streptococcal infections and their sequelae remains to be elucidated. If the streptococcal mitogen does depress the immune response it could confer to streptococci a survival advantage. Lymphocytes stimulated by the streptococcal mitogen might have a damaging effect on autologous cells, as has been demonstrated in vitro with PHA (36), and thus mediate tissue damage in some streptococcal sequelae. These possibilities are under investigation.

SUMMARY

The ability of streptolysin S preparations to induce high percentages of transformation in human peripheral blood lymphocytes was confirmed in a series of apparently healthy donors. Transforming activity was not demonstrated in the two media used for streptolysin S production, nor in control preparations in which a strain each of *Streptococcus viridans*, *Staphylococcus aureus* (nonhemolytic), and *Diplococcus pneumoniae* was substituted for the beta hemolytic streptococcal strain used for streptolysin S production.

The relation of the hemolytic activity to the lymphocyte transforming activity of streptolysin S preparations was studied by means of inactivation and fractionation experiments. Heating produced a loss in both activities, but more in the hemolytic than in the transforming activity. The transformation obtained with a heated preparation had a high degree of correlation with that obtained with the unheated preparation in a series of normal subjects and patients with various rheumatic diseases, whose lymphocytes were often less responsive to stimulation with streptolysin S preparations (both heated and unheated) than the lymphocytes of the normal subjects studied. Treatment of streptolysin S

preparations with chymotrypsin, vegetable lecithin, or trypan blue (the latter in minute amounts) resulted in preparations with no detectable hemolytic activity but with undiminished lymphocyte transforming activity. Chromatographic fractionations on DEAE-Sephadex columns yielded fractions endowed with transforming but not with hemolytic activity, and other fractions endowed with hemolytic but not with transforming activity. The recovery of the hemolytic activity was not complete and quantitation of the recovery of the transforming activity was not attempted.

These experiments indicate that the hemolytic and transforming activities of streptolysin S preparations are independent of each other, and specifically that they are the attributes of two different streptococcal products, one of which is streptolysin S. The other is a nonhemolytic streptococcal product present in streptolysin S preparations but previously unrecognized. Some implications of these findings are discussed.

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