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Isolation by Electrofocusing of Two Lymphocyte Mitogens Produced by *Staphylococcus aureus*

ARNOLD S. KREGER,[1] GIROLAMO CUPPARI, AND ANGELO TARANTA

Department of Medicine, New York University School of Medicine, New York, New York 10016

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Staphylococcus aureus strain DA352, grown in a diffusate of Todd-Hewitt broth, produced two extracellular nondialyzable lymphocyte mitogens having isoelectric points of 5.5 to 5.7 and 8.6 to 9.0. The mitogens were separable from one another by isoelectric focusing and could be isolated free of detectable amounts of other staphylococcal products by ethanol precipitation followed by isoelectric focusing. Dose-response curves with both mitogens showed a maximum per cent transformation in the range (90%+) obtained with phytohemagglutinin (PHA), a decrease of transformation with excess mitogen, and, with decreasing concentrations, a slope somewhat less steep than that obtained with PHA. Incubation with undigested or pepsin-digested pooled human gamma globulin enhanced the activity of the basic mitogen.

Ling and co-workers reported that filtrates of *Staphylococcus aureus* cultures grown without agitation for 4 to 5 days in Parker 199 medium stimulated human peripheral blood lymphocytes in vitro to "transform" into large, blastlike cells capable of mitosis (19-21). The stimulation appeared similar to that obtained with phytohemagglutinin (PHA). The crude active material was nondialyzable, soluble in 25% (w/v) trichloroacetic acid and in saturated ammonium sulfate, precipitable by 80% (v/v) acetone, and was inactivated by Pronase and by autoclaving, but not by heating for 15 min at 100 C. Although gel filtration of the culture filtrates suggested that transforming activity might be associated with molecules of various sizes (21), no separation into distinct components was reported then or since. Therefore, the mitogenic activity of staphylococcal culture filtrates has been customarily attributed to "staphylococcal mitogen," implying a single substance. We report that *S. aureus* strain DA352 produces two potent mitogens, one acidic and the other basic, which appear to be distinct from other known staphylococcal extracellular products.

MATERIALS AND METHODS

Mitogenic activity was estimated by two methods. The first, based on incorporation of tritiated thymidine, was developed by E. Gazit of Children's Hospital, Philadelphia, Pa. (*personal communication*). Portions (0.1 ml) of samples, filtered on membrane filters (Millipore Corp.) and diluted with isotonic phosphate-buffered saline (PBS), *p*H 7, were mixed with 0.2 ml of whole human blood and 1 ml of tissue culture medium in screw-cap tubes (16 by 125 mm) and incubated at 37 C in 5% carbon dioxide. The tissue culture medium contained 86 ml of modified McCoy's 5a medium (Grand Island Biological Co., Grand Island, N.Y.), 1 ml of heparin (2,000 units/ml), 1 ml of L-glutamine (29.2 mg/ml), 2 ml of an antibiotic mixture containing 5,000 units of penicillin and streptomycin per ml, and 10 ml of fetal calf serum (Grand Island Biological Co.) After 3 days of incubation, 1 μCi of thymidine-*methyl*-^3H (New England Nuclear, Boston, Mass.) in 0.2 ml of modified McCoy's 5a medium was added to each tube. After another 18 hr of incubation, the tubes were chilled and the cells were washed three times with 5 ml of 2% (v/v) acetic acid. The pellets were then washed twice with 3 ml of 5% (w/v) trichloroacetic acid (1 hr at 5 C for each washing) and dissolved in 0.5 ml of hydroxide of hyamine 10X (Packard Instrument Co., Downers Grove, Ill.). A 15-ml amount of Bray's scintillation fluid (4) was added to each dissolved pellet, and the solutions were counted in a Beckman LS-100 scintillation counter. PHA (reagent grade, Burroughs Wellcome, Greenville, N.C.) and negative controls were processed and counted simultaneously with the test samples.

The second method, based on morphological examination of the treated lymphocytes, was as previously described (29) with two modifications: the concentration of lymphocytes was adjusted to 5×10^5 instead of 10^6/ml, and the 4-ml portions of lymphocyte suspension were transferred into screw-cap tubes (16 by 125 mm) instead of flat-bottom bottles.

[1] Present address: The Bowman Gray School of Medicine, Department of Microbiology, Winston-Salem, N.C. 27103.

RESULTS AND DISCUSSION

Crude staphylococcal mitogen was produced by growing *S. aureus* strain DA352 (obtained from P. B. Smith, Center for Disease Control, Atlanta, Ga.) with either vigorous or gentle shaking in a diffusate of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) prepared by dialyzing one volume of five-times-concentrated (150 g/liter) Todd-Hewitt broth against five volumes of distilled water for 3 days. The inoculum was prepared by washing the staphylococci contained in one volume of a stationary-phase broth culture and suspending them in one volume of sterile PBS. For incubation with vigorous shaking, 2-liter Erlenmeyer flasks containing 625 ml of diffusate medium were inoculated with 0.1 ml and were incubated for approximately 18 hr at 37 C on a rotary shaker operating at 220 cycles/min. For incubation with gentle shaking, flasks containing 1,250 ml of medium were inoculated with 0.2 ml and incubated on a shaker operating at 60 cycles/min. The culture filtrate was obtained by centrifugation followed by membrane filtration (Millipore). Vigorously shaken cultures produced more total mitogenic activity (and relatively more basic mitogenic activity) than gently shaken cultures; however, they also contained staphylococcal delta hemolysin, which was not found in gently shaken cultures. Alpha and beta hemolysin activities were not detected in either culture filtrate. Staphylococci grown with vigorous shaking produced more mitogenic activity than cells grown by the method of Ling et al. (20, 21).

Isoelectric focusing in a gradient (*p*H 3 to 10) of filtrate from a vigorously shaken culture showed two distinct and well separated peaks of mitogenic activity having isoelectric points of approximately 5.5 to 5.7 and 8.6 to 9.0 (Fig. 1). Essentially similar results were obtained with the blood of three donors. In a similar experiment, fractions were diluted 1:100 and assayed with one donor's lymphocytes by the morphology method, which revealed two peaks in the same *p*H regions. The separation of the acidic mitogenic peak from the acidic hemolytic peaks was definite but that of the basic mitogenic and hemolytic peaks was not. Therefore, a more shallow gradient

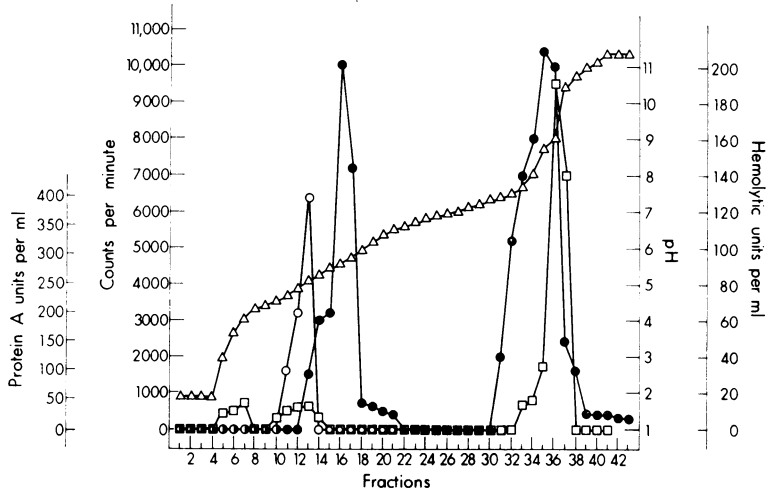

FIG. 1. *Isoelectric focusing of the culture filtrate of S. aureus strain DA352. The gradient was prepared from a less dense solution consisting of 54.2 ml of dialyzed (1% glycine) culture filtrate and 0.8 ml of 40% ampholine (pH 3 to 10; LKB Instruments, Rockville, Md.) and a more dense solution consisting of 38.8 ml of dialyzed culture filtrate, 1.7 ml of 40% ampholine (pH 3 to 10), and 25 g of sucrose. The total sample contained approximately 130 mg of protein, as determined by the Lowry method (22), using crystalline bovine plasma albumin as the standard. Focusing was done at 4 C for approximately 2 days in a 110-ml electrolysis column (LKB Instruments) with a final potential of 600 v. Fractions (3 to 4 ml) were examined for delta hemolysin activity (□) and protein A content (○), and 0.1-ml portions of 20,000-fold dilutions of each fraction were assayed in duplicate for mitogenic activity by the tritiated thymidine method (●). The scintillation counter values obtained were corrected by subtracting the negative control value (350 counts/min). The phytohemagglutinin (PHA) controls (0.1-ml portions of a 20-fold dilution of a 5-ml vial of dried PHA reconstituted with 5 ml of water) gave approximately 100,000 counts/min. The pH (△) was determined at approximately 4 C. Alpha and beta hemolysin activities were not detected in the fractions. The ampholine-sucrose gradient was not mitogenic by itself.*

(*p*H 7 to 10) was prepared, on which an incomplete but definite separation was achieved. The hemolytic activities could be inhibited by incubation with lecithin, with normal human serum (characteristics of staphylococcal delta hemolysin), or with anti-staphylococcal serum (Lederle Laboratories, Pearl River, N.Y.), but the mitogenic activities could not. Two mitogenic peaks having isoelectric points of approximately 5.5 and 8.8 were also detected in filtrates obtained from gently shaken cultures.

The report by Forsgren and Sjöquist (11) that staphylococcal protein A components have isoelectric points in the range of *p*H 4.95 to 5.25 and our unpublished observations that a crude preparation of protein A was mitogenic and that crude preparations of mitogens contain protein A suggested a possible identity of this protein with the acidic staphylococcal mitogen. The attractiveness of this hypothesis was increased by the knowledge that protein A reacts and precipitates with the Fc portion of gamma G-globulins (8–10), that lymphocytes carry immunoglobulins on their surfaces (30), and that anti-immunoglobulin sera can be mitogenic (27). Protein A might thus be mitogenic by combining with the gamma G-globulin determinants on the lymphocyte surface.

Two observations disprove the hypothesis that the acidic mitogen is protein A. First, the acidic mitogen and protein A are separable by isoelectric focusing (Fig. 1). Second, if the acidic mitogen were protein A, incubation of the mitogen with gamma G-globulin would be expected to result in precipitation of the mitogen and loss of its activity, whereas incubation with pepsin-digested gamma G-globulin, which does not contain the Fc portion of gamma G-globulin, would have no effect. However, incubation with undigested or pepsin-digested human gamma globulin did not suppress the activity of either mitogen and, in fact, markedly increased the activity of the basic mitogen (Table 1). The mechanism for this increase is not known at present.

The addition of ammonium sulfate to 90% saturation (630 g/liter), or of 2.5 volumes of chilled ethanol or acetone to 1 volume of culture filtrate resulted in precipitates containing most of the lymphocyte-stimulating activity. Electrofocusing of the ethanol precipitate obtained from filtrate of a gently shaken culture and dissolved in 1% glycine also revealed two active peaks with isoelectric points similar to those reported above. The two peak mitogen fractions were free of any detectable staphylococcal alpha hemolysin (3), beta hemolysin (14), delta hemolysin (17), leukocidin (31), protein A (28), esterase (16), lipase (24), protease (18), deoxyribonuclease (1), coagulase (1), hyaluronidase (1), ribonuclease (12), staphylokinase (2), lysozyme (15), acid phosphatase (23), and alkaline phosphatase (7) activity.

TABLE 1. *Enhancement of staphylococcal basic mitogen activity by human gamma globulin*

Mixture[a]	Counts per min of 100-fold dilution
Acidic mitogen	5,338
Acidic mitogen + 1.25 mg of gamma globulin	5,238
Acidic mitogen + 0.31 mg of gamma globulin	7,282
Acidic mitogen + 0.08 mg of gamma globulin	6,934
Acidic mitogen + 0.14 mg of PGG	6,859
Acidic mitogen + 0.04 mg of PGG	6,219
Basic mitogen	1,384
Basic mitogen + 1.25 mg of gamma globulin	10,167
Basic mitogen + 0.31 mg of gamma globulin	6,761
Basic mitogen + 0.08 mg of gamma globulin	5,602
Basic mitogen + 0.14 mg of PGG	6,025
Basic mitogen + 0.04 mg of PGG	4,412

[a] The mitogens in 10 ml of filtrate obtained from a culture grown with gentle shaking were precipitated with ethanol, dissolved in 2 ml of phosphate-buffered saline (PBS), and electrofocused in a gradient, *p*H 3 to 10. Portions (0.1 ml) of the acidic and basic mitogen peak fractions were incubated for 1 hr at 37 C with various amounts of gamma globulin or pepsin-digested gamma globulin (PGG) (25) contained in 0.9 ml of sterile PBS. The mixtures were then diluted with PBS, and 0.1-ml samples were assayed in duplicate by the tritiated thymidine method. The data shown are the means of the two assays. The gamma globulin and PGG were not mitogenic. Commercially available human gamma globulin (Immunology Inc., Glen Ellyn, Ill.) also increased the activity of the basic mitogen. The gamma globulin and PGG were assayed by the Lowry method (22) with crystalline bovine plasma albumin as the standard.

Our findings show that the two mitogens we isolated are distinct from staphylococcal alpha and delta hemolysins and leukocidin, partially purified preparations of which were reported (5, 6) to be mitogenic.

Ling and co-workers (19–21) considered the lymphocyte stimulation produced by staphylococcal culture filtrate to be most likely immunologically nonspecific, like that induced by PHA. Dose-response curves obtained by the morphology method with the staphylococcal mito-

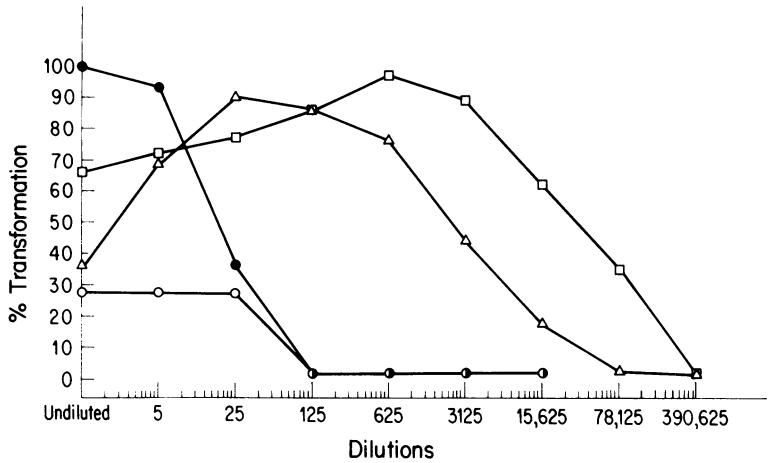

FIG. 2. *Percentage transformation as a function of mitogen concentration. Portions (0.1 ml) of fivefold dilutions of solutions of phytohemagglutinin (PHA) (●, 5-ml vial of dried PHA reconstituted in 5 ml of water), purified protein derivative (○, 2,500 U.S. tuberculin units/ml), acidic staphylococcal mitogen (△, fraction number 16 in Fig. 1), and basic staphylococcal mitogen (□, fraction number 35 in Fig. 1) were assayed in duplicate for transforming activity by the morphology method. The test samples were incubated with the lymphocytes for 5 days, with the exception of the PHA samples, which were incubated for 3 days. Negative controls showed approximately 2% transformation.*

gens, PHA, and tuberculin [purified protein derivative (PPD), second test strength, Merck Sharp and Dohme, West Point, Pa.] and with lymphocytes obtained from a PPD-sensitive donor were generally consistent with this interpretation for both staphylococcal mitogens (Fig. 2). The maximum per cent transformation obtained with them was almost as high as that obtained with PHA. The slopes of the dose-response curves obtained with the two mitogens were similar to each other though somewhat less steep than that obtained with PHA. The per cent transformation decreased when the mitogens were present in excess of an optimum transforming concentration. The same pattern of inhibition by excess mitogen has been observed with some (26) but not all (13) PHA preparations. The dose-response curve of PPD showed a maximum much lower than that obtained with PHA and extending over a broad plateau. Neither staphylococcal mitogen showed similar plateaus.

The two mitogens purified by electrofocusing (fractions 16 and 35 in Fig. 1) and dissolved in PBS were stable at 4, 0, and −70 C for at least 1 month, were partially inactivated by heating for 30 min at 60 C and above, and were completely inactivated by autoclaving. They were partially inactivated by incubation for 30 min at 37 C with Pronase (100 μg/ml) but not with trypsin, chymotrypsin, or papain.

Work is in progress to purify and characterize the two staphylococcal mitogens further.

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