Tuberculin Antigens Active in Human Lymphocyte Blastogenesis[1] (36877)

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We recently have demonstrated (1) that discrete pools of tuberculoprotein and tuberculopolysaccharide antigens can be prepared from unheated culture filtrates of tubercle bacilli by continuous-flow electrophoretic separation. The isolated antigens were found to have retained their original electrophoretic mobility and preciptinogenicity and studies of their reactivity in immune responses to the tubercle bacillus have been initiated. The purpose of this report is to describe the *in vitro* blastogenic response of human lymphocytes to these antigens.

Materials and Methods. Preparation of antigens. A concentrated, dialyzed, unheated filtrate from 8-week old cultures of the H37Rv strain of *Mycobacterium tuberculosis* was prepared as described previously (2). Separation of this material by continuous-flow electrophoresis (1) yielded 3 antigen pools. On chemical analysis, Pool I was found to contain the tuberculopolysaccharides; hexose and pentose carbohydrates comprised approximately 93% of its weight. Similarly, the tuberculoproteins constituted approximately 95% of Pool III. Pool II, although mainly proteinaceous, was contaminated with a considerable amount of polysaccharide and, consequently, was not used in the study. Immunoelectrophoretic analysis of Pools I and III, using a reference antiserum[2] and the methods recommended for the mycobacterial reference system (2), demonstrated (Fig. 1) that they contained, respectively, the cathodic tuberculopolysaccharides and the anodic tuberculoproteins of the culture filtrate preparation. Stock solutions of Pools I and III and the unseparated culture filtrate were prepared to contain 5 mg of non-dialyzable solids/ml of 0.85% NaCl, sterilized by membrane filtration and stored at −20° for use as test antigens in cell cultures.

Study subjects. A group of 16 healthy hospital employees served as blood donors for the study. All donors were tested intradermally with intermediate-strength tuberculin PPD (Merck, Sharp, and Dohme, Inc., West Point, Pennsylvania) either 1 month before or immediately after the blood sample was withdrawn; the skin reactions were examined and measured at 48 hr after injection. The PPD-positive group was composed of 4 male and 1 female white subjects and 1 male and 1 female black individuals; negative reactions ($<$ 5 mm) to PPD were exhibited by 7 female and 2 male white subjects.

Preparation of cell cultures. Leukocyte-rich plasma was obtained from approximately 30 ml of heparinized venous blood after gravity sedimentation for 1 to 2 hr at room temperature. As described previously (3), the cells were sedimented by centrifugation at 100*g* for 12 min and were resuspended to contain 1×10^6 lymphocytes in 3 ml of culture medium containing 80% medium 199 (Grand Island Biological Co., Grand Island, New York) and 20% autologous, platelet-depleted,

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[2] Mycobacterial Reference Antiserum (Lot #001) obtained through the United States–Japan Cooperative Medical Science Program from the Geographic Medicine Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014.

TABLE I. Comparison of Blastogenic Reactivity to Polysaccharide and Protein Components of Unheated Mycobacterial Culture Filtrate in Lymphocytes from Positive and Negative Tuberculin Reactors.

Group[a]	Blastogenic response (S/C ratio)[b] obtained with		
	Culture filtrate	Pool I (polysaccharide)	Pool III (protein)
PPD positive (7)	179.3 ± 42.1	15.4 ± 4.9	146.8 ± 37.2
PPD negative (9)	25.4 ± 7.2	1.9 ± 0.2	14.4 ± 5.4

[a] Values in parentheses represent number of subjects/group.
[b] Values represent mean ± standard error.

plasma. Triplicate 3 ml cultures in 16 × 125 mm, sterile, plastic tubes were prepared for each test substance and for the unstimulated controls.

Preliminary experiments with lymphocytes from representative PPD-positive and -negative subjects established that the unheated culture filtrate, at a concentration of 5 μg of non-dialyzable solids/ml of medium, induced blastogenesis to approximately the same degree as 5 μg of PPD/ml of medium. Accordingly, this concentration also was used to test the activity of the antigen pools in all experiments. After addition of antigens, the cultures were incubated at 37° in an upright position for 4 days.

Lymphocyte blastogenesis. The antigen-induced blastogenic response of the cultured lymphocytes was measured by their incorporation of ^{3}H-thymidine (Amersham-Searle Corp., Arlington Heights, Illinois). On the 4th day of incubation, 0.3 μCi was added to each culture and the cultures were incubated for an additional 3 hr after which they were processed for radioisotopic assay as described by Moorhead *et al.* (4). Trichloroacetic acid-precipitable radioactivity was measured in a scintillation counter and blastogenesis was expressed as a stimulated to control ratio (S/C) which was calculated by dividing the mean disintegrations/min of cultures to which the test substance was added (S) by that of the unstimulated controls (C). Blastogenesis also was confirmed occasionally by morphologic examination of Wright-stained smears made from selected cultures.

Results. The influence of the tuberculopolysaccharide and tuberculoprotein antigen pools on cultures of lymphocytes from 7 PPD-positive and 9 PPD-negative subjects is shown in Table I. Statistical analysis of the differences in mean S/C ratios between the reactor and non-reactor groups indicated that a significant blastogenic response was induced when lymphocytes of PPD reactors were exposed to the culture filtrate preparation ($p < 0.005$) and the pool of protein antigens ($p < 0.005$) but not when they were cultured with pooled polysaccharide antigens. Furthermore, within the reactor group, no significant difference was found when the S/C ratios obtained with the culture filtrate and the protein antigen pool were compared; the values obtained with the pool of polysaccharide antigens, however, were significantly less ($p < 0.005$) than those of the culture filtrate preparation and the pooled protein antigens.

When the responses of the PPD-positive subjects to the test substances were examined individually, as illustrated in Fig. 2, the activity of the culture filtrate preparation was found to be closely and consistently related to its protein components. It was obvious that the pattern of responses to the culture filtrate was reproduced almost completely by that of the pooled proteins. Furthermore, it was apparent with each individual that the response to the culture filtrate preparation could be attributed to its component protein antigens; approximately 80% of the S/C values obtained with the unseparated culture filtrate was found in the protein pool while only 7% was detected in the pool of polysaccharide antigens.

Discussion. The results of this study provide strong evidence that tuberculin-induced blastogenesis of human lymphocytes

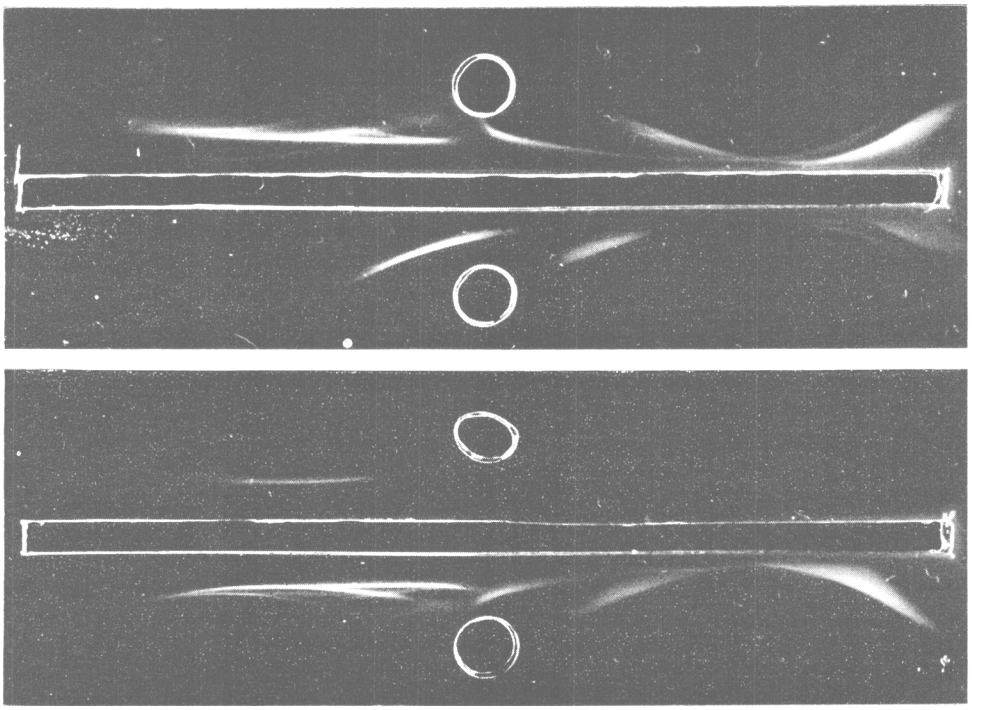

FIG. 1. Immunoelectrophoretic separation patterns of tuberculopolysaccharide (Pool I) and tuberculoprotein (Pool III) antigens. Troughs contained mycobacterial reference antiserum and the sample wells contained from top down: unseparated culture filtrate; Pool I, Pool III, and unseparated culture filtrate. Anode was on the left side; samples were separated at room temperature for 90 min in 1% lonagar No. 2 using a discontinuous (pH 8.6) barbital buffer system and 1.67 mA/slide. Slides were photographed after incubation at room temperature for 48 hr.

is a reflection of cellular reactivity to the tuberculoproteins. As indicated by immunoelectrophoretic and chemical analyses, it seems reasonable to attribute the minimal reactivity detected with the tuberculopolysaccharides to their trace contamination by the protein antigens. These findings, thus, confirm an earlier report by Heilman and McFarland (5) who demonstrated morphologically that preparations of the polysaccharide I and II antigens from tuberculin lacked blastogenic reactivity for human lymphocytes when compared to the tuberculoprotein antigens contained in PPD-S. The data also are consistent with those obtained recently by Chaparas and his associates (6) who found that a polysaccharide fraction (GA) of tuberculin failed to induce blastogenesis of lymphocytes from sensitized guinea pigs even though it provoked delayed hypersensitivity skin reactions, inhibited macrophage migration *in vitro* and stimulated production of migration inhibitory factor. The failure (7) of this same polysaccharide to elicit delayed-type skin reactions in tuberculosis patients remains unexplained but, at least, agrees with the present and prior (5) demonstrated lack of human blastogenic reactivity to the tuberculopolysaccharide antigens.

These results also further validate the effectiveness of separation of the tuberculoproteins and tuberculopolysaccharides by continuous-flow electrophoresis. The relatively clear distinction of the antigen pools on immunoelectrophoretic analysis was paralleled by their marked differences in blastogenic reactivity. A similar distinction between the pools in their ability to induce lethal tuberculin shock in sensitized guinea pigs has been described in a preliminary report (8); the tuberculopolysaccharides induced shock as effectively as the unseparated culture

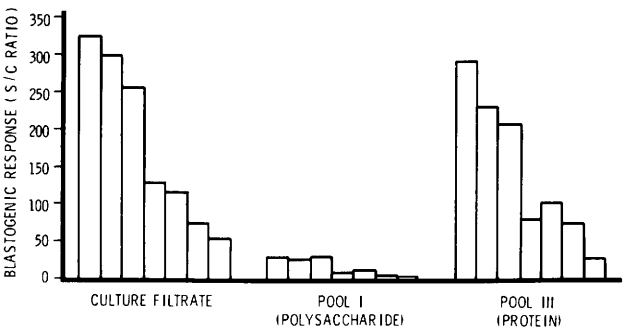

FIG. 2. Comparison of blastogenic response of lymphocytes from PPD-positive individuals to tuberculopolysaccharide and tuberculoprotein components of unheated mycobacterial culture filtrate. Subjects were ranked in descending order on the basis of S/C values obtained with unseparated culture filtrate; their respective values with the pooled antigens then were plotted in the same order.

filtrate preparation while the tuberculoproteins were inactive. On this basis, the antigen pools appear to be suitable starting materials for subfractionation to isolate the individual biologically reactive antigens.

Finally, it also is pertinent to comment on the degree of blastogenic reactivity to the culture filtrate and tuberculoprotein preparations exhibited by lymphocytes of the subjects in the PPD-negative group. The possibility exists that some individuals in this group may have had prior sub-clinical experiences with the tubercle bacillus or related mycobacteria and might have exhibited positive reactions on skin testing with higher concentrations of PPD prepared from human-type tubercle bacilli or other mycobacterial species. Antigenic cross-reactive lymphocyte blastogenic responses have been detected in healthy individuals by Chaparas and his coworkers (9). Some of their subjects exhibited negative skin reactions to PPD of *M. tuberculosis* but reacted to PPD of *M. intracellulare*; they had significant blastogenic responses to these preparations and to culture filtrate antigens of *M. scrofulaceum*, *M. kansasii*, and *M. fortuitum*. As an alternative explanation, it is possible that the reactivity of the PPD-negative donors might reflect their prior experiences with other microorganisms. Antigenic cross-reactivity between mycobacteria and other bacterial genera has been described recently in the primary binding test using sera of animals (10) and man (11). Obviously, isolation of the blastogenesis-inducing tuberculoprotein antigen and definition of its immunologic specificity are required to clarify the blastogenic reactivity of PPD-negative individuals.

Summary. Discrete pools of tuberculopolysaccharide and tuberculoprotein antigens, prepared by continuous-flow electrophoretic separation of an unheated culture filtrate of *M. tuberculosis* (H37Rv strain), were tested for induction of lymphocyte blastogenesis in cultures of blood leukocytes from healthy individuals who reacted to an intermediate skin test dose of tuberculin PPD. Approximately 80% of the blastogenic activity of the unseparated culture filtrate was found in the pool of protein antigens while only 7% was detected in the polysaccharide pool. As indicated by immunoelectrophoretic and chemical analyses, the minimal activity of the pooled polysaccharides was attributed to their trace contamination by the protein antigens. It was concluded that tuberculin-induced lymphocyte blastogenesis reflects cellular reactivity to tuberculoprotein antigens.

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