BINDING BETWEEN COMPONENTS OF THE TUBERCLE BACILLUS AND HUMORAL ANTIBODIES*

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Humans and experimental animals infected with tubercle bacilli may have detectable circulating antibody to a number of bacterial antigens as well as delayed skin sensitivity to tuberculin (1). Their significance during disease or their possible role in the pathogenesis of tuberculosis, however, is not clearly understood. Two factors in particular have prevented this understanding: (*a*) relatively few of the chemical constituents of the tubercle bacillus which are responsible for the induction of circulating antibodies in infected individuals have been characterized with certainty, and (*b*) a sensitive, primary binding type of test procedure to detect and quantitate small amounts of antibody to antigenic portions of the tubercle bacillus has not been developed.

A variety of serological methods have been applied to demonstrate the presence of antibodies in animals and humans infected with the tubercle bacillus. These include complement fixation, agar diffusion, hemagglutination, the Coombs indirect agglutination methods, and immunoelectrophoresis (2). None of these tests has been completely successful, and all have limitations in terms of sensitivity, specificity, or reproducibility. Each of these tests to detect antibody depends upon measuring events which may or may not occur after the primary reaction between antigen and antibody has taken place. In previous reports (3, 4) it was found convenient to consider such tests as secondary or tertiary manifestations of a primary interaction between antigen and antibody, according to whether they take place in vitro or in vivo. A negative secondary or tertiary reaction does not necessarily mean the absence of antibody (5–8), because secondary and tertiary events depend upon multiple variables other than the primary antigen-antibody bond. In addition, there is great variation among antisera as regards their efficiency in eliciting a given secondary manifestation such as precipitation (9), hemagglutination (10), or complement fixation (11).

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The essential requirements for developing a sensitive primary binding test system are (*a*) availability of either purified antigen or antibody, (*b*) a method to separate antigen-antibody complexes from free antigen, and (*c*) a sensitive and accurate method for detecting and measuring the amount of antigen or antibody present in the separated fractions. The degree of sensitivity desired usually requires the use of isotopic or fluorescent labeling.

In a previous study the primary interaction between antisera from patients with tuberculosis was studied by precipitation of ^{131}I-labeled antigen-antibody complexes with anti-human γ-globulin (12). Although binding by many of the antisera was noted, the antigen employed was not clearly defined or purified, thereby severely limiting the sensitivity and accuracy of the test.

The experiments described in the present report were undertaken to test the feasibility of developing primary binding tests using a purified component of the tubercle bacillus. By means of a combination of fractionation and purification procedures such as sonification, ultracentrifugation, and preparative electrophoresis in polyacrylamide gel, a component of the tubercle bacillus was isolated and partially purified. Because of the known complexity of the structure of the tubercle bacillus, this isolated component is believed to be only one of a multitude of potential antigenic components present in this organism. However, the primary interaction between this component and antibody was detected in sera from rabbits that had received repeated injections of sonicated killed tubercle bacilli, and also in sera from rhesus monkeys that had been protected with BCG vaccine and subsequently challenged with virulent organisms. Some of its properties were of unusual interest. Using a quantitative type of inhibition test, this component was found to differ from several other components derived from the tubercle bacillus and could be distinguished clearly from antigen mixtures derived from other mycobacteria. Some of its physicochemical characteristics were determined and are the subject of this report.

When the term *antigen* is employed in this paper, it is meant to refer to a substance with capacity to bind specifically to antibody. This usage should be differentiated from other frequent uses of *antigen* either as a substance with capacity to stimulate the production of circulating antibodies, whether or not these have protective or pathogenic properties, or as a substance with capacity to elicit a positive tuberculin type of skin reaction.

Materials and Methods

Mycobacterium tuberculosis, Strain 5159.—These organisms were kindly provided by Doctors L. Schmidt and R. C. Good, National Center for Primate Biology at Davis, Calif. Cells harvested from pellicle cultures on Proskauer-Beck medium were separated from culture medium by filtration through Whatman No. 1 paper and washed repeatedly with sterile distilled water. They were then heated to 70°C for 1 hr. Guinea pigs were inoculated with heated cells and examined after 6 wk to confirm sterility of the preparation.

Procedure for Preparing Crude Antigen.—

Disruption of cells by sonification: 40–50 ml suspensions of whole killed mycobacteria in distilled water were placed in a stainless steel chamber that was firmly sealed around a Sonifier

probe tip (Branson cell disruptor, Branson Instruments, Inc., Danbury, Conn.) The chamber was immersed in ice water, and sonification was carried out for 10 min, followed by a 5 min cooling period, until the organisms had been sonicated for a total of 60 min. Portions of the whole sonicate were used to immunize rabbits as described below, and other portions were subjected to ultracentrifugation.

Ultracentrifugation was carried out in a Beckman model L-2 instrument for 120 min at 35,000 rpm. The supernatant fluid separated from a distinct pellet constitutes the material referred to throughout this report as the crude antigen.

Other Mycobacteria and Cell Derivatives.—Live BCG cells (bacillus of Calmette and Guérin), obtained from Lederle Laboratories, Pearl River, N.Y., were heated to 70°C for 1 hr, after which they were washed with sterile distilled water. The following were generously supplied by other investigators: a sonicate of heat-killed *Mycobacterium fortuitum* organisms prepared and provided by Dr. R. Kalinske, La Jolla, Calif.; arabinogalactan and tuberculin-active peptide which had been purified from the human tubercle bacillus strain Aoyama B (13, 14), from Dr. Y. Yamamura, Osaka, Japan; NT, a protein component isolated from "old tuberculin," and PLP, a purified phospholipid component of BCG,[1] from Dr. G. Middlebrook, Baltimore, Md.; and PPD, from Parke, Davis and Co., Detroit, Mich. The whole mycobacterial preparations were sonified for 60 min as described above.

Other Microorganisms.—The following organisms were obtained from Dr. I. Crawford, La Jolla, Calif., and grown in nutrient broth (Difco Laboratories, Detroit, Mich.): *Escherichia coli* K-12; *Shigella dysenteriae* SH; *Pseudomonas putida*, and *Bacillus subtilis* 168. Organisms were grown at 37°C overnight, after which the cells were washed and resuspended in small volumes of sterile distilled water. They were then sonified for 60 sec. Group A hemolytic streptococci, supplied by Dr. B. F. Anthony, La Jolla, Calif., were harvested from Todd-Hewitt medium after overnight incubation at 37°C, heated to 56°C for 30 min, washed with distilled water, and sonified for 60 sec as above.

Rabbit Sera.—New Zealand white rabbits were immunized with portions of the whole sonicate that was prepared from the 5159 strain of mycobacteria. Each animal received initial total injections of 4 ml of sonicate suspended in equal parts of incomplete Freund's adjuvant, followed by two monthly injections of 1 ml each. The dry weight of the sonicated material was approximately 30 mg/ml. Rabbits were bled at 30 day intervals, and these antisera will be referred to as anti-5159. Control rabbit antisera to bovine serum albumin were prepared as previously described (4).

Monkey Sera.—Sera from rhesus monkeys (*Macaca mulatta*) were provided by the National Center for Primate Biology, Davis, Calif. A group of nine tuberculin-negative monkeys with normal chest roentgenograms were injected intravenously with 1.0 ml of 1×10^7 viable units of live BCG organisms. 90 days after vaccination the animals were challenged intratracheally with 1000 viable units of *M. tuberculosis* strain 5159. A second group was not vaccinated with BCG but was similarly infected with live 5159 organisms.

Chest roentgenograms, both anteroposterior and lateral views, were taken at monthly intervals after vaccination. 10 ml of blood was drawn from each animal prior to, and at monthly intervals after, vaccination, and at 2, 4, 8, and 12 wk after challenge. Surviving animals were killed 90 days after challenge and examined by necropsy.

Reagents.—Papain, bovine pancreas deoxyribonuclease and ribonuclease, calf thymus deoxyribonucleic acid, and bakers' yeast ribonucleic acid were obtained from Worthington Biochemical Corp., Freehold, N.J. Pronase was obtained from Calbiochem, Los Angeles, Calif., pepsin, from Nutritional Biochemicals Corp. Cleveland, Ohio; and crystallized BSA, from

[1] *Abbreviations used in paper:* AG, arabinogalactan; BSA, bovine serum albumin; NRS, normal rabbit serum; PLP, phospholipid component of BCG; RGE, radio-gel electrophoresis; RGG, rabbit γ-globulin; SAS, saturated ammonium sulfate; SAS/2, 50% saturated ammonium sulfate; TAP, tuberculin-active peptide; TCA, trichloroacetic acid.

Armour Pharmaceutical Co., Kankakee, Ill. Protein A, a cell wall derivative of the *Staphylococcus aureus*, was kindly provided by Dr. J. Sjöquist, Uppsala, Sweden. Rabbit and monkey γ-globulins were prepared by diethylaminoethyl cellulose chromatography (15).

Radioisotope Labeling.—Some of the test antigens were labeled with ^{131}I by a modification of the chloramine-T method (16). Samples of mycobacterial components were dialyzed in 0.5 M sodium chloride prior to labeling. Proteins labeled with ^{131}I contained approximately 1.5 μc $^{131}I/\mu$g nitrogen and are referred to in this report as I*Ag.

Protein Assay.—The nitrogen content of the cell extracts was determined by a modification of the micro-Kjeldahl method, using a Technicon AutoAnalyzer (17). For some protein determinations the method of Lowry et al. (18) was used.

Analytic Nonlinear Gradient Polyacrylamide Electrophoresis.—This procedure was performed as described by Wright.[2] It is a modification of the conventional disc acrylamide electrophoresis procedure originally developed by Ornstein (19) and Davis (20). Stock and working solutions were the same as used for the conventional procedure, except that gel percentages were 3.75%, 4.75%, 7%, and 12%. The stock Tris-glycine buffer, pH 8.3, was diluted 1:5. The ammonium persulfate solution was adjusted to 0.145%. Pyrex tubes, 12 cm in length and 6 mm in diameter, were marked at 3.5, 4.5, 5.5, 7, and 11 cm from their upper ends. Tubes were filled to the 7 cm mark with 12% acrylamide solution, and immediately the 7% gel solution was layered onto the 12% solution to the 5.5 cm mark. The 4.75% gel was then layered to the 4.5 cm mark, followed by the 3.75% gel to the 3.5 cm mark. The tubes were water-layered, and the gels were allowed to polymerize. They were then inserted into a buffer compartment, and 0.2–0.6 ml of sample containing approximately 300 μg protein in 40% sucrose was layered over the 3.75% gel by displacement of buffer over the top of the gel. Fig. 1 compares a nonlinear gradient gel preparation with the conventional method. Electrophoresis was carried out at room temperature with a current of 5 ma/tube for 90–120 min, or until a bromphenol blue tracking dye reached the 11 cm mark. After the gels were removed from the glass tubes, they were stained as follows: for protein, with Coomassie brilliant blue (0.06% of aqueous dye in 20% trichloroacetic acid), for lipoprotein, with oil red O (in a solution of equal parts of methanol saturated with oil red O and 20% TCA); for polysaccharides and glycoproteins, by the periodic acid–Schiff reaction; and for mucopolysaccharides, with alcian blue (0.1% in 5% acetic acid). Details of the procedure and staining methods have been reported[3] (21–23).

Preparative Nonlinear Gradient Acrylamide Electrophoresis.—Preparative gradient acrylamide electrophoresis was carried out using an electrophoresis glass column 14 cm long and 18 mm in diameter (Buchler Instruments, Inc., Fort Lee, N.J.). The glass was marked at 2.5, 4.0, 5.5, and 7.5 cm from its upper end. Gel solutions were added as in the analytic procedure, except that each gel was polymerized under water before adding the next gel. A schematic representation of the column holding the gel layers and the location of electrodes is presented in Fig. 2. A dialyzing membrane prevented loss of eluted protein into the electrode solution. Electrophoresis was carried out for 10–12 hr at 4°C, 20 ma, and 400–600 v. 3 ml samples were eluted and collected from the column at 5 min intervals by means of an automatic fractionating device (Fractophorator, Buchler Instruments, Inc., Fort Lee, N.J.). The absorbance of the eluates was examined in a Beckman DU spectrophotometer, and appropriate peaks were pooled, dialyzed in distilled water, concentrated by lyophilization, and examined by the analytic gradient acrylamide method.

Estimation of Molecular Weight.—Molecular weights of some of the pooled fractions obtained following electrophoresis were estimated according to their elution characteristics from a polyacrylamide gel column (Bio-Gel P, Bio-Rad Laboratories, Richmond, Calif.). A P-30

[2] Wright, G. L., Jr. 1969. Separation, isolation, and characterization of mycobacterial antigens by polyacrylamide electrophoresis. To be published.

size gel was selected for this purpose because of its optimal resolving capacity for substances with molecular weights ranging between 3,000 and 30,000. The gel was equilibrated with Tris-glycine buffer (1:5 dilution of stock) and poured into a column 9 mm in diameter, 60 cm in length, and 35 ml in volume. Samples applied to the column were a 1 ml mixture of cytochrome *c* (mol wt 12,500) and I*Ag in 40% sucrose. The fractions eluted were tested for radioactivity and the presence of cytochrome *c*. A similar sample was applied to a filtration cell

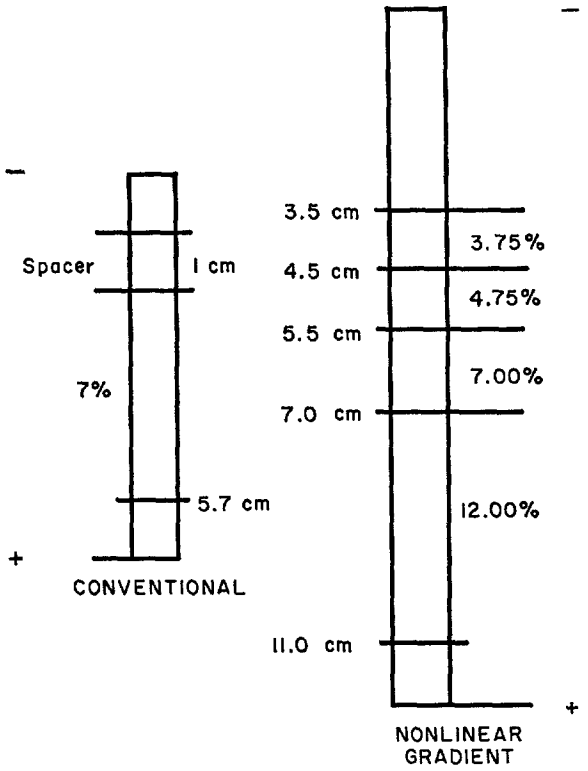

FIG. 1. Schematic representation of a nonlinear gradient polyacrylamide analytic preparation compared with the conventional method. *Courtesy of G. L. Wright, Jr.*[3].

(Amicon Corp., Cambridge, Mass.), using a membrane designed to retain molecules greater than 10,000 mol wt (UM-10).

Immunoelectrophoresis.—This was performed according to Scheidegger (24). Rabbit anti-monkey antisera specific for IgG and goat anti-rabbit IgG (anti-RGG) were made by immunization of rabbits and goats with IgG mixed in incomplete Freund's adjuvant. An anti-rabbit Fc antiserum was prepared by absorbing the anti-RGG serum with rabbit Fab produced by digestion of RGG with pepsin (25). Rabbit anti-human Fab and anti-human Fc antisera were kindly provided by Dr. H. Spiegelberg, La Jolla, Calif.

Circulating Antibody Measurements.—

(*a*) *Radio-gel electrophoresis*: A 0.1 ml sample of antiserum was incubated for 1 hr at 37°C and overnight at 4°C with 40 μl of I*Ag containing 1–4 μg N I*Ag/ml. Microscope slides were

coated with a 1 mm layer of 2% Ionagar (Consolidated Laboratories, Inc., Chicago, Ill.), and a trough 1.5 cm in length was cut perpendicular to the long axis of the slides. Electrophoresis of the I*Ag-serum mixture was performed using 35–40 v and 40 ma in Veronal buffer at pH 8.3. Radioactivity was detected by exposing fixed and dried preparations to sensitive X-ray film (Kodak Medical X-ray film NS-54T) for 24 hr. Slides were then stained with amido black. Details of the procedure and control experiments to establish specificity of the reaction have been described (4).

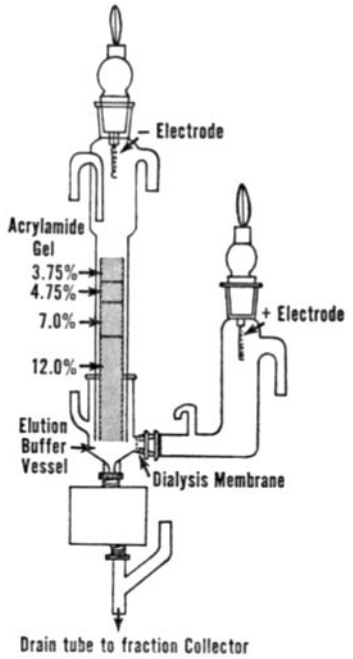

FIG. 2. Schematic representation of a nonlinear gradient polyacrylamide preparative gel column and apparatus employed for purification of crude antigen.

(*b*) *Radioimmunoelectrophoresis and radioimmunodiffusion* were performed as previously described (26, 27).

(*c*) *Ammonium sulfate test (ABC-33):* The capacity of antisera to bind antigen was measured by precipitating ^{131}I-labeled antigen (I*Ag)–antibody complexes with 50% saturated ammonium sulfate (SAS/2). Duplicate 0.5 ml aliquots of serum dilutions were incubated with 0.5 ml I*Ag at 4°C for 18 hr, after which 1 ml SAS was added. After incubation and centrifugation, the precipitates were washed with SAS/2 and radioactivity in the precipitates was determined. Results were expressed as the percentage of I*Ag specifically bound by a 1:10 dilution of a given antiserum. In some experiments results were expressed as micrograms of I*Ag N bound/ml of undiluted serum. These values were calculated after determining the reciprocal of the dilution of antiserum required to precipitate 33% of the I*Ag added and are referred to as ABC-33 values. Details of the procedure and calculations have been reported (9, 28).

(*d*) *Anti-rabbit γ-globulin (anti-RGG) to precipitate I* antigen-antibody complexes:* Binding

of antigen to antibody was sometimes measured by Feinberg's method of precipitating I*Ag-antibody complexes with antiserum containing known amounts of anti-RGG as previously described (4). The antiserum was prepared by repeated injections of goats with RGG mixed with incomplete Freund's adjuvant. Otherwise, the procedure and calculations were carried out as for the ammonium sulfate test.

(*e*) *Measurement of inhibition of I*Ag-antibody reaction by unlabeled sonicates and components of microorganisms:* Serial dilutions of various unlabeled sonicates of microorganisms and mycobacterial components were tested for their capacity to inhibit binding of a given I*Ag to a rabbit anti-5159 antiserum. To carry out this study, the dilution of anti-5159 antiserum in 1:10 normal rabbit serum that would bind 40–50% of a given amount of I*Ag was determined. Duplicate 0.5 ml aliquots of serial dilutions of the unlabeled sonicates or cell components tested were added to tubes containing 0.5 ml aliquots of anti-5159. To serve as controls, 0.5 ml aliquots of borate buffer were added to a series of 0.5 ml aliquots of antiserum. After overnight incubation, 0.5 ml I*Ag was added to all tubes, and they were incubated again overnight. On the third day, 1.5 ml SAS was added to each tube and the ammonium sulfate test was carried out as described above. For each dilution of unlabeled material the percentage of I*Ag bound to anti-5159 was calculated. The degree of inhibition by the sonicates and components tested was compared with the inhibition obtained by adding known amounts of unlabeled test antigen. If the unlabeled antigen was identical with the labeled antigen, small amounts completely inhibited the I*Ag-antibody reaction. Unlabeled cross-reacting or unrelated antigens lacked part or all of such an inhibitory capacity. Details of this method have been described (9, 28).

RESULTS

Radio-gel Electrophoresis.—Circulating antibodies to ^{131}I-labeled crude antigen were demonstrated by the RGE test in rabbit anti-5159 antisera and in sera from BCG-immunized rhesus monkeys; examples of the reactions are shown in Fig. 3. When serum-I*Ag preparations from normal animals were tested by RGE, all the radioactivity migrated from the point of application toward the anode (A and C), some of it faster than albumin. However, when serum I*Ag preparations from immune rabbits or the BCG-protected monkeys were studied, the RGE test became positive, because some radioactivity migrated toward the cathode combined with γ-globulin (B and D), indicating binding of antigen by circulating antibody. Positive RGE reactions were specific and could be inhibited by prior incubation of antisera with unlabeled crude antigen (see E, Fig. 3), but not by unrelated proteins such as BSA. Also shown in Fig. 3 is a similar preparation (F) stained with amido black to indicate the natural migratory behavior of serum proteins under these conditions. Serum D was drawn from a BCG-protected animal 60 days after intratracheal challenge with virulent tubercle bacilli. This monkey was protected from clinical disease and when killed 30 days later had no evidence of active tuberculosis at necropsy. Binding of antigen to antibody was confirmed by other primary tests, such as radioimmunoelectrophoresis and radioimmunodiffusion.

Preparative Gradient Acrylamide Electrophoresis.—In order to purify the crude antigen, electrophoresis in and elution from a preparative gradient polyacrylamide column were undertaken. When the crude antigen was applied to

such a column, and the absorbance of samples eluted over a period of hours was examined, the elution curve consisted of an early, clearly defined peak, followed by another that was relatively broad and asymmetric (Fig. 4). When ^{131}I-labeled crude antigen was applied to such a column, the radioactivity eluted from the column was about 20–30% of the amount originally applied, and the first peak alone represented approximately 15%.

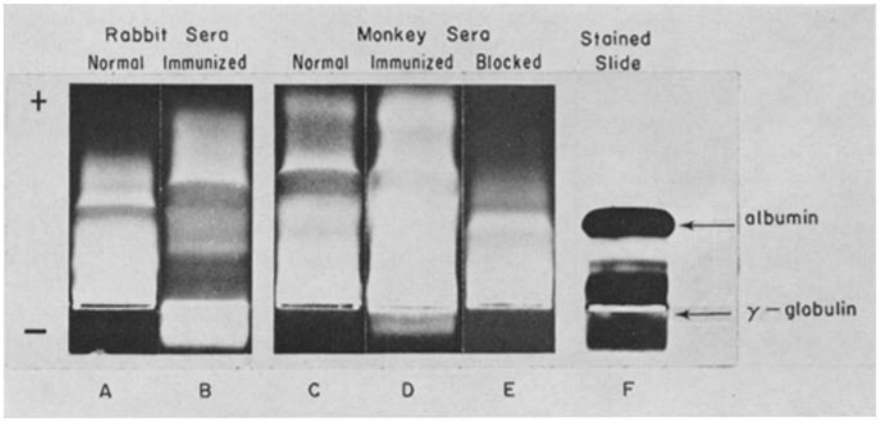

FIG. 3. Radio-gel electrophoresis films showing binding of ^{131}I-labeled crude Ag by rabbit and monkey antisera. Serum in I*Ag preparation A was from a normal rabbit; in B from a rabbit immunized with the 5159 sonicate; in C, from a normal monkey; in D, from a BCG-protected monkey 60 days after intratracheal challenge with virulent tubercle bacilli. Film E represents inhibition of the reaction shown in D, by prior incubation of monkey serum with unlabeled crude antigen. The RGE test is positive when some radioactivity migrates toward the cathode combined with γ-globulin as in B and D. Section F is a similar preparation after staining with amido black to indicate natural migratory behavior of serum proteins under these conditions.

When the first peak was pooled, concentrated, and labeled with ^{131}I, it was also found to have binding activity as measured by the RGE test. Even though it has not been established that material from this peak represents a homogeneous purified substance, it will be referred to throughout this report as "purified" Ag to distinguish it conveniently from the original crude antigen. Films from RGE preparations after this purified ^{131}I-labeled Ag had been incubated with serum from a normal and an immunized rabbit are shown in Fig. 5. In contrast with Fig. 3, where activity of the free crude material was spread from the point of application over the entire anodal area, most of the free or uncombined ^{131}I-labeled purified Ag migrated farther than albumin. Positive RGE reactions were inhibited by prior incubation of serum with trace amounts of unlabeled crude Ag as well as with unlabeled purified Ag.

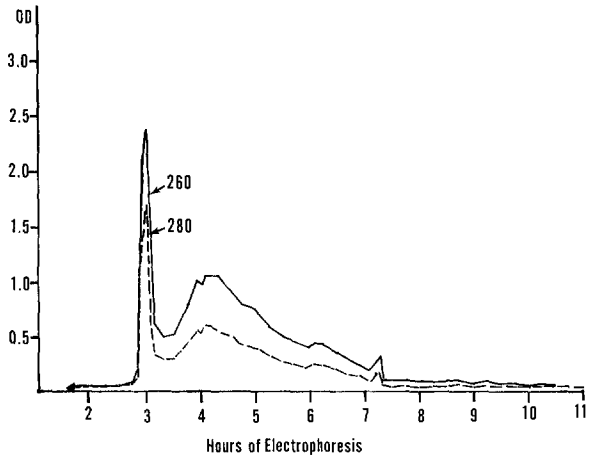

FIG. 4. Elution curve following electrophoresis from a preparative acrylamide column. The sample was crude antigen derived from *M. tuberculosis* strain 5159.

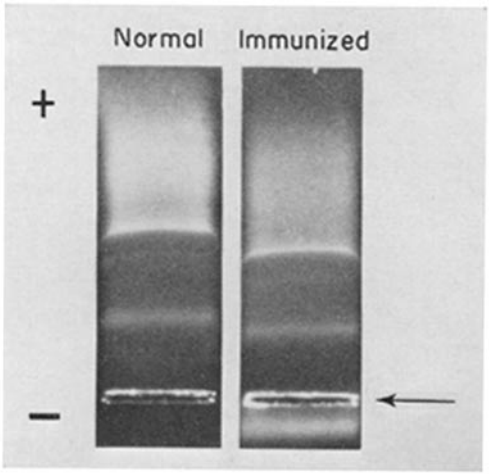

FIG. 5. RGE preparations after ^{131}I-labeled purified Ag was incubated in serum from a normal and an immunized rabbit. The majority of free or uncombined radioactivity migrates farther toward the anode than does albumin.

Binding of Labeled Antigen to the Fab Component of γ-Globulin.—Experiments were performed and established that the binding by the ^{131}I-labeled purified Ag to γ-globulin was a true immunological reaction. Such controls were necessary because of recent observations that protein A from *S. aureus* binds nonspecifically to the Fc fragment of γ-globulin rather than to the Fab or antigen-

combining portion thereby only simulating an antigen-antibody interaction (29, 30).

Enzymatic digestion of whole monkey and rabbit sera followed by immunoelectrophoresis was performed to separate and identify the Fc and Fab portions of the γ-globulin molecule. The standard method of papain digestion (31) was used, except that whole serum instead of purified γ-globulin was treated with papain (1:100, w/w) in 0.1 M phosphate buffer, pH 7.5. The cysteine concentration was 0.05 M, EDTA was 0.002 M, and digestion was carried out at 37°C for 2 hr. This modification allowed papain digestion to be carried out easily on numerous samples and small volumes (e.g. 0.5 ml) of test antisera. Using specific antisera, it was demonstrated by immunoelectrophoresis that separation of the Fab and Fc portions of γ-globulin had taken place. By radioimmunoelectrophoresis it was demonstrated that the I*Ag binding was confined to the precipitin band formed by the Fab portion of γ-globulin, and was not the same as the nonspecific I* protein A binding, which was limited to the precipitin band formed by the Fc portion.

Analytic Gradient Polyacrylamide Electrophoresis.—The crude and purified antigens were studied by the Wright method of analytic gradient polyacrylamide electrophoresis (see Fig. 1), and the gels were stained for protein, lipid, polysaccharides, and mucopolysaccharides. This method of analysis was employed because it has been shown that more bands are detected and there is enhanced resolution of bands as compared to the conventional method using a 7% gel[2].

When crude antigen was applied to such a column and electrophoresis carried out, at least 24 protein-staining bands were observed throughout the gel layers. Polysaccharides were identified in 12 bands throughout the gels; lipids, in four bands only in the 3.75% and 4.75% gels; and mucopolysaccharides were diffusely smeared through all the gels, but a portion also appeared as a clearly defined band at the anodal end of the column. When purified Ag was analyzed, there were one major and four minor protein-staining bands. There was one weak polysaccharide band, no reactions for lipid, and a prominent single band that stained for mucopolysaccharides. Some of the staining patterns for crude and purified Ag after analysis in acrylamide gels are illustrated in Fig. 6. When purified antigen was labeled with ^{131}I before electrophoresis and the gel was applied to sensitive X-ray film, most of the radioactivity appeared in the single major band that stained strongly for mucopolysaccharides.

Estimation of Molecular Weight of ^{131}I-Labeled Purified Ag.—The purified material was the fastest migrating portion of the crude antigen in the 12% polyacrylamide gel and was therefore expected to be of relatively low molecular weight. When it was applied to a Bio-Gel P-30 column, whose void volume, determined by a marker (blue dextran 2000, Pharmacia, Uppsala, Sweden), was 19 ml, the eluting volumes were 37 ml for cytochrome *c* and 42 ml for the major portion of radioactivity. When labeled purified Ag was filtered through

a filtration cell, a major portion was retained by a UM-10 membrane, which retains molecular weight substances greater than 10,000. Accordingly, the ^{131}I-labeled purified Ag was considered to lie in the molecular weight range between 9,000 and 12,000.

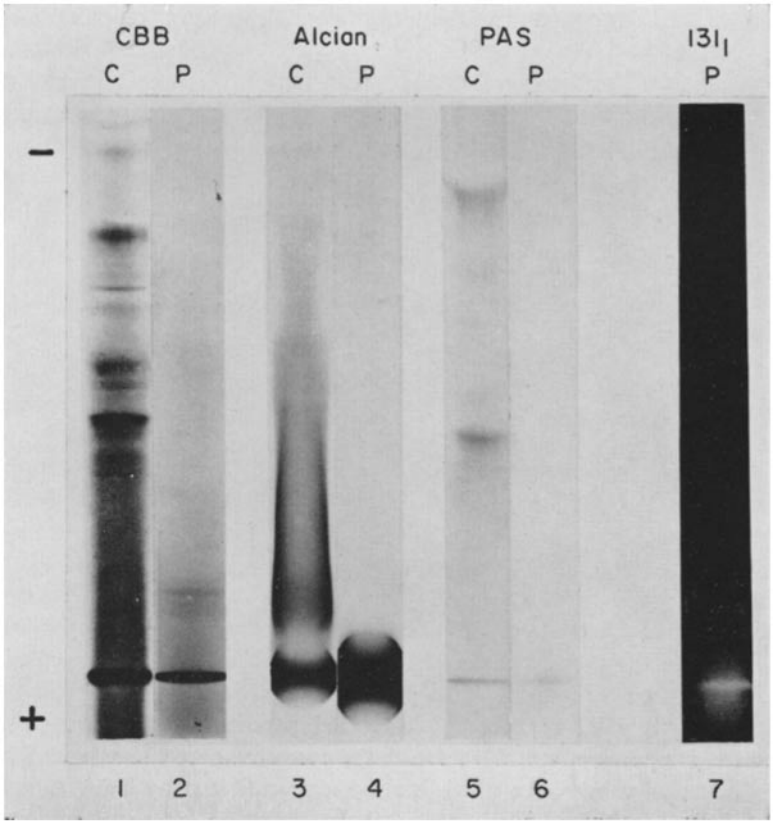

FIG. 6. Staining patterns of crude (C) and purified (P) Ag after analytic gradient polyacrylamide electrophoresis. Protein was stained with Coomassie brillant blue (CBB) (Nos. 1 and 2), mucopolysaccharides with alcian blue (Nos. 3 and 4), and polysaccharides with the periodic acid–Schiff stain (PAS) (Nos. 5 and 6). When ^{131}I– purified Ag was applied to the analytic column and the gel was exposed to X-ray film, radioactivity appeared in a single band (No. 7).

Precipitation of ^{131}I-Labeled Antigen-Antibody Complexes by SAS/2 and Anti-RGG.—Comparisons were made of the solubility characteristics of ^{131}I-labeled crude and purified antigens in SAS/2. Duplicate 0.5 ml aliquots of these antigens diluted in 1:100 normal rabbit serum (NRS) in borate buffer were added to equal volumes of 1:10 NRS which served as a carrier. NRS was obtained

from individual normal animals. After the addition of 1 ml SAS, the mixtures were incubated and centrifuged, and the resulting precipitates were resuspended in SAS/2 and centrifuged again. The percentage of radioactive counts originally added that remained in the precipitates was then determined. As seen in Table I, ^{131}I-labeled purified Ag proved to have suitable although not ideal characteristics for use in the ammonium sulfate test when the diluent was NRS from individual normal rabbits. However, when sera from individual normal monkeys or pooled serum from normal rabbits were used, the percentage of labeled antigen in the precipitates was 15.2–16.6%. For this reason, the ammonium sulfate test was not used to quantitate antigen binding by monkey sera. The possible significance of the increased binding of antigen by sera from individual normal monkeys and pooled NRS is discussed below.

TABLE I

Comparison of aolubility of ^{131}I-Labeled Antigen in SAS/2 and 10% Trichloroacetic Acid (TCA)

Precipitation by	I* crude Ag	I* purified Ag
	%	%
SAS/2 (rabbit serum)	52.6*	7.6*
		15.2‡
SAS/2 (monkey serum)		16.6
10% TCA	80–90	55–80

* Carrier was serum from individual normal rabbit.

‡ Carrier was pooled sera from normal rabbits.

Similar tests were made to determine precipitability of the antigens in 10% TCA, because the percentage of antigen bound to antiserum was calculated and corrected according to the TCA precipitability of the I*Ag. The use of this calculation was valid because the corrected percentage of untreated I*Ag bound to an antiserum was the same as the corrected percentage bound to the same antiserum using I*Ag which had been precipitated by TCA and resuspended in borate buffer. Furthermore, the supernatant after precipitation by TCA did not bind to antiserum either by the ammonium sulfate test or by RGE. The basis for expressing data according to TCA precipitability of I*Ag has been discussed (Table II in reference 9 and 28).

Effect of Enzyme Reactions with Antigen.—The following experiments were carried out to test the capacity of ^{131}I-labeled antigens to react with antibody after treatment with a number of enzymes. 3 ml aliquots of a Pronase solution in 0.15 M sodium phosphate buffer, pH 7.4, were added to 3 ml aliquots of labeled crude Ag, labeled purified Ag, and I*BSA. On a weight basis, Pronase was approximately 20% of the I*Ag. The digest was incubated at 37°C for 24 hr, then heated to 80°C for 10 min. Pronase-treated aliquots of I*Ag were added to aliquots of a 1:10 dilution of anti-5159 or anti-BSA, and the amount

of I*Ag bound to antiserum was determined by precipitation of ^{131}I-labeled antigen-antibody complexes by SAS/2 or anti-RGG. The percentages of I*Ag bound to 1:10 dilutions of anti-5159 or rabbit anti-BSA for the three antigens before and after Pronase treatment are presented in Table II. The binding capacities of ^{131}I-labeled antigens which had only been incubated and heated to 80°C but not treated with Pronase were lowered by about 10%. These data suggest that the major portion of the binding activity of the three labeled antigens was Pronase-sensitive.

The absorbance of purified Ag as eluted from a preparative acrylamide column was higher at 260 mμ than at 280 mμ, and the effect of DNase and RNase on the binding of the labeled antigens was tested because of the possibility that nucleic acids or nucleotides contributed to the binding properties of the antigens. DNase and RNase, 100 μg each in a total of 0.2 ml, were added to 4.3 ml

TABLE II

Comparison of Enzyme-Treated and Untreated ^{131}I-Labeled Antigens

Treatment	Radioactivity after precipitation of I* antigen-antibody complexes		
	I* crude Ag	I* purified Ag	I* BSA
	%	%	%
Before Pronase	57.3	37.9	97.4
After Pronase	12.4	3.5	16.6
Before DNase and RNase	60.5	42.0	96.1
After DNase and RNase	61.5	41.9	95.5

of the above labeled antigens and incubated in the presence of 0.5 ml magnesium chloride so that the final concentration of the latter was 0.01 M. Antigen-enzyme mixtures were incubated at 37°C for 1 hr, then dialyzed in distilled water at 4°C. When the effect on binding of antigen to antiserum was tested, no changes were noted for the DNase- and RNase-treated antigens, suggesting that DNase- and RNase-sensitive components did not contribute to the binding by untreated labeled antigens. DNA and RNA were exposed to DNase and RNase under similar conditions to verify the activity of these enzymes.

Inhibition of the ^{131}I-Labeled Purified Ag:Anti-5159 Reaction by Unlabeled Microorganisms and Mycobacterial Components.—Inhibition studies of the labeled purified Ag:anti-5159 reaction as measured by the ammonium sulfate test (9, 28) were employed to evaluate antigenic similarities and differences among unlabeled microorganisms and mycobacterial components. The rabbit anti-5159 sera used as the reagent bound 1.3–2.2 μg N of labeled purified Ag/ml (ABC-33 values) and were diluted to bind only 40–50% of 0.02 μg N of the labeled test Ag. Comparisons were made of the inhibitory effects of progressive increments of unlabeled crude and purified antigens on the ^{131}I–purified Ag:

anti-5159 reaction. As illustrated in Fig. 7, the reaction was inhibited by the same amount of unlabeled crude Ag as by purified Ag when antigen concentrations were based on nitrogen content. However, since the purified antigen had

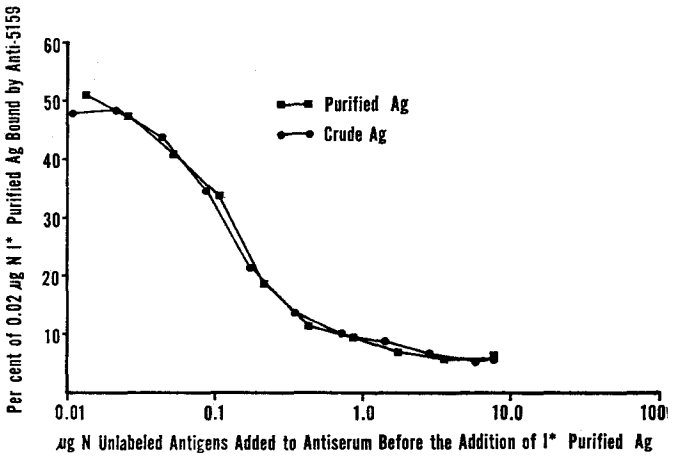

FIG. 7. Inhibition of ^{131}I-labeled purified Ag:anti-5159 reaction by unlabeled purified Ag and crude Ag. Inhibition by each is the same when compared on a nitrogen basis.

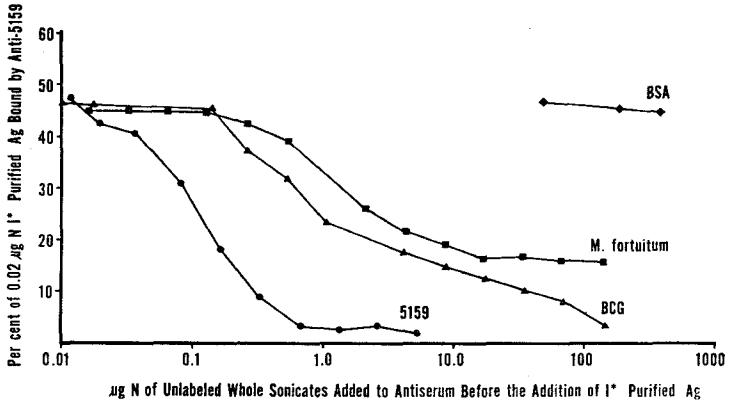

FIG. 8. Inhibition of ^{131}I-labeled purified Ag:anti-5159 reaction by unlabeled sonicates of 5159 strain of *M. tuberculosis*, of BCG, and of *M. fortuitum*. BSA failed to inhibit the reaction.

a higher nitrogen content per unit weight than did the crude antigen, a greater total amount of crude than purified Ag was required to achieve the same inhibition.

The inhibitory effect of unlabeled sonicates of two other mycobacteria, BCG and *M. fortuitum*, was compared with that of crude 5159 Ag. As seen in Fig. 8,

0.7 μg N of unlabeled crude Ag completely inhibited the reaction, but 270 times this amount (190 μg N) of BCG was needed to achieve comparable inhibition, and the *M. fortuitum* sonicate did not completely inhibit the reaction even when 190 μg N was added. Both BCG and *M. fortuitum* showed partial inhibition at lesser concentrations. BSA failed to inhibit the reaction at any concentration.

Five other microorganisms were sonified and similarly tested. As seen in Fig. 9, *E. coli* and *S. dysenteriae* SH, two Gram-negative enteric bacteria, partially inhibited the reaction when added in large amounts. *P. putida*, a Gram-negative fluorescent pseudomonad, also partially inhibited the reaction, and *B. subtilis* 168, a Gram-positive spore-forming bacillus, had the strongest inhibitory capacity of those tested. The shape of the inhibition curves suggests that some deter-

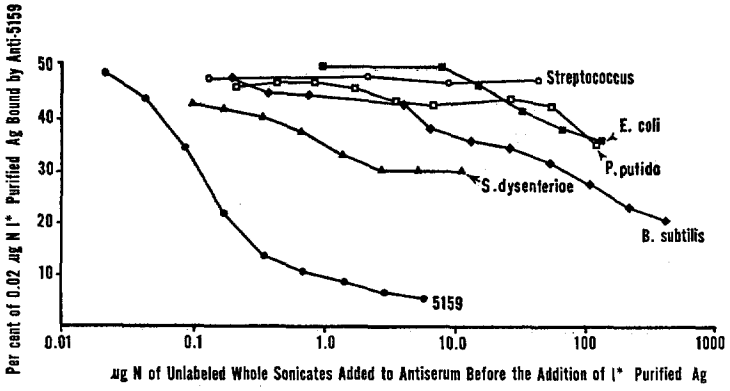

FIG. 9. Inhibition of ^{131}I– purified Ag:anti-5159 by unlabeled sonicates of nonmycobacterial microorganisms. Partial inhibition was noted with sonicates of *E. coli*, *P. putida*, *B. subtilis*, and *S. dysenteriae*. None was noted with a sonicate of streptococcus.

minants associated with these organisms were capable of binding some antibody populations in anti-5159. However, inhibition was not complete, reflecting the known significant antigenic differences. The streptococcus, also Gram-positive, was the only organism tested that failed to cause any inhibition. Similarly, the culture medium in which the bacteria were grown caused no inhibition.

As seen in Fig. 10, the inhibitory capacity of purified Ag derived from 5159 was compared with other mycobacterial components kindly provided by other investigators. NT, a protein component derived from old tuberculin, produced partial but not complete inhibition. TAP prepared from the Aoyama B strain of mycobacteria closely resembled purified Ag when small amounts were added, but with increasing concentrations there was only partial inhibition. AG, which is primarily a polysaccharide antigen from Aoyama B organisms, produced slight inhibition at a concentration of 2 mg. If data for inhibition by the AG component were expressed on a weight basis, the slight inhibition observed

could be accounted for by nitrogen, which was merely a trace constituent (less than 0.5%) of the whole component. Not shown in this figure, PLP, a phospholipid component which had been prepared from BCG organisms, caused no inhibition when as much as 1.3 mg was added. As expected, relatively crude commercially prepared PPD produced almost complete inhibition when excess amounts were added. None of the components tested was identical with the ^{131}I-labeled purified Ag.

Circulating Antibodies in Sera from BCG-Vaccinated Monkeys.—Sera from nine monkeys that were intravenously vaccinated with BCG and later chal-

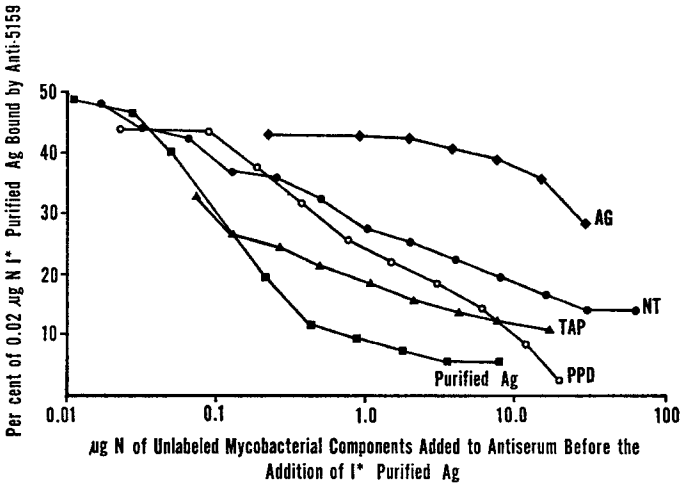

FIG. 10. Inhibition of ^{131}I–purified Ag:anti-5159 reaction by unlabeled mycobacteria components. AG is primarily a polysaccharide component, and TAP is a protein derivative of human tubercle bacilli, Aoyama B strain. NT is a component derived from old tuberculin.

lenged with live 5159 organisms were studied by the RGE test, using ^{131}I–purified Ag. Sera were obtained before vaccination and at periodic intervals before and after challenge. As seen in Table III, sera from six animals had positive reactions 77 days after vaccination and before challenge, and RGE tests following challenge appeared to indicate an enhanced antibody response. Sera from the remaining animals had positive RGE reactions after challenge. RGE tests performed on consecutive bleedings from one monkey are illustrated in Fig. 11. A positive reaction was seen at day 77, which was before challenge, and in sera from all subsequent bleedings. Two animals failed to show antibodies until 173 days after vaccination or 90 days after challenge, suggesting that the 5159 challenge was the stimulus for the primary production of circulating antibody. However, this question cannot be answered at present, because these sera were obtained from monkeys that were used in an unrelated experiment in which the

TABLE III

Circulating Antibodies in Sera from BCG-Vaccinated Monkeys Determined by RGE Test

	Vaccinated BCG				Challenged 5159				
Bleeding No.	1	2	3	4	5	6	7	8	9
Days before and after vaccination	−7	21	36	49	77	97	118	146	173
Days after challenge					−6	14	35	63	90
Monkey	*RGE reactions*								
1	−	*	−	−	+	+	+	+	+
2	*	−	*	−	+	+	+	+	+
3	−	−	−	−	+	+	+	+	+
4	−	−	−	−	−	−	−	−	+
5	−	−	−	−	+	+	+	+	+
6	−	−	−	−	+	*	+	+	+
7	−	−	−	−	−	−	+	+	+
8	−	−	−	−	+	+	+	+	+
9	−	−	−	−	−	−	−	−	+

* Not done.

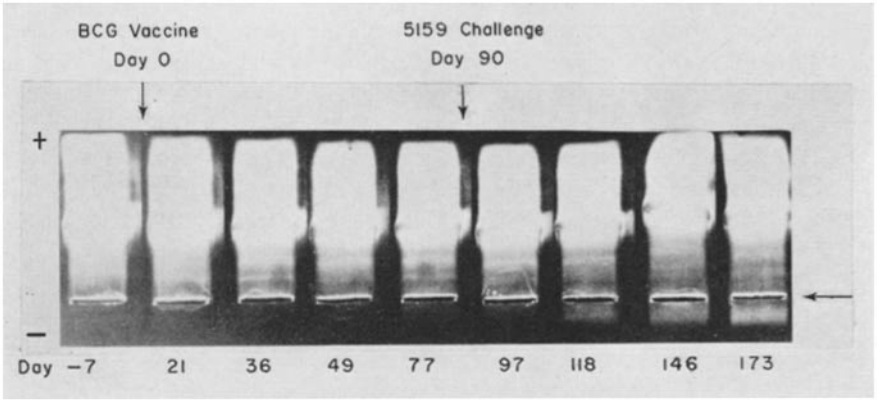

FIG. 11. RGE films made from consecutive bleedings from an individual monkey. Sera were obtained before and after BCG vaccination and before and after challenge with live 5159 organisms. A positive reaction is seen 77 days after BCG vaccination and before the 5159 challenge. Reactions from subsequent bleedings show enhancement of circulating antibody response.

protocol did not require BCG-protected animals that were not subsequently challenged with live organisms. All nine BCG-protected animals were reported to have normal chest roentgenograms during this entire period and to be free of disease when examined at necropsy 90 days after challenge. None of the sera from control animals which were not BCG-protected but which were challenged with virulent organisms had a positive RGE test. Three control animals died

of disease within 30 days of challenge; 5 died within 60 days; and one animal, when killed 90 days after challenge, had extensive tuberculous disease at necropsy. It was not clear whether sera from these animals would have demonstrated humoral antibodies had they survived longer periods of time.

DISCUSSION

Many constituents of the tubercle bacillus have been isolated and studied in a variety of ways. Most previous studies have focused on the search for components having immunogenic activity, and some were found that would react with circulating antibodies and/or elicit delayed tuberculin-type skin sensitivity (32–35). Because of the complex nature of this organism, many fractionation schemes have been devised. Intact cells and culture filtrates have been subjected to chemical fractionation (36–39), and some components have been isolated by a variety of methods including enzyme digestion, electrophoresis, gel filtration, and chromatography (40–48). As a result, many fractions have been isolated and partially defined with respect to physical structure and biological properties, but none has been applied to development of a primary test to detect and measure humoral antibodies.

The present studies were undertaken to find a substance or substances that could be used in primary binding types of tests that would be expected to distinguish between different strains of mycobacteria. It was preferable for this purpose to have a soluble rather than a particulate antigen, which, in addition, would be amenable to further analysis and fractionation. Accordingly, whole washed tubercle bacilli were fractionated by the use of sonic energy. Because of the complexity of the structure of the tubercle bacillus, sonification was carried out for 60 min, which is longer than required to disrupt most microorganisms (49). Although considerable destruction of organisms occurred, portions of the sonicate remained insoluble and were separated from the supernatant by ultracentrifugation. This soluble supernatant, consisting of many potential antigenic substances, was shown to be a mixture of proteins, polysaccharides, lipids, and mucopolysaccharides of varying electrophoretic mobilities and molecular sizes.

When this crude material was further purified, a fraction was isolated that bound antibodies in antisera from immunized rabbits and rhesus monkeys. Binding by this "purified" component was inhibited after it was treated with Pronase, indicating that it was susceptible to proteolysis. When it was analyzed by polyacrylamide gradient electrophoresis, one major and three minor protein-staining bands, a single faint polysaccharide band, and a prominent mucopolysaccharide band were identified. When the purified antigen was labeled with ^{131}I, most of the radioactivity was confined to the single mucopolysaccharide band, which was also the major protein-staining band (see Fig. 6). Other properties were solubility in SAS/2, molecular weight in the 9,000–12,000 range, and migration by electrophoresis at pH 8.3 that was more anodal than albumin.

These findings strongly suggested that the major antigenic determinants isolated in ^{131}I-labeled purified Ag were associated with anionic mucopolysaccharides, and studies are in progress to define their molecular structure further.

RGE, which was used throughout these experiments to detect circulating antibodies, is a primary test that has been shown to be sensitive, specific, and reproducible (4). When sera from rhesus monkeys were tested using ^{131}I-purified Ag, BCG-protected animals had circulating antibody and normal animals did not. As discussed above, the available information does not permit proper evaluation of these antibodies. Although RGE was a valuable screening type of qualitative test, quantitative procedures would be of greater value because they could be used to compare the *amount* of antigen bound by antisera obtained at serial bleedings, especially in human disease.

The ammonium sulfate test was used for this purpose for rabbit antisera, but as presently carried out could not be applied to measure binding by monkey sera, because of the relatively large amounts of antigen bound by sera from normal monkeys. Reasons for this "nonspecific" type of binding are not clear, but it may possibly be "specific" and result from shared antigenic groups associated with 5159 organisms and components of other organisms (Fig. 9) to which rhesus monkeys have been exposed.

Inhibition curves as shown in Figs. 7–10 reflected some of the immunological similarities and differences known to exist among microorganisms. For example, sonicates of BCG and *M. fortuitum* contained antigenic determinants capable of binding some populations of anti-5159 antibodies which have the capacity to bind ^{131}I-purified Ag (Fig. 8). However, *M. fortuitum* appeared to be completely lacking in at least some determinants present in ^{131}I-purified Ag, because there was a plateau in the inhibition curve when more than 20 μg N of the sonicate was added to the system prior to the addition of ^{131}I-purified Ag. BCG sonicates had antigenic groups similar to those in *M. fortuitum*, but also contained groups related to ^{131}I-purified Ag that were not present in sonicates of *M. fortuitum*. The flat slope of the BCG inhibition curve between 20 and 200 μg N of unlabeled BCG sonicate suggested "cross-reactivity" rather than identity between antigens in BCG and those in the 5159 sonicate. The possibility that BCG contained trace amounts (0.05%) of antigenic groups identical with those in ^{131}I-purified Ag was not excluded.

The plateau observed in the *M. fortuitum* curve (Fig. 8) indicated heterogeneity of antigenic determinants in ^{131}I-purified Ag capable of reacting with anti-5159. Had the latter been homogeneous, unlabeled *M. fortuitum* molecules that were capable of inhibiting binding to 18% would have inhibited the reaction completely, as did the 5159 sonicate. Heterogeneity of this component was again illustrated in Fig. 10, where the curves suggested anti-5159 antibodies capable of reacting with at least two determinants in ^{131}I-purified Ag, but with only one of these determinants present in TAP.

Inhibition studies also showed (Fig. 9) that the labeled purified Ag: anti-5159

reaction was partially inhibited by sonicates from a variety of microorganisms not closely related to mycobacteria, suggesting that shared antigenic groups exist in purified Ag derived from 5159 and in other, nonmycobacterial organisms. It is interesting to speculate whether shared antigenic groups may account in part for reports of enhanced resistance to tuberculosis in experimental animals following vaccination with nonmycobacterial organisms such as *Hemophilus pertussis* and *Brucella abortus* (50, 51). Recently microbicidal activity of peritoneal macrophages for *Listeria monocytogenes* and *Salmonella typhimurium*, associated with morphological changes of macrophages, has been demonstrated in BCG-vaccinated mice (52). The possibility that this phenomenon may be influenced by immunological responses against antigenic components shared by all these organisms should be considered.

The inhibition experiments demonstrated a capacity to define subtle similarities and differences among mycobacteria, mycobacterial components, and other microorganisms. Additional studies of the inhibitory effects of appropriate microorganisms to differentiate antigenic groups between pathogenic and nonpathogenic mycobacteria and between individual pathogens are in progress. Similarly, direct primary binding tests with ^{131}I-labeled purified Ag derived from 5159 organisms suggest the advisability of isolating comparable antigens from other mycobacteria pathogenic to man, such as H37Rv, to be compared with components from nonpathogenic mycobacteria such as H37Ra and BCG.

SUMMARY

Studies were undertaken to find a substance or substances for use in primary binding types of tests to detect humoral antibodies in rabbits and monkeys exposed to the tubercle bacillus that would distinguish between strains of mycobacteria. The antigen employed was a component of the 5159 strain of *Mycobacterium tuberculosis* that was obtained following sonification, ultracentrifugation, electrophoresis, and elution from a preparative polyacrylamide column. When the antigen was labeled with ^{131}I, specific binding was observed in sera from immunized rabbits and BCG-protected rhesus monkeys by the radio-gel electrophoresis and ammonium sulfate tests.

This component was partially characterized, and its major antigenic determinants were associated with anionic mucopolysaccharides. Electrophoretically at pH 8.3 it migrated anodally to albumin, its molecular weight was between 9,000 and 12,000, and it was soluble in 50% saturated ammonium sulfate. Binding to antibody was destroyed after treatment with Pronase, but not after DNase or RNase.

Inhibition of the reaction, as measured by the ammonium sulfate test, between the ^{131}I-labeled component and antisera from rabbits that had been immunized with sonicated 5159 organisms, was studied. These experiments demonstrated a capacity to define subtle similarities and differences among different

mycobacteria and mycobacterial components. Some microorganisms not clearly related to mycobacteria also partially inhibited this reaction, suggesting that they shared antigenic groups with the component derived from 5159 organisms. The studies described suggested the advisability of using direct primary tests and purified components of mycobacteria to differentiate further the antigenic groups between individual pathogenic mycobacteria and between pathogenic and nonpathogenic organisms.

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