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Antigenic Differences Between Extracts of Actively Replicating and Synchronized Resting Cells of *Mycobacterium tuberculosis*

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Protein extracts were prepared from aerobically replicating cells of *Mycobacterium tuberculosis* and from synchronized non-replicating cells derived from the sediments of non-agitated cultures. Although both preparations share a number of antigens, the extracts of the non-replicating cells also contain antigenic components that are not shared by the replicating cells, and which can be isolated and visualized by immunoaffinity chromatography and immunoelectrophoretic techniques.

It was recently demonstrated that tubercle bacilli that settle slowly through the oxygen gradient of an unagitated culture stop replicating but do not lose their viablity during several weeks of residence in the accumulated sediment (20). During this process the bacilli in the supernatant medium continue to replicate logarithmically; the generation rate decreases from that of a well aerated culture until it is balanced by the settling rate, establishing a fairly constant supernatant population density. When the bacilli that have accumulated in the sediment are resuspended in fresh medium and diluted sufficiently to assure adequate aeration, they exhibit synchronized replication after an 8-h lag period during which ribonucleic acid, but not deoxyribonucleic acid, is synthesized (21). Inasmuch as actively replicating tubercle bacilli that are subjected suddenly to an oxygen depletion rapidly lose viability (22), the fact that the organisms that had settled slowly through the gradient remained viable suggested that some induction processes were involved. It was speculated that these might be reflected in qualitative differences in antigenic composition between actively replicating *Mycobacterium tuberculosis* and the synchronized resting cells (21). Experiments to be reported here demonstrate that antigens do exist in synchronized resting cells, which cannot be demonstrated in extracts of actively replicating tubercle bacilli.

MATERIALS AND METHODS

Cultivation of bacilli and preparation of crude antigens. *M. tuberculosis* H37Rv was used for all studies. Four different media were used.

Dialyzed broth with glucose and Tween 80 (DYGUT) was prepared by dissolving 24 g of TB broth base (Difco) in 60 ml of water with heat. The concentrated solution was dialyzed at 70°C against 2,000 ml of water for 18 h. Since the dialysis tubing retained the Tween 80 in the basal medium, it was necessary to reconstitute the dialysate by adding 10 ml of a 10% stock solution of the detergent to yield a final concentration of 0.5 mg/ml. The dialysate was distributed in 200-ml amounts to 10 tall, square, screw-capped bottles (60 mm by 60 mm by 170 mm) and autoclaved at 121°C for 15 min. Upon cooling, each bottle of medium was supplemented with 3 ml of sterile 50% glucose solution (final concentration, 7.5 mg/ml).

Dialyzed broth with glycerol and Tween 80 was prepared in the same way as dialyzed broth with glucose and Tween 80 except that glucose was omitted and 10 g of glycerol was added per 200 ml of medium, which was autoclaved in 500-ml baffled nephelo flasks (Bellco) instead of bottles.

Dubos Tween albumin broth was prepared from Dubos broth base (Difco) supplemented with one-tenth volume of Dubos medium albumin (Difco) according to the manufacturer's instructions. The final medium contained 50 mg of albumin fraction V per ml, 0.2 mg of Tween 80 per ml, and 7.5 mg of glucose per ml, and was distributed in 200-ml amounts to the tall, square, screw-capped bottles.

Dubos broth with glycerol consisted of Dubos Tween albumin broth supplemented with glycerol to a final concentration of 10 mg/ml. It was distributed in 200-ml amounts to the baffled nephelo flasks.

The flasks and bottles of medium were each inoculated with 2.5×10^8 to 5×10^8 viable bacilli from resting unagitated cultures of *M. tuberculosis* H37Rv grown in dialyzed broth with glucose and Tween 80.

The glycerol-free cultures (i.e., dialyzed broth with glucose and Tween 80, or Dubos Tween albumin broth) in bottles were used to prepare the resting sedimentary bacilli. They were incubated at 37°C,

mixed once after 3 days, and reincubated without any further agitation for 19 days before harvesting of the resting bacilli.

The glycerol-enriched media (i.e., dialyzed broth with glycerol and Tween 80, or Dubos broth with glycerol) in flasks were used to prepare actively replicating cells. These cultures were incubated at 37°C with no agitation other than a single daily manual shake, until optical density measurements at 580 mm indicated a bacillary concentration of about 5×10^7 per ml. They were then agitated continuously on a rotary incubator-shaker (Psychrotherm, New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C and 120 rpm until the bacillary concentration had increased about 10-fold, and were harvested as actively growing logarithmic phase cells.

The supernatant culture medium from the non-agitated cultures was carefully aspirated to avoid disturbing the resting cells in the sediments. These sedimented bacilli were suspended in load buffer [0.005 M tris(hydroxymethyl)aminomethane-phosphate, pH 8.0) and harvested by centrifugation at $4,300 \times g$. On the other hand, the entire contents of the agitated culture flasks (actively replicating cells) were centrifuged at $4,300 \times g$. Thereafter, both types of cells were treated in the same manner.

The centrifuged cells were separated from supernatant fluids, resuspended in fresh load buffer, and centrifuged again at $4,300 \times g$. The cells were then removed and suspended in fresh load buffer containing 0.01% Tween 80, transferred to calibrated centrifuge tubes, and centrifuged at $850 \times g$. The volume of packed cells was noted, and the wash was discarded. The cells were suspended in an equal volume of plain load buffer and disrupted by sonic treatment at 0°C as described previously (9).

Sonic extracts were added to a suspension of diethylaminoethyl (DEAE)-cellulose (Sigma Chemical Co., St. Louis, Mo.), containing 250 mg of DEAE for each milliliter of packed bacilli as measured before sonic treatment, in 100 ml of cold load buffer. After mixing, the DEAE with adsorbed bacillary proteins was permitted to settle at 5°C. The cloudy, lipid-rich supernatant was discarded. The DEAE was washed and permitted to settle several times in fresh, cold load buffer until the supernatant was only slightly opalescent. The DEAE was then resuspended in load buffer, transferred to a chromatographic column, and allowed to settle. The packed column was washed with load buffer until the effluent was clear; the washes were discarded. The bound protein was eluted with 0.5 M NaCl buffer (equal portions of load buffer and 1 M NaCl in 0.05 M phosphate, pH 6.5), using 30 ml of the buffer mixture per gram of DEAE. The eluate was sterilized by membrane filtration (Millipore Corp.).

These crude protein extracts were assayed for protein by the method of Lowry et al. as described by Layne (16), and dialyzed against appropriate buffers for immunoelectrophoresis and for immunization of rabbits.

Immunization of rabbits. Male New Zealand white rabbits were used throughout. Immunization was achieved by using Freund complete adjuvant in which the whole cells of *M. tuberculosis* used were derived from the same cultures as were used to prepare a given cell sonic extract antigen. A portion of washed packed bacilli was suspended in 10 volumes of distilled water and steamed for 20 min. After centrifugation, the supernatant was discarded and the cells were resuspended in water and steamed again for 20 min. The cells were again separated by centrifugation and resuspended in water to yield a suspension corresponding to 15 mg/ml (dry weight). A 6-ml volume of the crude soluble protein antigen (1 mg of protein per ml of phosphate-buffered saline) was mixed with 6 ml of incomplete Freund adjuvant (Difco, Detroit, Mich.) and 0.4 ml of homologous whole cell adjuvant suspension and converted to a homogeneous paste by repeated flushing through a 20-gauge needle. The initial inoculum consisted of 1.0 ml into each of four subcutaneous sites and 0.1 ml into each of two intradermal sites. Animals were boosted after the first 5 weeks with 1 ml in each of two subcutaneous sites, and thereafter at 4- to 5-week intervals with 0.1 ml intravenously and 0.4 ml subcutaneously. Working samples of serum were derived from blood drawn 1 week after each boost.

Immunoaffinity chromatography. The procedure described is for 1.0 ml of serum. Larger columns were prepared in the same manner, with all amounts proportionately increased.

Immune serum was rendered 50% saturated in ammonium sulfate and stored overnight at 5°C. The suspension was centrifuged at $1,100 \times g$, and supernatant fluid was discarded. The precipitated globulin was dissolved in sufficient bicarbonate buffer (0.1 M $NaHCO_3$ in 0.5 M NaCl) to bring the volume back to original 1.0 ml, and dialyzed against the same buffer. The globulin was coupled to CNBr-activated Sepharose 4B (Pharmacia) by the manufacturer's instructions. On a Buchner funnel, 0.5 g of activated gel was swollen and washed with 100 ml of 10^{-3} M HCl. The gel was transferred to a screw-capped test tube, mixed with 1.0 ml of the globulin solution and 1.25 ml of bicarbonate buffer, and incubated overnight on a rocker at 5°C. The mixture was allowed to settle, and the supernatant fluid was removed. The remaining slurry was resuspended in 7.5 ml of 0.1 M glycine and allowed to stand at 5°C for 1 h. The suspension was then filtered on a Buchner funnel and washed with 5.0 ml of bicarbonate buffer. Non-covalently adsorbed protein was removed by three wash cycles, each cycle consisting of 5.0 ml of 0.1 M acetate buffer in 1 M NaCl (pH 4) and 5.0 ml of 0.1 M borate buffer in 1 M NaCl (pH 8). The Sepharose-globulin immunoabsorbent was suspended in phosphate-buffered saline (0.01 M sodium phosphate buffer [pH 7.5] in 0.9% NaCl), and the slurry was poured into a 1.0-cm-diameter column. The column was stored in 0.1% NaN_3 at 4°C.

Antigen was loaded onto the column equilibrated with phosphate-buffered saline. Unbound protein was immediately washed out with phosphate-buffered saline. Non-specifically bound products were eluted with a wash of 2 M NaCl in phosphate buffer (pH 7.2) (23). Bound antigens were recovered with a wash of 3 M NaSCN. The eluates in high-salt solutions were desalted on columns of Sephadex G-25 M.

Immunoelectrophoretic methods. All electrophoresis was performed on a basic electrophoresis unit (LKB 2117 Multiphor), according to published proce-

dures (3). Barbital buffer (pH 8.6) (buffer salt mixture, type B-2; Harleco, Philadelphia, Pa.) was supplemented with calcium lactate (0.4 g/liter), Trizma base (Sigma) (44.3 g/liter) and sodium azide, 1.0 g/liter). This was diluted 1:5 for use throughout the electrophoretic experiments, including preparation of agarose and all other reagents (13).

Glass plates (70 by 100 by 1.5 mm; Bio-Rad) were cleaned and precoated with 0.1% agarose (agarose powder, Bio-Rad). Gels were prepared by pouring 0.155 ml of 1.0% agarose per cm^2 onto coated glass plates placed on a leveling table.

Following are the categories of immunoelectrophoresis (IEP) used in this work (3). (i) Rocket. The plate was poured with serum incorporated in the agar. Antigen was placed in the wells, and IEP was carried out in one direction, allowing the antigen to migrate through the serum-containing agarose.

(ii) Crossed. This technique employs electrophoresis in two directions. Antigen was placed in a well 2.0 cm from the bottom and side of the plate. First the antigen was subjected to electrophoresis across plain buffered agarose along the width of the plate. The unused agarose was cut away starting 2.5 cm from the base of the plate and replaced with serum agarose. Second-dimension IEP along the length of the plate then allowed the separated proteins to migrate into the serum agarose.

(iii) Line. Antigen was incorporated in a 0.5-cm strip of buffered agarose across the width of the plate, 2.5 cm from the bottom. The first 2.5 cm was filled with plain buffered agarose, and the remaining 7.0 cm was filled with homologous serum agarose. IEP was carried out in one direction, allowing the antigen to migrate as a line through the serum-containing agarose.

(iv) Combined line-and-rocket. The plate was prepared as for line IEP; reference antigen was incorporated in a strip of buffered agarose, and homologous serum agarose was poured above the line. Test antigens were placed in wells below the antigen line. IEP was carried out in one direction.

After electrophoresis, the gels were pressed, washed in saline and distilled water, pressed again, and dried at 60°C. Plates were stained in Crocein scarlet by the method of Crowle and Cline (4).

Samples of the United States-Japan cooperative Medical Sciences Program reference antigens MARS 003 (*M. tuberculosis* culture filtrate) and MARS 004 (*M. tuberculosis* cell extract) were provided by the Trudeau Institute, Saranac Lake, N.Y. (7, 14).

RESULTS

Yields of *M. tuberculosis* averaged 0.24 ml (packed cell volume) per 200 ml of medium for the unagitated sedimented resting bacilli and 1.2 ml per 200 ml of medium for the agitated growing bacilli. The resting cells yielded an average of 9.2 mg of crude protein per ml of packed cells, and the actively replicating cells yielded 8.1 mg of crude protein per ml of packed cells.

On testing both protein from resting bacilli (PRB) and protein from aerobically growing bacilli (PAG) samples against the homologous and heterologous antisera (i.e., SRB and SAG) by combined line-and-rocket IEP, a number of common precipitin lines were observed (Fig. 1 and 2). However, since we were not interested in antigens common to both protein extracts, no attempt was made to determine the total number of antigenic components of each product. Instead, the two protein extracts were passed through affinity columns prepared from the respective heterologous antisera to remove common antigens. When this was done, it was seen that the resting cell extract, PRB, retained antigenic components after absorption of common antigens on the heterologous SAG column, but the converse was not true, i.e., absorption of PAG on the SRB column removed all antigens detectable by either serum. Thus, both actively growing and resting cells shared a number of common antigens, but the resting cells yielded additional antigenic components which appeared unique to those cells.

Further studies were directed toward isolating and testing antigens that appeared to be unique to resting bacilli (URB). The flow sheet for production of URB is presented in Fig. 3. Briefly, rabbits were immunized (step 1) with PRB or (step 2) with PAG. The serum from PAG-im-

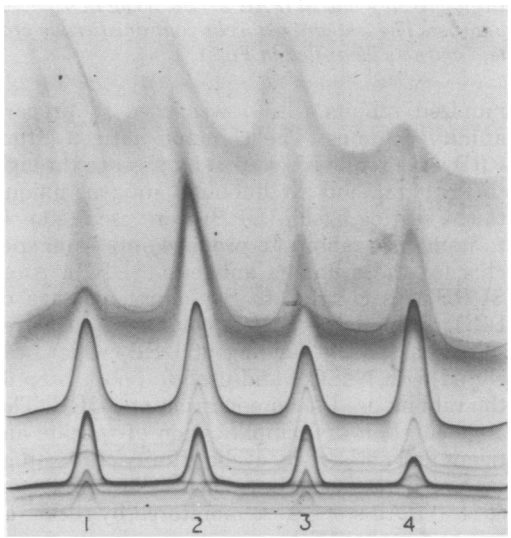

FIG. 1. *Combined line-and-rocket IEP of extracts of M. tuberculosis against a reference system derived from resting bacilli. The upper portion of the slide contained antiserum (SRB 73-196/204) raised against crude protein from resting bacilli, 0.053 ml of serum per ml of agarose. The line antigen trough contained homologous crude protein from resting bacilli (PRB 39/14), 150 μg in 0.5 ml of agarose. The test antigen wells contained crude protein: from actively growing bacilli—(well 1) AG 6/29 (10 μg), and (well 3) AG 16/6 (10 μg); from resting bacilli— (well 2) RB 12/17 (10 μg), and (well 4) RB 20/19 (4.8 μg).*

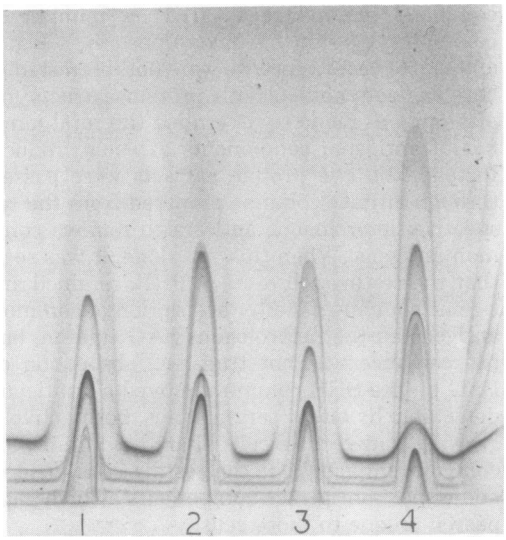

FIG. 2. *Combined line-and-rocket IEP of extracts of M. tuberculosis against a reference system derived from actively growing bacilli. The upper portion of the slide contained antiserum (SAG 81-166) raised against crude protein from actively growing bacilli, 0.053 ml of serum per ml of agarose. The line antigen trough contained homologous crude protein from actively growing bacilli (PAG 27/26), 30 μg in 0.5 ml of agarose. The test antigen wells contained crude protein extracts identified in Fig. 1.*

munized rabbits (SAG) was used to prepare affinity columns. The common antigens from PRB were removed (step 3) by passage through this column, and the unbound antigens unique to the resting bacilli (URB) were used (step 4) to immunize rabbits to produce antiserum specific for these unique antigens (SURB). After SURB was coupled to Sepharose, passage of PRB through the SURB column (step 5) resulted in specific binding of URB, which was eluted with NaSCN and used to boost (step 6) the rabbits used for preparation of SURB. The augmentation and amplification of specific antigens and antisera by cyclic affinity chromatography have been discussed in detail by Anderson et al. (1). All steps were monitored by rocket or line-and-rocket IEP.

The SAG-Sepharose column used to bind common antigens in the initial studies consisted of globulin derived from 12 ml of serum raised against protein from actively growing bacilli, coupled to 6 g of BrCN-Sepharose. This column was capable of removing the common antigen from 1.28 mg of crude PRB.

The unbound URB derived from a portion of one batch of PRB (PRB 39/14) was employed as the line antigen and as one of the test antigens in a combined line-and-rocket IEP, using antiserum against whole crude PRB (Fig. 4). The other test antigens included two concentrations of the untreated resting bacillus product (PRB 39/14), a second batch of resting bacillus protein (PRB 12/17), and two different batches of protein from actively growing bacilli (PAG 27/26 and PAG 16/16). The reference URB precipitin line formed approximately 7.5 mm above the load trough. The homologous URB produced a marked deflection of the line and only faint traces of additional rockets near the trough. The crude PRB preparations also caused steep deflections of the line, as well as a number of rockets associated with common antigens. The two PAG preparations, on the other hand, yielded only the common antigen rockets, but no deflection of the main URB line, which crossed the common rockets.

The URB antigen was used to immunize rabbits to produce a serum largely free of antibody to the common antigens. The initial SURB-Sepharose column used for step 5 (Fig. 3) had a very low capacity and was not an efficient agent for isolating URB. Therefore the rabbits used for SURB production were boosted in step 6 only twice with this product, and thereafter with the unbound URB as in step 4.

Combined line-and-rocket IEP was performed on a number of antigen preparations, employing the SURB to eliminate the bulk of the common antigen lines seen with antiserum to the crude PRB. (For these studies we used the lowest concentrations of SURB and PRB or URB that gave a sharp reference line approximately 15 mm above the antigen line trough. Although

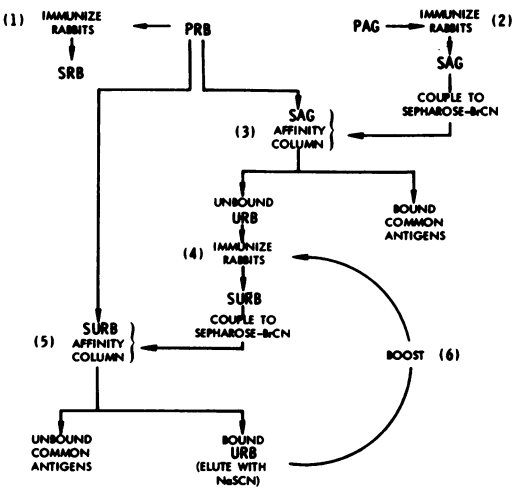

FIG. 3. *Flow sheet for isolation of antigens unique to resting cells of M. tuberculosis by cyclic immunoaffinity chromatography. All steps were monitored by rocket or line-and-rocket IEP.*

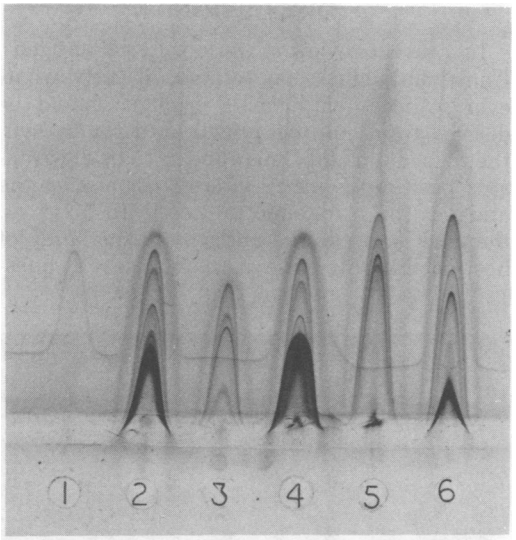

FIG. 4. *Combined line-and-rocket IEP of extracts of M. tuberculosis against a reference line antigen prepared by removing common antigens from a resting bacillus preparation by immunoaffinity absorption. The upper portion of the slide contained antiserum (SRB 83-356) raised against crude protein from resting bacilli, 0.053 ml of serum per ml of agarose. The line antigen trough contained homologous protein from resting bacilli after removal of common antigens on a SAG-Sepharose column (URB-34977) in a concentration corresponding to 85 μg of original crude protein per 0.5 ml of agarose. The test antigen wells contained: (1) homologous URB-34977, derived from 30 μg of crude protein; (2, 4, and 6) crude protein derived from batches of resting bacilli PRB 39/14 (16 μg), PRB 39/14 (4.7 μg), and PRB 12/17 (11 μg), respectively. Wells 3 and 5 contained crude protein from batches of actively growing bacilli, PAG 27/26 (10 μg) and PAG 16/6 (10 μg), respectively.*

increasing the concentrations of both components in a proportional manner kept the system in balance, the result of using greater amounts of each was to attenuate the sensitivity of the line-and-rocket system by decreasing the relative effect of a given amount of antigen in a well). An example is illustrated in Fig. 5. In this preparation the reference crude antigen PRB 39/14 was used in the line trough, and also in one of the test wells. The URB antigen derived from PRB 39/14 was used in another well, as were two other batches of crude PRB (52/16 and 55/15), a sample of PAG (46/16), and one of the United States-Japan culture filtrate antigen, MARS 003. The common peaks have been largely eliminated by using the specific SURB; deflections of the line are produced only by products from resting cells, but not from actively growing bacilli or the United States-Japan pellicle culture filtrate.

To be sure that differences in culture medium or simple batch variations, rather than the mode of growth, were not responsible for the absence of URB in actively growing cells, samples of crude proteins extracted from bacilli grown in media containing different basal compositions, different carbohydrates, and with and without albumin were compared in test antigen wells against the SURB-PRB 39/14 line-and-rocket reference system. The PRB antigen line trough contained 40 μg of protein in 0.5 ml of agarose, and the SURB concentration was 0.026 ml per ml of agarose in the upper segment of the plate. To standardize the expression of results, the specific activity (A) of the antigens with respect to the URB line system was calculated from the formula $A = (P - L)/LC$, where P is the height of the line displacement peak above the antigen line load trough, L is the distance of the precipitin line from that trough, and C is the amount (in micrograms) of the protein in the 0.01 ml of antigen solution that was added to a well. The relative activity (R) was calculated from $R = A_t/A_r$, where A_t is the specific activity of the test sample and A_r is the specific activity of the

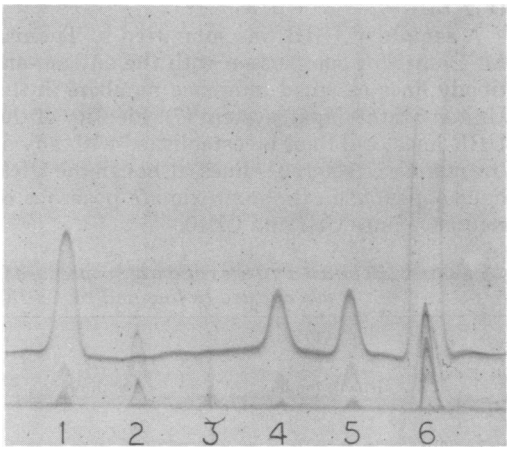

FIG. 5. *Combined line-and-rocket IEP of extracts of M. tuberculosis against antibody to unique resting bacillus antigen. The upper portion of the slide contained antiserum (SURB 89-258) raised against the unique components of a protein extract of resting bacilli, 0.026 ml of serum per ml of agarose. The line antigen trough contained crude protein from resting bacilli (PRB 39/14), 20 μg in 0.5 ml of agarose. Test antigen wells 1, 4, and 5 contained crude protein derived from batches of resting bacilli PRB 39/14 (2 μg), PRB 52/16 (2 μg), and PRB 55/15 (2 μg), respectively. Well 2 contained crude protein from actively growing bacilli, PAG 46/16 (5.5 μg). Well 3 contained United States-Japan reference culture filtrate antigen MARS 003 (10 μg). Well 6 contained protein from resting bacilli after removal of common antigen, URB 207 (derived from 14 μg of crude protein).*

reference sample, PRB 39/14. The mean specific activity of the reference antigen was 0.68 units per μg of protein. The results are presented in Table 1. Although the relative activity of the URB component detected in crude PRB products varied from batch to batch, it was detected in significant amounts in every batch tested, regardless of which basal medium and enrichments were used for growth of the bacilli. On the other hand, URB components were not detected in the PAG products. It should be noted that PAG 46/16 was prepared from a pool of 10-ml cultures in screw-capped test tubes (20 by 150 mm) under continuous rotation at 250 rpm, since tubercle bacilli do not grow well in Tween-containing media when glycerol is absent and the cultures are shaken in flasks (20). A relatively small URB component was detected in the United States-Japan cell extract (MARS 004) but not in the culture filtrate (MARS 003).

A careful examination of the line-and-rocket IEP peaks obtained with URB revealed suggestion of a double line at the rocket tips. When a sample of PRB was subjected to crossed IEP against SURB, the presence of two components with almost identical migration was confirmed (Fig. 6).

A sample of URB was submitted to Thomas M. Daniel for comparison with the antigen-antibody lines assigned reference numbers in the United States-Japan system (7). Identity of the URB lines could not be established with any of the numbered reference lines, although the URB lines appeared in the approximate positions of reference lines CE7 and CE10.

DISCUSSION

In a recent review of mycobacterial antigens, Daniel and Janicki have stated, "Clearly, whatever source of antigen is used, it should be derived from cultures grown on a totally synthetic medium" (8). This appears to be a reasonable position if one is interested in using the antigens for taxonomic purposes. If, however, the goal is to use the antigens in the study of host-bacillus interactions, there arises the ques-

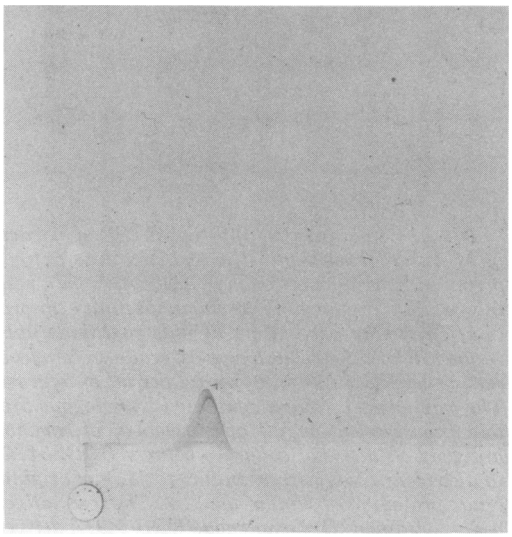

FIG. 6. *Crossed IEP of a crude protein extract of resting bacilli (PRB 39/14) against antibody to the unique resting bacillus antigen (SURB 89).*

TABLE 1. *Relative activities of antigens prepared from tubercle bacilli grown under different conditions, when tested by line-and-rocket IEP against a specific antiserum (SURB)*

Growth mode	Batch no.	Medium				Relative activity[a]
		Base	Enrichments (mg/ml)			
			Glucose	Glycerol	Albumin	
PRB	39/14	Dubos	7.5	0	5.0	1.00
	41/17	Dubos	7.5	0	5.0	4.13
	43/9	Dubos	7.5	0	5.0	0.93
	52/16	TB broth	7.5	0	0	0.51
	55/15	TB broth	7.5	0	0	0.53
	49/13	TB broth	0	50	0	0.47
PAG	27/26	Dubos	7.5	50	5.0	<0.01
	6/30	TB broth	0	50	0	<0.01
	46/16	TB broth	7.5	0	0	<0.01
Pellicle (U.S.-Japan)[b]						
Cell extract MARS 004		Proskauer-Beck	0	20	0	0.06
Culture filtrate MARS 003		Proskauer-Beck	0	20	0	<0.01

[a] Relative activity is the ratio of the specific activity of test antigen to that of the reference antigen (PRB 39/14).

[b] These antigens were prepared for the U.S. Tuberculosis Program of the United States-Japan Medical Science Program and distributed for the National Institute of Allergy and Infectious Diseases by the Trudeau Institute.

tion of comparability of antigen composition of bacilli grown in a synthetic environment with that of bacilli grown in a more complex host environment.

Most studies of mycobacterial antigens have been directed towards products derived from surface cultures of the bacilli on a glycerol-rich protein-free synthetic medium such as that of Sauton or of Proskauer and Beck. This choice has been dictated largely by issues of practical convenience for the investigator, since it permits harvest of high yields of cells, ease of separation of cells from culture medium, minimum manipulation of cultures, and avoidance of a need to distinguish between antigenic substances of bacillary origin and those extraneous products contributed by the medium itself.

Antoine and Tepper have reported that the presence of glycerol and the relatively restricted nitrogen source in Sauton medium favor accumulation of lipid and polysaccharide in aging pellicles of *M. tuberculosis* (2). This, in turn, undoubtedly leads to heterogeneity of physiological state of the bacilli in various regions of the pellicle, as lipid restricts penetration of nutrients and/or oxygen. Turcotte (18, 19) has reported a general decline in numbers of antigens detectable in extracts of aged pellicles of *M. tuberculosis*; Janicki et al. (15) have suggested that their results in similar experiments reflected a quantitative, rather than qualitative change in antigenic composition. The heterogeneity of physiological age of members of a population of bacilli in a pellicle makes it difficult to interpret these phenomena.

The use of well dispersed cultures would offer the advantage of assuring a more uniform exposure of bacilli to nutrients, and thus better control of physiological homogeneity. Tubercle bacilli can be grown logarithmically in a dispersed state in a modified Sauton medium containing Tween 80 and albumin, with constant agitation. However, when the same organisms are grown in this glycerol-containing medium with only intermittent agitation, a metabolic imbalance occurs as oxygen is depleted, which results in death and autolysis of the bacilli (22). On the other hand, cultures of *M. tuberculosis* in Dubos Tween albumin medium which are never agitated exhibit a steady net linear growth. This represents a balance between normal logarithmic growth in the upper layers of the medium, and settling of bacilli through a self-induced O_2 gradient (20). The bacilli that settle to the bottom of the culture have adjusted their metabolism on passage through that gradient and have stopped replicating, but retain their viability. They are in a homogeneous physiological state, as reflected in induction of synchronized replication when the bacilli in the sediment are diluted in fresh medium (21). The requirement for an 8-h period of RNA synthesis before cell division occurs suggested a difference in enzyme composition between the resting and actively replicating organisms. The existence of a chronic stable phase in human clinical tuberculosis suggested that the resting organisms from a nutrient-rich but oxygen-poor region of the culture might reflect the chemical composition of organisms in a host lesion more accurately than would actively growing, well aerated bacteria.

The studies reported here were designed to explore differences in chemical composition of resting and actively growing tubercle bacilli as they might be reflected in antigenic composition. TB broth base was selected for initial studies since it is a very rich and complex mixture of nitrogen sources and supports the luxuriant growth of *M. tuberculosis*, even without glycerol (17). This basal medium was dialyzed, and only the dialysate was used to minimize the introduction of non-bacillary macromolecular substance. As may be seen in Table 1, however, the presence or absence of albumin, glucose and/or glycerol did not significantly alter the occurrence of the dominant antigens that appeared unique to resting bacilli. The occurrence of these antigens is determined by the adaptation to conditions of limited aeration.

Prior applications of immunoaffinity chromatography to mycobacterial products have been directed toward absorption of desired antigenic components on agarose that was coupled to homologous immune globulin that had been raised against the preselected antigen(s) of interest. The desired antigens were then eluted with appropriate chaotropic agents (5). Daniel and Anderson prepared their antiserum by immunization of goats with United States-Japan reference antigen 5 that had been purified by physicochemical means (6). This antigen was selected because it is unique to *M. tuberculosis* and *M. bovis* and is not present in other mycobacterial species. Monospecific antiserum has also been raised against a single antigen-antibody line cut out of a complex pattern of lines in a BCG counterimmunoelectrophoresis experiment (11, 12). This serum could have been used for preparation of an immunoaffinity column. Ferguson et al. (10) have employed a cyclic immunoaffinity system, first coupling a crude sonic extract of BCG to Sepharose to bind specific globulins from immunized rabbits, and then eluting the globulins to prepare affinity columns for isolation of antigens from a BCG extract.

In the present study, the presence of common antigens obscured the search for antigens that

might have been unique to the resting or the actively growing bacilli. Therefore the extracts were passed through immunoaffinity columns prepared from the heterologous immune serum to remove the dominant common antigens and permit detection of those that were unique to a given preparation, and thus not bound to the heterologous column. In this way two antigens were detected, of very similar electrophoretic mobility and antigenicity, which appeared to be unique to the resting bacilli (URB). It is of course possible that these antigens do occur in actively growing cells also but in amounts too low to be detected in our system, i.e., less than 1/100 of the concentration seen in resting bacillus extracts. Similarly, the growing bacilli may also have unique antigens unshared by resting bacilli, but undetected to date. Preliminary experiments suggest the presence of traces of additional antigens unique to resting bacilli. Production of crude antigens is presently being scaled up to provide sufficient quantities of these products to permit further exploration of this question.

The presence of a small component of URB antigen in the United States-Japan pellicle-derived reference product MARS 004 probably reflects the heterogeneity of population in the pellicle. The concentration of this component, in terms of the total protein in the MARS 004 product, is only about 1/16 that seen in a comparable amount the reference URB product.

It is noteworthy that the two URB line components could not be shown to correspond to any of the previously numbered reference lines in that system.

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