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Identification of a *Mycobacterium bovis* BCG 45/47-Kilodalton Antigen Complex, an Immunodominant Target for Antibody Response after Immunization with Living Bacteria

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Increased protection against a virulent challenge with *Mycobacterium tuberculosis* is induced mainly by a previous immunization with living avirulent mycobacteria, usually *Mycobacterium bovis* BCG. Only a transient and marginal protection is obtained after immunization with bacterial extracts or dead bacteria. Both living and heat-killed bacteria share a number of common antigens. In order to identify mycobacterial molecules which are dominant antigens during immunization with living bacteria, a two-step selection method was used. Two groups of guinea pigs were immunized either with living or with heat-killed BCG. Sera were then collected and used to select and counterselect antigens present in BCG culture filtrates. Each major fraction eluted from a series of high-pressure liquid chromatography columns (gel filtration, DEAE, and reverse-phase chromatography) was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on polyvinylidene difluoride sheets. The molecules present on twin immunoblots were stained with antibodies raised in guinea pigs immunized either with living or with heat-killed BCG. Cross-reactive antigens stained in twin immunoblots were eliminated. Major antigens interacting with antibodies raised after immunization only with living bacteria were further purified. A complex of 45- and 47-kDa major molecules (45/47-kDa complex) was thus identified and further purified. The complex was found to interact only with antibodies present in sera of guinea pigs immunized with living bacteria and not at all with antibodies raised after immunization with dead bacteria. The 45/47-kDa antigen complex molecules were resolved on two-dimensional electrophoresis in three major and seven minor proteins detected with silver staining. All the molecules interacted with the antibodies present in sera of guinea pigs immunized with living BCG. The three major proteins (two at 47 kDa and one at 45 kDa) were amino-terminal sequenced. The sequence A-P-E-P-A-P-P-V-P-P-A-A-A-A-P-P-A, which was not previously reported, was the same for these three molecules. By using a competitive enzyme-linked immunosorbent assay, the concentrations of the 45/47-kDa antigen complex were measured in BCG culture filtrates, freeze-dried BCG, and dried heat-killed BCG; they were, respectively, 2, 0.01, and 0.001% of the total mass. The low or very low values compared with the high antibody concentration emphasized the ability of the 45/47-kDa complex delivered through live BCG to trigger an antibody response.

Investigation of the interactions between pathogenic mycobacteria and components of the host immune system has been adopted as an approach that may lead to the development of novel diagnostic reagents and to a better understanding of the molecular mechanisms involved in mycobacterial disease pathogenesis and immunity (5). Mycobacteria contain numerous chemically different components that could take part in such interactions. A selection of antigens to be investigated must be made on the basis of not only their capacity to interact with a given component of the immune response but also their potential ability to play a role during an active infection and/or during the pathogenesis of the disease.

An increased resistance against a challenge with pathogenic living *Mycobacterium tuberculosis* is obtained mainly after a previous immunization with living avirulent mycobacteria, such as *Mycobacterium bovis* BCG (6). Both classical (32) and more recent (22) studies have pointed out that immunization with dead bacteria or bacterial extracts is quantitatively less potent than injections of living bacteria in protecting experimental hosts against a challenge with virulent *M. tuberculosis*.

It has been reported that nonreplicating living mycobacteria contain antigenic components that are not present in the replicating cells (31). Similarly, the growing bacilli may also have unique antigens that are not shared by resting bacilli or dead bacteria but have not been detected to date. It may be hypothesized that some molecules are expressed on bacilli in higher concentrations in vivo than in vitro. Whatever the exact mechanism, an increase in the number or concentration of specific immune effectors may reflect the increased production of some mycobacterial molecules during active growth of mycobacteria in the host's environment.

In the context of this hypothesis, guinea pigs were immunized with living or heat-killed BCG, and their respective sera were used as screening tools in order to detect and to select only those mycobacterial antigens recognized as major targets of antibodies raised after immunization with living bacteria.

The present paper reports the biochemical characterization of a complex of antigens, present in the BCG culture medium filtrate, which interact only with antibodies present in serum of guinea pigs immunized with living BCG.

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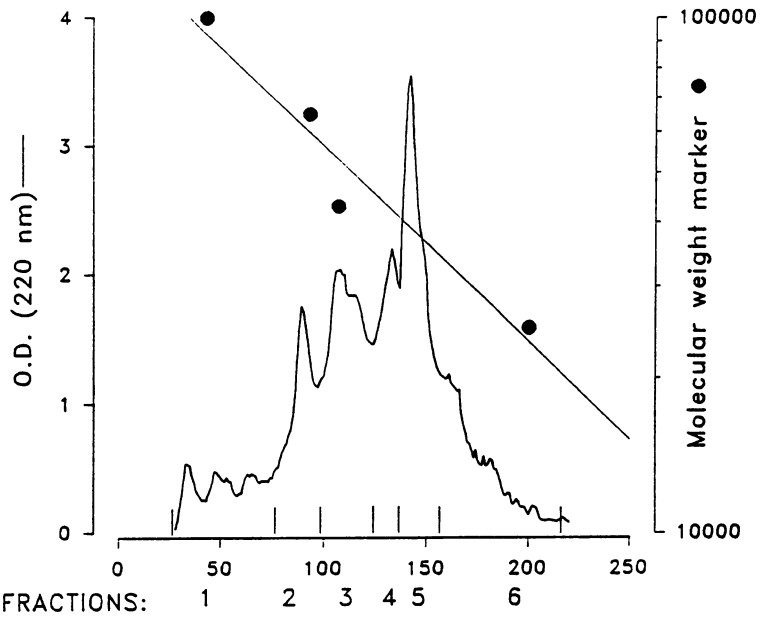

FIG. 1. Molecular filtration of material present in Sauton medium after 14 days of BCG growth. The synthetic Sauton medium was collected on day 14 of *M. bovis* (var. BCG) growth. The molecules present in the media were extensively washed with deionized water containing butanol (4%) on a PM_{10} membrane (Amicon). After a 10-fold concentration, the material was freeze-dried. The freeze-dried crude material was passed through an Si300 gel filtration preparative column (Serva). The six major fractions were collected according to optical density (OD) profile (λ 220 nm), extensively washed on a PM_{10} membrane, and freeze-dried.

MATERIALS AND METHODS

Guinea pigs. Groups of 12 to 15 female guinea pigs (outbred Hartley, weighing 250 to 300 g at the beginning of the experiments) were immunized only once with living BCG or with heat-killed BCG. The living BCG (containing 2×10^7 viable units in 0.2 ml of saline solution and less than 10% dead bacilli) was injected intradermally into two sites on the flanks. Two milligrams of heat-killed BCG (120°C, 30 min) were mixed in 0.5 ml of a mixture of saline and incomplete Freund's adjuvant (Difco) and injected intramuscularly.

Sera of different groups of guinea pigs were collected 7 to 12 months after immunization. They were passed through a 0.22-μm-pore-size filter, aliquoted, and stored at −20°C. Assays with different pools of sera obtained after immunization with living bacteria (five pools) or with dead bacteria (six pools) gave very similar results following the same immunization protocol. For each immunization protocol, the reported results were obtained with a representative pool of sera.

The titers of these different serum pools were measured on crude BCG filtrate and on purified protein derivative (PPD) in enzyme-linked immunosorbent assays (ELISAs). They were found to be in the same range (around 1/4,000) whatever the immunization procedure and more elevated for two pools of sera obtained after immunization with dead bacteria.

Source of antigens. BCG (strain $1173P_2$) was cultured in round flasks containing 130 ml of synthetic Sauton medium (15). The culture medium was harvested after 14 days and filtered through a gauze and a 0.22-μm-pore-size filter. The medium was intensively washed at 4°C with deionized water containing butanol (4%) on a PM_{10} Amicon membrane and concentrated around 10-fold. The concentrated medium containing molecules with molecular masses above 10 kDa was freeze-dried and stored at −20°C.

Purification procedure. (i) Molecular sieve column. A preparative Si300 column (3-μm particle size, 50 by 750 mm; Serva) was equilibrated with a saline buffer (50 mM Na_2HPO_4 adjusted to pH 7.5 with KH_2PO_4) containing butanol (4%). The column flow was 1.25 ml/min; the maximal pressure of 45 bars (45×10^5 Pa) was never reached.

The crude material was prepared in a buffer-butanol solution at a concentration of 50 mg/ml and ultracentrifuged at $40,000 \times g$ for 2 h to eliminate any insoluble material. After filtration (0.22-μm-pore-size filter), aliquots (10 ml) containing 500 mg of material were stored at −20°C. They were refiltered (0.22-μm-pore-size filter) after being thawed and just before the injection into the column. The optical density profiles at 220 nm were recorded (Fig. 1). The six main fractions as determined by optical density profiles were extensively washed at 4°C with deionized water containing butanol (4%), concentrated on a PM_{10} membrane, and freeze-dried. After being freeze-dried, the fractions were weighed and stored at −20°C. Fraction 2, which contained the majority of molecules interacting with the antibodies of guinea pigs immunized with living bacteria, was then applied to a DEAE ion-exchange column.

(ii) DEAE column. A preparative column (DEAE-TSK 5PW, 21.5 by 150 mm; LKB) was equilibrated with a saline buffer of low ionic concentration (10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.5], 10 mM NaCl) containing butanol (4%). The flow rate was 6 ml/min with a maximum pressure of 30 bars (30×10^5 Pa). A linear gradient of NaCl (1 M maximum in the same buffer) was applied after injection of 100 mg of the above-described fraction 2 dissolved in 4 ml of starting saline buffer. Three major parts were collected according to optical density (220 nm). They were extensively washed at 4°C with deionized water-butanol (4%), concentrated on a PM_{10} membrane, and freeze-dried. Only fraction 1 containing the antigens recognized by the antibodies of guinea pigs

immunized with living bacteria was loaded onto the next column.

(iii) Reverse-phase column. An RP300 C8 column (10-μm particle size, 4.6 by 250 mm; Aquapore Brownlee Laboratory) was equilibrated with an ammonium acetate buffer (20 mM NH_4-$OOCCH_3$ [pH 6.5]) under a flow rate of 2 ml/min with a maximum pressure of 115 bars (115 × 10^5 Pa), and fraction 1 (7.5 mg) from DEAE column chromatography was dissolved in 1 ml of the ammonium acetate buffer and injected. The elution gradient containing 90% acetonitrile for higher concentrations was generated according to the profile shown in Fig. 3. The optical density profile at 220 nm allowed the separation of five major parts, which were concentrated under vacuum at 40°C to eliminate acetonitrile and freeze-dried.

(iv) Two-dimensional electrophoresis. Fraction 5 obtained after reverse-phase chromatography was subjected to two-dimensional electrophoresis on immobilized pH gradients (24). The first-dimension gel for isoelectric focusing was prepared with a mixture of Immobilines (pKs of 3.6, 4.6, and 6.2; Pharmacia) polymerized in the presence of 4% polyacrylamide on an hydrophilic plastic support (Gel Bond PAG; FMC Corporation). After being washed in deionized water, the gels were dried and cut into individual 5-mm-wide strips. The strips were rehydrated in 1% ampholine (Servalyt 2-6)–2% Nonidet P-40 until they swelled to 100% of the initial mass.

About 100 μg of fraction 5 in 4% ampholine–2% NP-40 was loaded onto each strip. Electrofocusing was carried out overnight at 2,000 V. Individual strips were immersed for 30 min in 10 ml of denaturating buffer containing 10% glycerol, 0.125 M Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate (SDS), and a trace of bromophenol blue. Each strip was then applied to the top of a 5% stacking gel of a vertical 10% SDS gel. The second-dimension run was carried out at only 10 mA until the front visualized by bromophenol blue came near the interface between stacking and separating gels. At this time the strip was removed by holding nylon yarns which had been punched previously at each extremity of the plastic support.

Immunoblotting. The antigens present in each part were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gels according to the method of Laemmli (18). Samples containing between 2 and 10 μg of freeze-dried material according to the purification step were loaded into the wells in 10 μl of buffer containing 5% 2-mercaptoethanol, 3% SDS, and a trace of bromophenol blue. After electrophoresis, the antigens present in each line were transferred on a polyvinylidene difluoride (PVDF) sheet (Millipore) by wet electrophoretic transfer (29). Similarly, after two-dimensional electrophoresis, the molecules were transferred on a PVDF sheet for immunoblotting or for N-terminal sequencing.

The detection of immunoblots was performed as described previously (17). In brief, the PVDF sheet was rapidly stained with a solution of Coomassie blue in order to mark molecular weight markers with a pencil. After destaining, the sheet was washed for 30 min with phosphate-buffered saline (PBS) containing Triton X-100 (3%) at 25°C and three times more with PBS alone for 5 min each. The sheet was blocked with nonfat dry milk (5%) in PBS for 1 h at 37°C and then extensively washed with PBS containing Tween 20 (0.2%).

The blocked and washed sheets were incubated in the presence of immune sera diluted (1/20) in PBS containing Tween 20 (0.2%) and nonfat dry milk (5%) for 1.5 h at 37°C. They were washed three times with PBS containing Tween 20 and incubated in the presence of an alkaline phosphatase conjugate directed against immunoglobulin from the first step. These antibodies against guinea pig immunoglobulin (Biosys) were diluted (1/2,500) in PBS–Tween 20 (0.2%)–nonfat milk (5%). After incubation for 1.5 h at 37°C, the PVDF sheets were washed three times in PBS–Tween 20 and were revealed for 5 to 10 min with a bromochloroindolylphosphate-nitroblue tetrazolium substrate (17).

Competitive ELISA. A competitive ELISA was used to measure the concentrations of the 45/47-kDa antigen complex present in different crude preparations. In brief, a potent polyclonal rabbit immune serum was obtained against the antigens of the 45/47-kDa complex by using a classical immunization procedure: injection of 50 μg of the complex mixed with incomplete Freund adjuvant and of 25 μg 1 month later. The antigen complex was immobilized on a plastic surface (1 μg/ml in carbonate buffer). The preliminary experiments indicated the optimal dilution of rabbit serum (1/8,000) to obtain a sensitive titration curve, i.e., the measurement of the remaining antibodies after their incubation with the fraction to be assayed. A curve with the 45/47-kDa complex to determine the 50% value was included in each assay. The sensitivity was in the 2-ng/ml range by using phosphatase-labelled antibodies directed against rabbit immunoglobulin G to determine the amounts of bound anti-45/47-kDa complex antibodies (21).

Measurement of DTH reactions. Two groups of guinea pigs which had been immunized 12 weeks previously with either living or heat-killed BCG were checked for their delayed type hypersensitivity (DTH) reactivity.

The DTH reactions were tested on the flanks of guinea-pigs which had been depilated 24 h previously. A standard PPD was injected into one flank in order to measure the DTH reactivity level of each guinea pig. This standard injection was 0.25 μg of PPD in 0.1 ml of a saline-Tween solution (PBS with 0.0005% Tween 80) in a strictly intradermal route. On the other flank, a known concentration of material to be tested was injected intradermally in 0.1 ml of a saline-Tween solution.

The DTH reactions were measured 28 h later. The longitudinal and transversal diameters of erythema were measured. The arithmetic means and standard deviations of results obtained for four guinea pigs immunized with either living or dead BCG were determined.

N-terminal sequencing. Amino-terminal sequences were determined by automatic Edman degradation with an Applied Biosystems 470A from pieces of a PVDF sheet on which the proteins had been electrotransferred. The PVDF pieces were cut after a light staining with Coomassie blue (19).

Nucleotide sequence accession number. The EMBL accession number for the sequence determined is P 80069.

RESULTS

Molecular sieve column. The freeze-dried material present in the BCG culture medium was loaded onto an Si300 column. The fractions (numbered 1 to 6) were collected according to the optical density profile, extensively washed, and concentrated on a PM_{10} membrane before being freeze-dried.

A similar amount (10 μg) of each of fractions 1 to 6 and 25 μg of the crude starting material (fraction 0) were submitted to SDS-PAGE on three gels run simultaneously. Two gels were transferred on PVDF sheets, and one gel was stained with Coomassie blue. Figure 2 shows the stained gel and

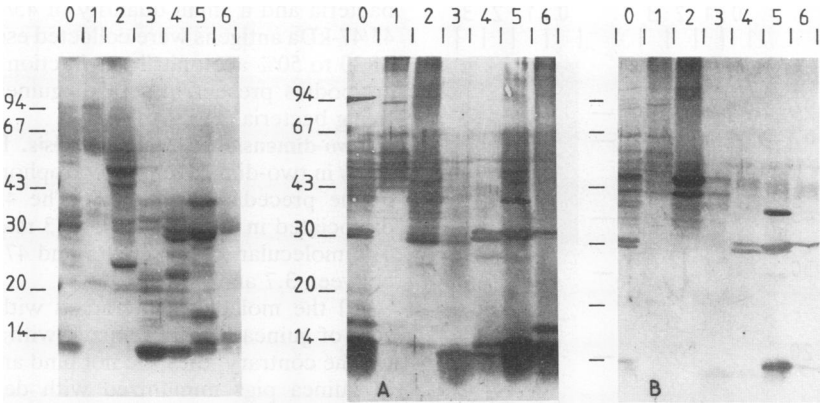

FIG. 2. Immunodetection of major antigens present in the fractions obtained after Si300 filtration. The crude starting material (lanes 0) and the six major fractions of Si300 filtration were analyzed on 10% polyacrylamide gels in the presence of SDS (lanes 1 to 6). For the panel on the left, the gel was stained with Coomassie blue. (A and B) After electrotransfers on PVDF membranes, the antigens present were detected with antibodies raised in guinea pigs immunized with dead (A) or living (B) BCG.

PVDF sheets revealed with antibodies obtained from guinea pigs immunized either with dead bacteria (A) or with living bacteria (B). Two 30-kDa antigens interacting with the two pools of antibodies were found in fractions 4, 5, and 6, and one 38-kDa molecule was found in fraction 5. Different antigens in the range of low molecular masses (10 to 16 kDa) in fractions 3, 4, 5, and 6 interacted mainly with antibodies of guinea pigs immunized with dead bacteria. In contrast, two antigens (45 and 47 kDa [45/47-kDa antigen complex]) present in fraction 2 were found to interact with antibodies contained in the sera of guinea pigs immunized with living bacteria. Fraction 2 was selected for the next purification step.

DEAE column. A sample of the preceding fraction 2 was injected onto a DEAE-TSK preparative column and eluted with an NaCl gradient. The optical density profile allowed the identification of three major parts (Fig. 3). Each part was extensively washed, concentrated on a PM_{10} membrane, and freeze-dried.

A sample (5 μg per well) of each part was analyzed by PAGE and immunoblot with sera obtained from guinea pigs immunized with dead (Fig. 4A) or living (Fig. 4B) bacteria. Fraction 1 of the DEAE column contained some minor antigens interacting with sera of animals immunized with dead bacteria: two faint bands at 10 and 14 kDa, one other small band at 52 kDa, and a weak smear around 67 to 94 kDa. In contrast, the same fraction 1 of the DEAE column contained two important major bands interacting with antibodies raised in guinea pigs immunized with living bacteria and strong smears in the high-molecular-mass range above the 67-kDa marker. Fraction 1 of the DEAE column was chosen for the next purification step. It is worthy of note that part 3 of the DEAE column was found to contain the

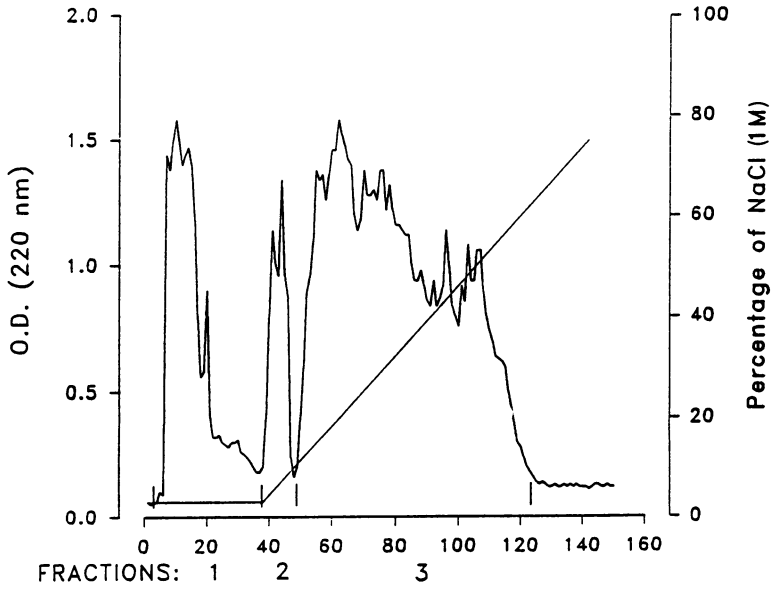

FIG. 3. Purification on a DEAE column of molecules present in fraction 2 of Si300 filtration. Fraction 2 of Si300 filtration was charged on a DEAE-TSK preparative column. The three major fractions were collected according to ionic strength. They were extensively washed with deionized water containing butanol (4%) on a PM_{10} membrane before concentration and freeze-drying.

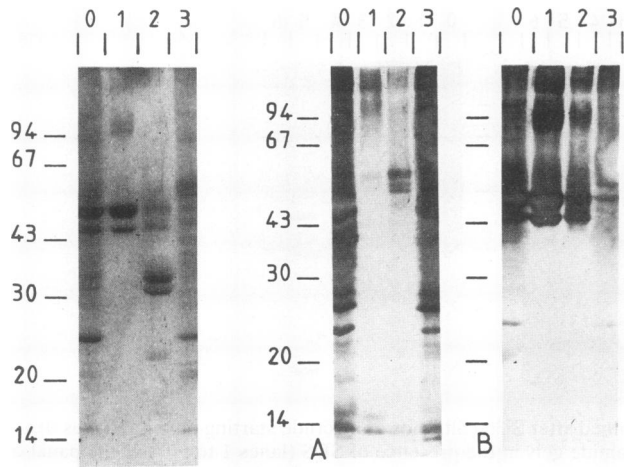

FIG. 4. Immunodetection of major antigens after DEAE fractionation. Fraction 2 of Si300 filtration starting material (lanes 0) and the three fractions obtained from DEAE chromatography (lanes 1 to 3) were analyzed on 10% polyacrylamide gels in the presence of SDS. For the panel on the left, the gel was stained with Coomassie blue. (A and B) After electrotransfer on PVDF membranes, the antigens were detected with antibodies raised in guinea pigs immunized with dead (A) or living (B) BCG.

majority of antigens interacting with antibodies obtained after immunization with dead bacteria.

Reverse-phase column. An RP300 column was loaded with the preceding fraction 1 from the DEAE column. The column was eluted with an acetonitrile gradient. Five major parts were collected, pooled, and concentrated under vacuum at 40°C to eliminate acetonitrile before being freeze-dried (Fig. 5).

Fraction 4 corresponding to 25 to 30% acetonitrile contained antigens in the 10- to 15-kDa range interacting with antibodies present in guinea pigs immunized with dead bacteria and a small quantity of 45/47-kDa antigens. These 45/47-kDa antigens were collected essentially in the presence of 30 to 50% acetonitrile in fraction 5; they interacted with antibodies present in sera of guinea pigs immunized with living bacteria (Fig. 6).

Two-dimensional electrophoresis. Figure 7 shows the patterns in two-dimensional electrophoresis of proteins present in the preceding fraction 5. The 45/47-kDa complex was dissociated in 10 closed spots (3 major and 7 minor spots). The molecular sizes were 45 and 47 kDa, and the pIs were between 3.7 and 4.1.

All the molecules interacted with antibodies present in sera of guinea pigs immunized with living bacteria (Fig. 8). On the contrary, they did not bind antibodies present in sera of guinea pigs immunized with dead bacteria. To verify protein transfer on the PVDF sheet, it was subsequently stained with a potent rabbit polyclonal antiserum directed against the crude freeze-dried BCG culture filtrate. This control experiment indicated the presence of the antigens of only the 45/47-kDa complex (data not shown).

N-terminal sequencing. A series of PVDF sheets on which the antigens had been electroblotted were stained lightly with Coomassie blue. Pieces of the PVDF sheets were carefully cut around each blot and accumulated. The three major blots, labelled 3, 5, and 8 in Fig. 7, were amino-terminal sequenced. Only 10 hydrolysis cycle residues were obtained for blot 5 (small amount of the protein), and 17 hydrolysis cycle residues were obtained for blots 3 and 8. The sequence (A-P-E-P-A-P-P-V-P-P-A-A-A-A-P-P-A; EMBL accession no. P 80069) was identical for blots 3 and 8 and for the 10 residues obtained for blot 5.

Presence of the 45/47-kDa antigen complex on bacteria. A rabbit polyclonal immune serum was prepared against the molecules present in the 45/47-kDa antigen complex. Assayed on an immunoblot on which a sample of the crude starting material has been electrotransferred after an SDS-PAGE run, this rabbit serum detected the 45/47-kDa doublet and a 30-kDa molecule with only a faintly visible reaction

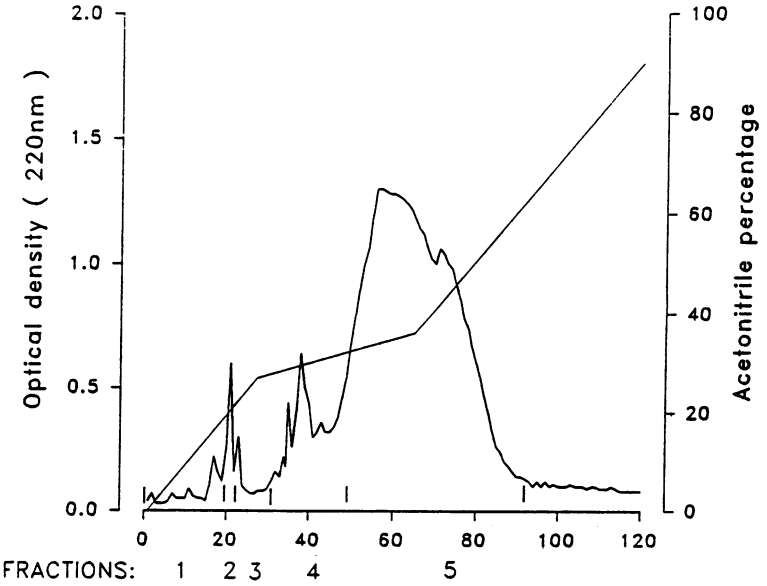

FIG. 5. Purification on a reverse-phase column (RP300) of molecules present in fraction 1 of DEAE fractionation. Fraction 1 of DEAE fractionation was loaded onto an RP300-C8 reverse-phase column. The five major fractions were eluted by an acetonitrile gradient and collected according to the optical density (O.D.) profile. They were concentrated under vacuum at 40°C before being freeze-dried.

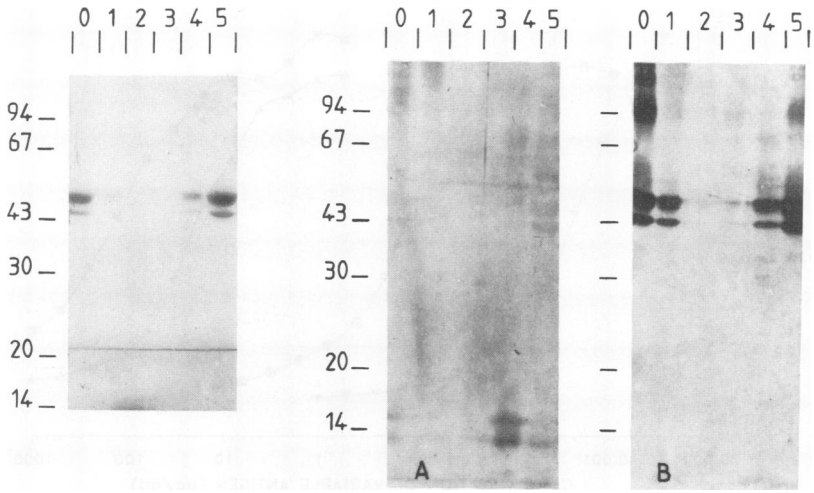

FIG. 6. Immunodetection of major antigens after reverse-phase fractionation. Fraction 1 of DEAE fractionation (lanes 0) and the five fractions obtained from reverse-phase chromatography (lanes 1 to 5) were analyzed on SDS–10% PAGE. For the panel on the left, the gel was stained with Coomassie blue. (A and B) After electrotransfer on PVDF membranes, the antigens were detected with antibodies raised in guinea pigs immunized with dead (A) or living (B) BCG. Fraction 5 contained the majority of 45/47-kDa molecules, with a minimum of cross-reacting antigens. The presence of 45/47-kDa molecules in fraction 1 related to a column overload must be noted.

(data not shown). The rabbit immune serum was used in a competitive ELISA to measure the amounts of similar antigens present in BCG culture filtrates (crude starting material), on dried living BCG, and on dried heat-killed BCG. The concentrations of immunoreactive molecules in these crude preparations were approximately 2% in the BCG culture filtrate, 0.01% on dried living BCG, and less than 0.001% on dried heat-killed BCG (Fig. 9).

DTH reactions. The two groups of guinea pigs were found to develop similar DTH reactivity when the standard PPD was injected. The standard PPD values (mean ± standard deviation) were 12.3 ± 0.6 mm for guinea pigs immunized with living BCG and 11.5 ± 0.8 mm for guinea pigs immunized with dead BCG. The DTH values after injection of 0.25 μg of the 45/47-kDa antigen complex were 12.5 ± 0.7 mm for guinea pigs immunized with living BCG and less than 5 mm for guinea pigs immunized with dead bacteria. To obtain DTH reactions in the same range as the PPD standard ones, for guinea-pigs immunized with dead bacteria it was necessary to inject 10 μg of the 45/47-kDa antigen complex (11.5 ± 0.7 mm). These results, 0.25 μg versus 10 μg of the same sample to elicit similar reactions, allowed the determination that the proteins were approximately 40-fold more potent to elicit DTH reactions in guinea pigs immunized with living BCG than in dead-BCG-immunized guinea pigs.

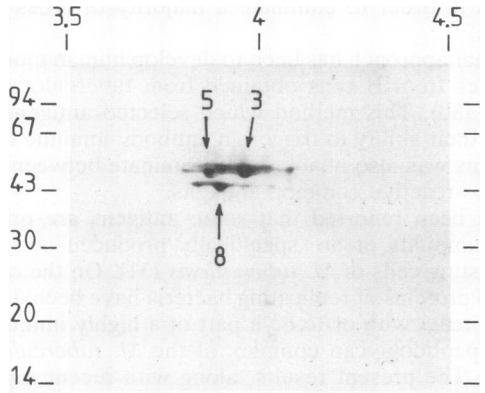

FIG. 7. Two-dimensional electrophoresis of the 45/47-kDa antigen complex. Fraction 5 of RP300-C8 reverse-phase chromatography was analyzed on two-dimensional electrophoresis. The electrofocusing of a 100-μg sample was performed on a narrow pH (3.5 to 4.5) Immobiline gel in the presence of NP-40 (2%) and ampholines (1%). The vertical SDS-PAGE was run, and the gel was silver stained before being photographed. An electroblotting on a PVDF sheet was performed, the proteins were slightly stained with Coomassie blue, and the corresponding pieces of PVDF were cut for the direct microsequencing procedure. The three major proteins, labelled 3, 5, and 8, were N-terminal sequenced. An identical sequence, A-P-E-P-A-P-P-V-P-P-A-A-A-A-P-P-A. . ., was obtained for each protein. Molecular size markers are indicated on the left.

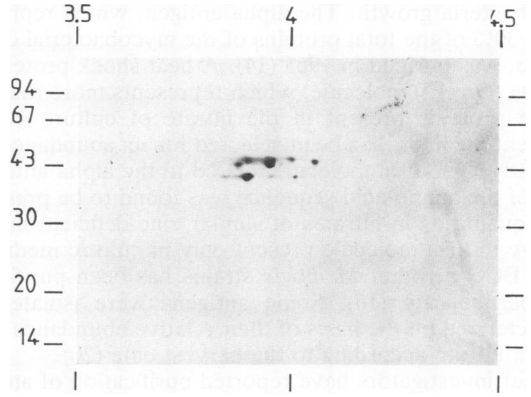

FIG. 8. Immunodetection of molecules present in the 45/47-kDa antigen complex. Fraction 5 of RP300-C8 reverse-phase chromatography was analyzed on two-dimensional electrophoresis and electroblotted on a PVDF membrane. The antigens present were detected only with antibodies raised in guinea pigs immunized with living BCG.

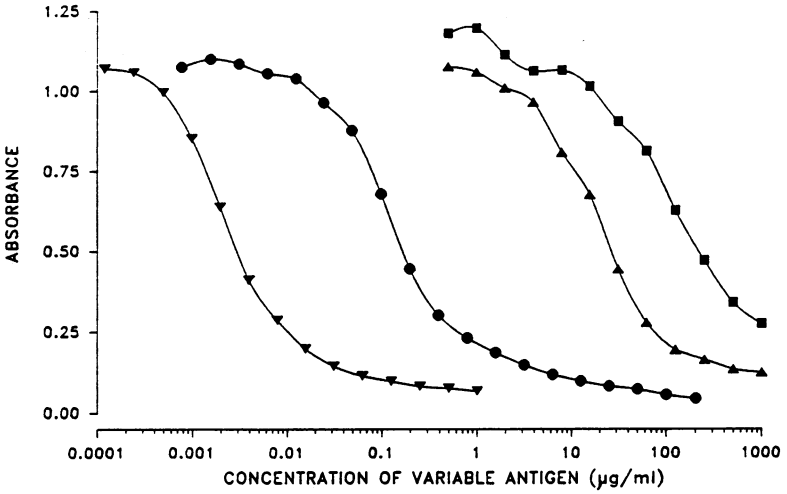

FIG. 9. Determination of the 45/47-kDa complex concentration by using a competitive ELISA. The molecules of the 45/47-kDa complex were fixed on polystyrene wells at 1 µg/ml in carbonate buffer. A polyclonal rabbit immune serum directed against the 45/47-kDa complex antigens was used at a constant concentration of 1/8,000. The titration curve obtained with the 45/47-kDa complex (▼) was used to measure the relative antigen concentrations present in crude BCG culture filtrate (●), on dried living BCG (▲) and on dried heat-killed BCG (■).

DISCUSSION

In the present work, two criteria were used to select the antigen fractions of the BCG culture filtrate to be further purified. Each chemical step of the purification procedure was chosen not only to enrich the fractions containing antigens able to interact with antibodies present in sera of animals immunized with living bacteria but also to deplete in the same fractions antigens interacting with antibodies induced after immunization with dead bacteria. The fraction containing the 45/47-kDa antigen complex did not interact with sera of animals immunized with heat-killed bacteria, at least on immunoblots.

To our knowledge, a biochemical purification of mycobacterial antigens which are recognized by antibodies of animals or humans in whom bacteria are actively growing has not been reported. Numerous papers have described mycobacterial antigens for their ability to interact with antibodies produced after immunization with dead bacteria or with bacterial extracts (7, 9, 33). Other antigens have been purified for their relative abundance in medium supporting mycobacteria growth. The alpha antigen, which represents about 30% of the total proteins of the mycobacterial culture filtrate, was purified in 1965 (14). A heat shock protein, the 64-kDa (Gro El) molecule, which represents more than 50% of the proteins present in the filtrate of culture in zinc-deficient medium, has been selected for its abundance (12). Similarly, a 32-kDa molecule related to the alpha antigen in term of the amino-acid sequence was found to be present in large quantities in filtrates of similar zinc-deficient medium (11). A 16-kDa molecule present only in culture medium of some BCG or other *M. bovis* strains has been purified for this particularity (16). Some antigens were isolated and characterized on the basis of their relative abundance in the culture filtrate according to the harvest date (20).

Other investigators have reported purification of antigens after their cloning in expression libraries screened with monoclonal antibodies (4, 26). As the screening probes were antibodies raised in mice immunized with extracts or dead mycobacteria, identical molecules were frequently identified and cloned despite apparently different strategies (13).

A recent paper has reported an attempt to purify mycobacterial antigens present in the *M. tuberculosis* culture filtrate on the basis of their ability to be retained on an immunoadsorbent column made with a pool of sera obtained from tuberculous patients (28). The major antigens which were semipurified by this method were certainly a mixture of specific and cross-reactive antigens. Many antigens present in culture medium after BCG growth are able to interact with antibodies of patients suffering from another infectious disease. The use of an immunoadsorbent obtained from sera of tuberculous patients does not select the antigens specific only to mycobacteria; in using this strategy, it would be important to run eluted molecules through a second immunoadsorbent made with sera of patients suffering from other diseases in order to eliminate a majority of cross-reactive antigens.

Another approach has been to develop human monoclonal antibodies from B cells obtained from tuberculous pleural exudate (30). This method which selected antigens on the basis of their ability to trigger an antibody immune response in humans was also unable to discriminate between specific and cross-reactive common antigens.

It has been reported that some antigens are present in greater amounts or are specifically produced on synchronized resting cells of *M. tuberculosis* (31). On the contrary, secreted proteins of replicating bacteria have been described to cross-react with or to be a part of a highly immunogenic protein peptidoglycan complex of the *M. tuberculosis* cell wall (3). The present results, along with recent reports (1, 20), pinpoint the importance of components secreted or released during growth of the bacteria and separated from the autolytic products inevitably present after 2 or 3 weeks in culture media. The 45/47-kDa antigens were found to be in larger relative concentrations in culture media harvested some days after the beginning of culture than after 4 to 12 weeks (results not reported). The choice of collecting media on day 14 of culture was based on the availability of enough material to be used without total loss during the different biochemical steps and of a relatively high proportion of the molecules in the starting material.

A rabbit immune serum prepared against the antigens of the 45/47-kDa complex was used in a competitive ELISA to

measure the concentrations of these antigens in crude preparations. The small amounts (about 2%) of antigenic material found in the BCG culture filtrate would explain the lack of their description in previous reports on the proteins present in culture filtrates (1, 20). Similarly, the very low concentration of these antigens on freeze-dried living BCG (about 0.01%) or on dried heat-killed BCG (about 0.001%) would explain the lack of antibody against the molecules when extracts or crude heat-killed BCG were used to immunize mice or rabbits. In an immunoblot performed from a two-dimensional electrophoresis of crude BCG culture filtrate, the 45/47-kDa antigen complex was among the main spots when they were screened with sera of guinea pigs immunized with living BCG. When sera of patients suffering from large pulmonary tuberculosis or relapse were assayed on similar blots, the 45/47-kDa antigen complex was also among the main spots recognized. These preliminary results obtained for 10 patients and 10 control sera have directed us to develop a quantitative assay of anti-45/47-kDa antigens to test the ability of these antigens to be used as a diagnostic reagent. The observations for guinea pigs and humans according to preliminary results indicate that this complex, which represents only a minor component of the bacteria or of their products, is highly immunogenic.

One of the next steps concerning the biochemical characterization of the 45/47-kDa antigen complex molecules will be to analyze the ability of separated proteins to aggregate with each other. Some of our results support such molecular interactions. In the immunoblot assays performed after the SDS-PAGE transfers, a smear situated in the vicinity of the 94-kDa marker was frequently observed despite only slightly stainable molecules on the corresponding gels after silver staining. This unstained but immunoreactive 94-kDa material was never observed in two-dimensional gels. The presence of 2% NP-40 and/or the electrofocusing itself suggested that this step could dissociate aggregated molecules. Regarding the small differences in pIs and molecular sizes between the molecules of the 45/47-kDa complex, posttranslational modifications of a protein coded by a single gene could explain the biochemical differences. On the contrary, the proteins of the 45/47-kDa complex could be the products of separate genes, as demonstrated for the 85 complex (34). Only the cloning of the gene(s) coding for the molecules of the 45/47-kDa complex will clarify this point.

With regard to the high percentage of proline present in the N-terminal sequence and the known partial hydrolysis of the proline peptidically bound, the N-terminal sequences were determined for only 17 hydrolysis cycles. The overall sequence shows no significant homology with any of the protein sequences from the compiled data banks (EMBL and GenBank), as determined with the FASTA program (23). Similarly, no identical sequence was reported for any molecule present in a recent compilation of mycobacterial protein antigens (35). Furthermore, the high percentages of alanine and proline (seven and eight residues, respectively, for 17 amino acids) were not previously reported for mycobacterial proteins. The reported proline-rich sequence of a 36-kDa *Mycobacterium leprae* antigen did not contain a particularly high percentage of alanine (27). The herein reported N-terminal sequence is grossly representative of the huge composition in amino acids of the 45/47-kDa complex, in which the quantities of prolines and alanines are equivalent, approximately 20% for each of these amino acids.

To obtain protection against an infectious challenge with a virulent *M. tuberculosis* strain, living avirulent bacteria (*M. bovis* var. BCG or R_1) must have been previously injected (6, 8). Other investigations have demonstrated the role of T lymphocytes in controlling primary infection with *M. tuberculosis* and supporting the protection induced by a previous immunization with a living avirulent strain. On the contrary, the role of T lymphocytes for the development of lung lesions, the pathogenesis of tuberculosis, has been recently reevaluated (10). Similarly, recent clinical observations of an increased frequency of tuberculosis cases among humans suffering from a T-lymphocyte depletion during human immunodeficiency virus infection emphasized the role of T lymphocytes not only in controlling tuberculosis but also in creating the typical tubercle lesions (2).

The present report demonstrates that antigens present at very low concentrations on living bacteria can be selected by using the specific effectors of the immune response as selective tools. This result pinpoints a previous report concerning a BCG antigen (named antigen L) which was selected on its high potency to reveal DTH response in living-BCG-immunized guinea pigs (25). The 45/47-kDa antigen molecules were also found to elicit the DTH response, a T-lymphocyte immune response, essentially or only in living-BCG-immunized guinea pigs. Therefore, the roles of T-lymphocyte epitopes present on the 45/47-kDa molecules will be explored regarding their helper function not only for antibody responses but also for disease pathogenesis or immunity. The capacity of this complex to be immunogenic when delivered through live BCG in guinea pigs emphasizes the importance of the immunization schedule and the choice of immunological tools when attempting to select mycobacterial molecules that play a role in the infectious process.

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