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Immunology

Isolation of a proline-rich mycobacterial protein eliciting delayed-type hypersensitivity reactions only in guinea pigs immunized with living mycobacteria

(*Mycobacterium bovis*/bacillus Calmette–Guérin/*Mycobacterium tuberculosis*/purified protein derivative)

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ABSTRACT **Effective protection against a virulent challenge with *Mycobacterium tuberculosis* is induced only by a previous immunization with living attenuated mycobacteria, usually bacillus Calmette–Guérin (BCG). Living and killed bacteria share a number of common antigens. To identify and to purify molecules that are dominant antigens during immunization with living bacteria, a two-step selection procedure was used. Quantitative delayed-type hypersensitivity (DTH) reactions elicited in guinea pigs immunized either with living or with killed BCG were used to select or counterselect antigens present in BCG culture filtrates. Each major fraction eluted from a series of HPLC columns (gel filtration, DEAE, reverse-phase chromatography) was assayed and titrated on guinea pigs of each group. A protein with an unusual amino acid composition (40% proline, 12% threonine) was purified and N-terminally sequenced. To our knowledge, the sequence Thr-Pro-Pro-Xaa-Glu-Xaa-Pro-Pro-Pro-Pro-Gln-Xaa-Val-Xaa-Leu has not been previously reported. The protein was 100-fold more potent on guinea pigs immunized with living bacteria than on guinea pigs immunized with dead bacteria to elicit a DTH reaction.**

Mycobacterium tuberculosis is a facultative intracellular bacillus—i.e., whereas it first replicates within host macrophages, it secondarily replicates outside the phagocytes—namely, in necrotic tissues and particularly on the walls of excavations developing in pulmonary caseous lesions (1). The host-protective immunity, effective essentially during the primary infection by *M. tuberculosis*, results from the "activation" of macrophages—i.e., their ability to inhibit the multiplication of phagocytized bacteria. A central question in the immunology of the primary tuberculosis infection concerns the early control of infection such that the multiplication of bacilli and their secondary dissemination can be prevented. Prior vaccination with an attenuated strain of *M. tuberculosis* or with the classical bacillus Calmette–Guérin (BCG) leads to an enhanced cell-mediated immunity (CMI) and a delayed-type hypersensitivity (DTH) state toward mycobacterial proteins or extracts (2). Because both of these immune reactions are mediated by T lymphocytes, it was thought likely that vaccines able to stimulate this cellular population would result in the effective activation of macrophages to control *M. tuberculosis* infection. But it is not so clear that CMI and DTH responses are mediated by the same or even overlapping T-cell populations. When an increased resistance against a challenge with a virulent *M. tuberculosis* strain is obtained after a previous immunization with living attenuated mycobacteria like BCG (3) or R_1Rv (4), the DTH reactivity and CMI develop "synchroneously." On the contrary, when heat-killed bacteria of the same strains are injected alone or with Freund's adjuvant, only a marginal protection or no protective response toward virulent bacteria is detectable despite the perfect expression of DTH reactivity toward tuberculin or purified protein derivative (PPD). Classical (5) and more recent studies (6) have pointed out that the immunization procedures using dead bacteria or bacterial extracts are unable to protect experimental hosts fully against a challenge with virulent *M. tuberculosis* despite the development of a true DTH reactivity toward PPD or other complex antigen preparations. Furthermore, mice and guinea pigs immunized with a resting viable vaccine, a streptomycin-dependent strain of *M. tuberculosis*, were protected poorly against i.v. challenge infection with virulent tubercle bacilli. On the contrary, a strong immunity was induced (7) when the same vaccinating strain was used concurrently with streptomycin supplied to the host to permit its temporary multiplication. These results support the hypothesis that "growing bacilli" may synthesize unique molecules not expressed by resting bacilli or dead bacteria, molecules that have remained undetected to date. In the very same view some molecules may be more expressed by bacilli replicating *in vivo* than *in vitro*. Whatever the exact mechanism, an increase in the number or frequency of specific immune effectors selectively triggered by infection with living bacteria may reflect the increased synthesis or immunogenicity of some molecules present on mycobacteria actively multiplying in the host's environment.

In the context of this hypothesis, guinea pigs were sensitized with living or with dead BCG. The intensity of their respective DTH reactions elicited by mycobacterial antigens was used to select and counterselect such antigens.

MATERIALS AND METHODS

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

Guinea pigs of the two groups were tested for their DTH reactivity between 2 and 12 months after immunization. It has been shown previously that their DTH reactivity remains in the same range during the period (8). Usually the immunized

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Abbreviations: DTH, delayed-type hypersensitivity; BCG, bacillus Calmette–Guérin; PPD, purified protein derivative; CMI, cell-mediated immunity; i.d., intradermal(ly); TU, tuberculin unit(s); PVDF, poly(vinylidene difluoride).

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guinea pigs were tested five to eight times during the period with an interval of at least 45 days between two tests.

Measurement of DTH Reactions. The tuberculin-type tests were performed on the flanks of guinea pigs plucked 24 hr previously. Four i.d. injections were performed on each flank to compare different dilutions of the experimental material with dilutions of a standard PPD (8). The procedure assesses the level of reactivity of experimental preparations against the control standard PPD, and it allows expression of the potency of the tested preparation in tuberculin units (TU)/mg. All dilutions and injections were performed in phosphate-buffered saline containing Tween 80 (0.05%) to avoid adsorption of the tested molecules on the vials, syringes, etc. (9).

For precision assays, eight guinea pigs immunized with living BCG and eight guinea pigs injected with dead BCG received four successive dilutions of the tested material and the control PPD in alternated fashion in order to inject each dilution on different anatomical sites. The assays so performed allowed calculation of the potency of tested materials with an accuracy of >10%.

The potency of intermediate fractions during biochemical steps was assayed on two or four guinea pigs of each group with only one concentration of tested material and one standard concentration of PPD (0.25 μg of PPD in 0.1 ml). This simpler procedure reduced the number of immunized animals and gave an approximation in the calculated strength or titer of around 30%.

DTH reactions were measured 28 hr after i.d. injections, noting the degrees of induration as well. The longitudinal and transverse diameters of erythema were recorded in mm, and the values were plotted against the logarithm of concentrations of tested material or PPD. A curve was drawn using a classical regression analysis for each tested material and for each group of immunized guinea pigs. Comparison with the values observed for the standard PPD allowed transformation of the results into conventional TU/mg (10).

Culture Material. BCG strain $1173P_2$ was cultured on synthetic Sauton medium and harvested after 14 days. Molecules above 10 kDa excreted into the culture medium were concentrated and freeze-dried as described (11).

Purification Procedure. *Molecular sieve column.* A preparative Si300 column, 3 μm, 50 × 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 630 psi (1 psi = 6.89 kPa) was never reached. The preceding crude material was prepared in buffer/butanol solution according to previous protocol (11) and injected onto the column. The fractions determined by optical density (λ = 220 nm) were extensively washed at 4°C with deionized water containing butanol (4%), concentrated on an Amicon PM_{10} membrane, and freeze-dried. After freeze-drying the fractions were weighed and assayed for DTH potency. The fraction that contained a high concentration of molecules that would elicit a DTH reaction in guinea pigs immunized with living BCG was loaded onto a DEAE ion-exchange column.

DEAE column. A preparative DEAE-TSK 5PW column, 21.5 × 150 mm (LKB), was equilibrated with 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 maximum pressure of 420 psi. A linear gradient of NaCl (1 M) was applied after injection of 100 mg of the preceding fraction dissolved in 4 ml of starting saline buffer. Major fractions were collected according to optical density (220 nm), washed, concentrated on a PM_{10} membrane, and freeze-dried. Only the fraction containing antigens of high potency in eliciting DTH reactions on guinea pigs immunized with living bacteria was loaded onto the next column.

Reverse-phase column. An RP300 C_8 column, 10 μm of 4.6 × 250 mm (Brownlee, Applied Biosystems), was equilibrated with ammonium acetate buffer (20 mM NH_4COOCH_3, pH 6.5) under a flow rate of 2 ml/min with maximum pressure of 1500 psi; 7.5 mg of the fraction from the DEAE column was dissolved in 1 ml of the ammonium acetate buffer and injected. For the higher concentrations the elution gradient contained 90% acetonitrile. The optical density scan at 220 nm allowed separation of major fractions, from which acetonitrile was eliminated by concentration under vacuum at 40°C, before freeze-drying. The major fractions were assayed for their DTH potency on guinea pigs immunized with either living or dead BCG.

N-Terminal Sequencing and Amino Acid Composition. The fraction 3 obtained after reverse-phase chromatography was N-terminally sequenced on 200 pmol (10 μg) by automatic Edman degradation by an Applied Biosystems 470A. A total composition in amino acids was obtained from 50 μg of the fraction 3 on a Beckman autoanalyzer.

Electrophoresis. Four micrograms of the fraction 3 of the preceding reverse-phase column was loaded into the wells of a classical 10% SDS/PAGE gel, in buffer with or without 5% 2-mercaptoethanol and 3% SDS according to Laemmli (12). After electrophoresis monitored by migration of bromophenol blue, different staining procedures were used to detect proteins either in the gel itself or after transfer on a poly(vinylidene difluoride) (PVDF) sheet. The classical protein staining procedures, Coomassie blue, silver nitrate, India ink, colloidal gold (Aurogold), Quick stain, etc., did not lead to detection of any band or blot, in contrast to perfect staining results on other control proteins. Accordingly, 1 μl of *N*-hydroxysuccinimidoiminobiotin hydromide (NHS, iminobiotin, ref. 21117; Pierce) was added at a concentration of 1 mg/ml in water to 4 μg of the fraction 3 into 2 μl of buffer (0.1 M $NaHCO_3$). The mixture was held 30 min at room temperature and dried on a vacuum centrifuge (Speedvac, Savant). The biotinylated molecules were loaded into wells of a classical 10% SDS/PAGE gel as were nonbiotinylated molecules and stained Rainbow protein molecular mass markers (Amersham). The gel of the track containing biotinylated molecules was transferred to a PVDF sheet on which phosphatase-labeled streptavidin (Boehringer Mannheim) was used to identify the bands with a classical procedure (13). The gel of the track containing nonbiotinylated molecules was cut into 20 pieces, each being eluted in 1 ml of water containing Tween 80 (0.05%), and tested for ability to elicit DTH reaction on guinea pigs immunized with living BCG.

RESULTS

Crude BCG Culture Filtrate. The freeze-dried material present in BCG culture medium was assayed for its DTH reactivity on guinea pigs injected with living bacteria or with dead bacteria. The reactivity of the two groups of guinea pigs was found to be very similar in tests with the standard PPD. But, when the material obtained from BCG culture filtrate was assayed, the apparent activity of the crude material was around 10-fold more potent on guinea pigs immunized with living bacteria than on guinea pigs immunized with dead bacteria (Fig. 1).

Molecular Sieve Column. The freeze-dried material was loaded onto an Si300 column, the different fractions being titrated for TU/mg on guinea pigs injected with living or with dead bacteria in comparison with the standard PPD. An estimation of the potency (TU/mg) was calculated for each fraction tested on the two groups (Fig. 2). The apparent potency, found by DTH reactivity on guinea pigs immunized with living bacteria, increased in the first fractions with maximum for the fraction 4 and a decrease with fractions 5 and 6. The activity of fraction 4 was evaluated as being around 75,000 TU/mg when assayed on guinea pigs immu-

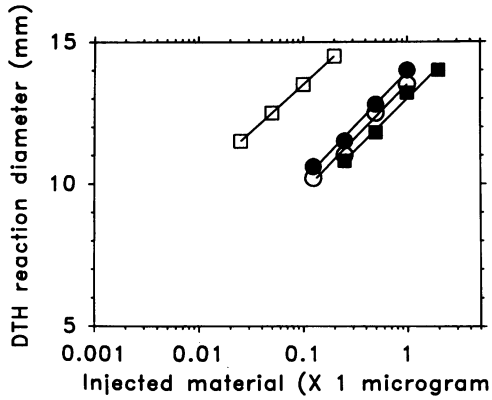

FIG. 1. Increased tuberculin potency of BCG culture filtrate titrated on guinea pigs immunized with living bacteria. The DTH reactions were elicited in guinea pigs immunized with living bacteria (open symbols) or with dead bacteria (closed symbols). Different amounts of the standard PPD (circles) or of the BCG culture filtrate (squares) were injected i.d. into eight guinea pigs of each group. Erythema was measured 28 hr later; the arithmetic means are plotted with the corresponding regression line. The SEM (around 10%) are not reported, to simplify the plots.

nized with living bacteria vs. 14,000 TU/mg on guinea pigs immunized with dead bacteria (Fig. 2).

DEAE Column. A sample of the preceding fraction 4 was injected onto a DEAE preparative column and eluted with a NaCl gradient. Known amounts of each fraction were assayed for their apparent tuberculin potency on both groups of guinea pigs. Fraction 1 was found to titrate at 180,000 TU/mg on guinea pigs immunized with living BCG but only at 5000 TU/mg on guinea pigs immunized with dead BCG (Fig. 3). Fraction 1 from DEAE was loaded onto the next column.

Reverse-Phase Column. An RP300 C_8 column was loaded with the preceding fraction 1 from DEAE. The column was eluted with an acetonitrile gradient (0–90%) according to the profile shown in Fig. 4.

Fraction 3, corresponding to 20–22% acetonitrile, possessed a very high potency on guinea pigs immunized with living BCG (550,000 TU/mg) but very low activity on guinea pigs injected with dead BCG, <3000 TU/mg (Fig. 4).

The material present in fraction 3 of the RP300 C_8 reverse-phase chromatography column was titrated more precisely on groups of eight guinea pigs per immunization group. The purified material was 100-fold more potent on guinea pigs

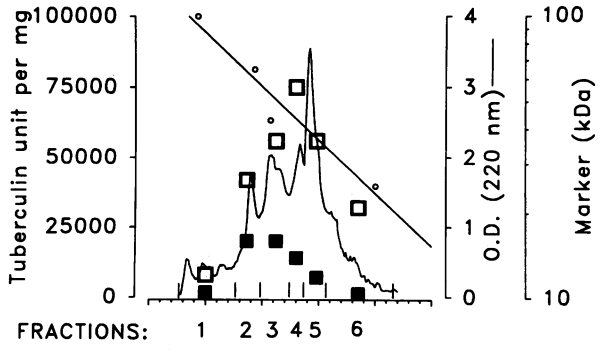

FIG. 2. Fractionation of BCG culture filtrate on a molecular filtration column. Different amounts of each fraction were assayed on guinea pigs immunized with living bacteria (open squares) or with dead bacteria (closed squares), which received also i.d. 10 TU of the standard PPD. The tuberculin potency was calculated by comparison with the values recorded for the standard PPD. The molecular mass markers used to calibrate the column were dextran 2000 (>10^6 Da), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa).

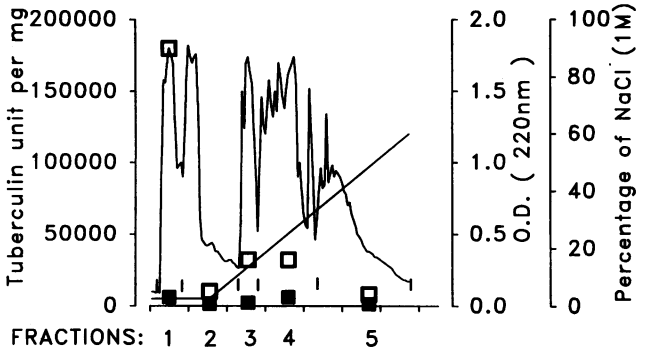

FIG. 3. Fractionation of the preceding Si300 fraction 4 on a DEAE column. Freeze-dried fraction 4 obtained from the Si300 separation step was loaded onto a TSK DEAE column. Known amounts of each fraction were assayed on guinea pigs immunized with living bacteria (open squares) or with dead bacteria (closed squares) in comparison with 10 TU of the standard PPD.

immunized with living bacteria than on guinea pigs injected with dead bacteria; the activities were, respectively, 450,000 TU/mg and 3000 TU/mg (Fig. 5).

PAGE. The molecules present in 4 μg of fraction 3 of the RP300 column were run on a SDS/PAGE gel, the gel being cut into 0.5-cm pieces, which were eluted in water with Tween 80 (0.05%). The presence of active material was tested directly on guinea pigs immunized with living bacteria (Fig. 6*A*). The front of activity was found in the 45-kDa range and the maximum was in the 55-kDa range. A biotinylated sample, run on the same gel, was transferred to a PVDF sheet and stained with phosphatase-labeled streptavidin and phosphatase substrate (Fig. 6*B*). The biotinylated molecules were found in the 55-kDa range. Other similar nonbiotinylated gels stained with Coomassie blue, silver nitrate, or Quick stain or corresponding PVDF blots stained with India ink or colloidal gold did not reveal any band or smear.

Amino Acid Composition and N-Terminal Sequencing. The global composition in amino acids indicated that proline was the major amino acid (40%), threonine (12.2%) being also in an unusually high concentration. Numerous amino acids were absent or present in <1% concentration: methionine, cysteine, tyrosine, phenylalanine, histidine, arginine. Similarly, the absence of absorbance at 280 nm on an UV spectrum between 195 and 310 nm, measured on a sample containing 1 mg/ml, indicated the absence of tryptophan. Of the other amino acids in the material, their concentrations were asparagine/aspartic acid, 1.2%; serine, 5%; glutamine/

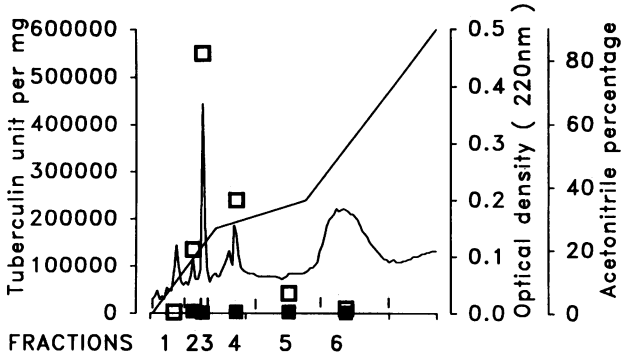

FIG. 4. Fractionation of the preceding DEAE fraction 1 on a reverse-phase column. Freeze-dried fraction 1 obtained from the DEAE separation step was loaded onto an RP300 C_8 column. Known amounts of each fraction were assayed on guinea pigs immunized with living bacteria (open squares) or with dead bacteria (closed squares) in comparison with 10 TU of the standard PPD.

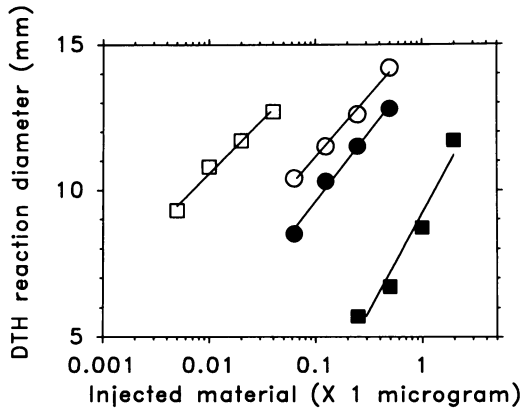

FIG. 5. High potency of the preceding fraction 3 from the reverse-phase chromatography separation only on guinea pigs immunized with living bacteria. The DTH reactions were elicited in guinea pigs immunized with living bacteria (open symbols) or with dead bacteria (closed symbols) with the standard PPD (circles) or with the fraction 3 from the reverse-phase chromatography separation (squares). The arithmetic means of eight measures per point were plotted with the corresponding regression lines.

glutamic acid, 11.5%; glycine, 7.7%; alanine, 8.9%; valine, 9.8%; isoleucine, 2.8%; leucine, 1.8%; and lysine, 1.6%.

An N-terminal amino acid sequence analysis was performed twice on fraction 3 with identical results. The sequence for 16 hydrolysis cycles contained four undetectable residues on hydrolysis steps 4, 6, 12, and 14. The sequence Thr-Pro-Pro-Xaa-Glu-Xaa-Pro-Pro-Pro-Pro-Gln-Xaa-Val-Xaa-Leu . . . (EMBL accession no. P80149) confirmed the high proportion of proline (six for 16 hydrolysis cycles).

DISCUSSION

The DTH reaction is known to be a strictly T-cell-dependent immune reaction (14, 15). It is well documented that T-cell receptor recognizes a peptide fragment that loads the groove of major histocompatibility complex class I or class II molecules (16, 17). These immunological findings, associated with the total disappearance of DTH reactivity of the fractions after incubation with subtilopeptidase A or pronase (data not shown), support the role of protein(s) or peptide(s) present in the fractions despite the absence of UV absorbance at 280 nm and of material that fails to stain with classical protein stains. The differences between biological tests and biochemical results were puzzling as far as the global composition in amino acids was obtained. The absence of histidine, tyrosine, phenylalanine, and tryptophan explained the absence of UV absorption at 280 nm; similarly, the failure to stain has to be related to the very low concentration of basic amino acids—absence of arginine and histidine, low concentration of lysine. The biotinylation of all protein(s) or peptide(s) present in the fraction through the N terminus and/or ε-NH_2 of lysine allowed us to reveal the protein(s) after transfer to a PVDF sheet with use of phosphatase-labeled streptavidin. The separation on SDS/PAGE showed that one unique band of biotinylated molecules was present in the range of the maximal DTH activity (Fig. 6). An isoelectric focusing gel (5% polyacrylamide/5% ampholytes, pH 2–11) loaded with a sample of the purified fraction was cut in 0.5-cm pieces after electrophoresis. The molecules eluted from individual pieces were tested for their DTH activity. The DTH activity was found essentially in one location and the adjacent one at pH 4 (data not shown). These results, SDS/PAGE, isoelectric focusing, and N-terminal sequencing, support the conclusion that only one protein species was present in the fraction.

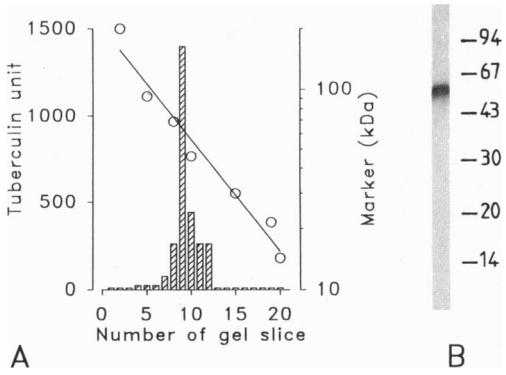

FIG. 6. Analysis of SDS/PAGE of fraction 3 of the reverse-phase chromatography separation. (*A*) A sample of fraction 3 obtained after the reverse-phase chromatography separation was loaded into one well of a 10% SDS/PAGE gel and adjacent wells received the stained molecular mass markers (Rainbow proteins, Amersham). After the run the gel of the track containing the sample was cut in 20 pieces, which were eluted in 1 ml of water containing Tween 80 (0.05%). The eluted molecules were assayed directly on guinea pigs immunized with living bacteria. (*B*) A sample of the fraction 3 obtained after the reverse-phase chromatography separation was biotinylated (NHS, iminobiotin; Pierce). The biotinylated sample was loaded into one well of a 10% SDS/PAGE gel and, after the electrophoresis, was transferred on a PVDF sheet that was stained with phosphatase-labeled streptavidin.

Regarding the molecular mass determination, the value of 55 kDa is certainly too large. It is well established that the classical SDS/PAGE method overestimates molecular mass when the percentage of proline is >10% in a given protein (18). The presence of 40% proline totally disturbs molecular mass determination by the SDS/PAGE technique. SDS/PAGE as used here was performed essentially to correlate biological activity recovered from gel slices with the corresponding blot of equivalent biotinylated molecules. The increase in molecular mass related to biotinylation was considered to be marginal considering the low percentage in lysine residues. At the most, three or four biotin residues were fixed per molecule; there was an increase in molecular mass of <1 kDa. After electrophoresis on SDS/PAGE the biotinylated and native molecules were certainly in the same range.

Regarding the N-terminal sequence, no identity was found with any of the protein sequences from the compiled data banks (EMBL; GenBank) as determined with the FASTA program (19) or from a recent compilation of mycobacterial protein antigens (20). The absence of detectable residues on hydrolysis steps 4, 6, 12, and 14 could be related to modified amino acids with poor solubility in the solvent used in the sequencer.

The experiments reported here were aimed at identifying mycobacterial antigens having the ability to reveal a DTH reactivity only or mainly in guinea pigs immunized with living bacteria. The fractionation procedure was chosen to select molecules in guinea pigs immunized with living bacteria but also to counterselect material that had the ability to reveal DTH reactivity in guinea pigs injected with dead bacteria. The second criteria explained the important differences in the activities according to immunization procedures (450,000 TU/mg vs. <3000 TU/mg).

It is known that factors such as the nature of the antigen, route, or presence of adjuvant influence immune responses (21–25). To test the influence of doses and route, groups of guinea pigs received i.d. or i.m. different doses of viable units of BCG (10^2–10^7) or dead BCG. The respective DTH reactivities toward PPD and the purified fraction were identical with those observed after the previous immunization procedures; only the intensity of the reaction was less important or

delayed after the low doses of antigen (data not shown). Similarly, to test the possible role of denaturation during heat killing of bacteria, a freeze-drying procedure outside the limit for persistence of obligatory linked water (1–2%) was used to kill the bacteria without heating (26). The results regarding DTH reactivity were identical with those obtained after immunization with heat-killed bacteria. The difference between the groups of guinea pigs regarding their DTH reactivity toward the fraction was always related to the living or dead state of the bacteria, never with the route, dose, presence of adjuvant, or denaturation by heat during the killing of bacteria. Recent experiments indicated that a group of similar molecule(s) is present in *M. tuberculosis* culture filtrate and, like the antigen obtained from BCG culture filtrate, elicits DTH only in guinea pigs infected with living *M. tuberculosis*.

The purification of a mycobacterial protein for its ability to differentiate between immunization with living bacteria and the injection of dead bacteria should prompt assay of its capacity to stimulate CMI *in vivo*.

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