Decreased Capacity of Recombinant 45/47-kDa Molecules (Apa) of Mycobacterium tuberculosis to Stimulate T Lymphocyte Responses Related to Changes in Their Mannosylation Pattern*

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The molecules secreted by Mycobacterium tuberculosis, Mycobacterium bovis, or BCG have been identified as major immunodominant antigens. Mass spectrometry analysis indicated similar mannosylation, a complete pattern from 1 up to 9 hexose residues/mole of protein, of the native species from the 3 reference strains. The recombinant antigen expressed in M. smegmatis revealed a different mannosylation pattern: species containing 7 to 9 sugar residues/mole of protein were in the highest proportion, whereas species bearing a low number of sugar residues were almost absent. The 45/47-kDa recombinant antigen expressed in E. coli was devoid of sugar residues. The proteins purified from M. tuberculosis, M. bovis, or BCG have a high capacity to elicit in vivo potent delayed-type hypersensitivity (DTH) reactions and to stimulate in vitro sensitized T lymphocytes of guinea pigs immunized with living BCG. The recombinant Apa expressed in Mycobacterium smegmatis was 4-fold less potent in vivo in the DTH assay and 10-fold less active in vitro to stimulate sensitized T lymphocytes than the native proteins. The recombinant protein expressed in Escherichia coli was nearly unable to elicit DTH reactions in vivo or to stimulate T lymphocytes in vitro. Thus the observed biological effects were related to the extent of glycosylation of the antigen.

The immune protection against tuberculosis can be achieved only by prior vaccination with a living vaccine (1–3). An explanation of this phenomenon could be that living bacilli release protective antigens, which are not present in sufficient amounts in dead bacteria or which are not appropriately presented to T lymphocytes. The T lymphocyte immune response plays a key role to control tuberculosis as is also demonstrated by the high sensitivity of AIDS patients to mycobacterial infections (4). Some of the proteins secreted by Mycobacterium tuberculosis during its growth have been proposed as potential immunodominant antigens to be included in a future vaccine (5, 6) or to improve diagnostic tests. Small quantities of immunogenic proteins detected in mycobacterial culture filtrate slow the growth of bacteria and safety precautions needed to work with virulent bacteria generated serious obstacles in obtaining and/or evaluating these antigens.

The development of recombinant DNA systems for efficient expression of mycobacterial genes in Escherichia coli appeared as an attractive alternative for obtaining larger amounts of mycobacterial antigens important for immune responses. However, recent reports on post-translational modifications of mycobacterial antigens such as acylation and glycosylation (7, 8) emphasized the importance of comparing structure and biological properties of native bacterial products to those obtained by recombinant DNA technology.

In the present paper, we describe the purification and biochemical characterization of Apa also known as the 45/47-kDa complex, a major immunodominant antigen secreted in vitro by the bacteria of the M. tuberculosis complex (i.e. M. tuberculosis, Mycobacterium bovis, and BCG). The gene apa encoding this antigen in M. tuberculosis has been expressed in Mycobacterium smegmatis and E. coli. The recombinant protein showed significant changes in the mannosylation patterns which were correlated with a lower or no potency to elicit in vivo DTH reactions on guinea pigs immunized with living BCG or stimulate their sensitized T lymphocytes in vitro.

MATERIALS AND METHODS

Bacteria and Growth Conditions

M. tuberculosis H37Rv virulent strain (14 001 0001), M. bovis AN5 (14 002 003), and M. bovis BCG strain 1173P2 reference strains were obtained from the “Center National de Référence des Mycobactéries” (Institut Pasteur). M. smegmatis mcΔ155:apa harboring the plasmid containing the apa gene from M. tuberculosis (H37Rv) (pLA1) has been previously described (9). The mycobacterial strains were grown on synthetic Sauton medium at 37 °C (10). Culture media, harvested after 6 days (M. smegmatis mcΔ155:apa) and 15 days (M. tuberculosis H37Rv, M. bovis AN5, M. bovis BCG) were filtered through a 0.22-μm filter in a glove box before their handling for biochemical procedures. The M15 E. coli host strain, pQE60 expression vector, and Qiagen REP4

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The nucleotide sequences reported in this paper for the 45/47-kDa molecules coding gene have been deposited in the GenBank data base under accession number AF013589 for the BCG sequence and X80268 for the M. tuberculosis sequence.

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1 The abbreviations used are: BCG, Bacillus Calmette Guénan; NNTA, nickel-nitritroacetic acid; PAGE, polyacrylamide gel electrophoresis; PVPD, polyvinylidene difluoride; PBS, phosphate-buffered saline; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF, matrix-assisted laser deionization time-of-flight; DTH, delayed-type hypersensitivity; TU, tuberculin units.

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**Decreased Antigenicity of Apa Related to Modified Glycosylation**

**DNA Techniques**

E. coli plasmid DNA was isolated using the Promega purification protocol. DNA manipulations were performed by standard procedures (11). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and New England Biolabs. Restriction fragments and polymerase chain reaction products were purified with a Wizard purification system (Promega).

**Purification of Mycobacterial Antigens**

The culture filtrates were extensively washed at 4 °C with 4% butanol in deionized water on a PM-10 Amicon membrane then concentrated about 10-fold and freeze-dried. The crude material from M. tuberculosis, M. bovis AN5, M. bovis BCG, and M. smegmatis mc²155:apa (50 mg/ml) was suspended in 50 mM sodium phosphate, pH 7.5, containing 4% butanol. Samples were centrifuged, at 40,000 × g for 2 h to remove insoluble material then applied on a AcA54 Ultrigel (2 × 87 cm) column equilibrated in the same buffer.

The fractions containing the 45/47-kDa species and eluted as a single broad peak were pooled and further purified by ion-exchange high performance liquid chromatography (DEAE-TSK-5PW, 21.5 × 150 mm; Amersham Pharmacia Biotech). The column was eluted with 10 mM sodium phosphate, pH 7.5, 10 mM NaCl, and 4% butanol, at a flow rate of 6 ml/min (maximum pressure, 55 bars). The proteins were eluted with a linear gradient of NaCl from 10 mM to 1 M. The 45/47-kDa species eluted at low salt concentration were concentrated after extensive washes on PM-10 membranes, then chromatographed on a reversed phase Aquapore RP300 C8 column (75 mm; 5 μm; 4.6 mm, Applied Biosystems Brownlee column) equilibrated with 20 mM ammonium acetate, pH 6.5. The elution was made with an acetonitrile gradient (0–90%) in the same buffer, under a flow rate of 2 ml/min with a maximum pressure of 115 bars.

**Expression in E. coli and Purification of rApa**

Recombinant Apa was expressed in E. coli by use of the Qiaexpressionist system followed by nickel-nitrotriacetic acid (Ni-NTA) purification (Qiagen), and ion exchange chromatography (Source 15Q-Pharma, Freiburg, Germany). The apa DNA from M. tuberculosis was amplified by polymerase chain reaction from pL434-2 (9). The apa sequence was modified to create an NcoI site in the reverse sequence (5'-CATGCGGTAGTACGTTGACCCACCATGACA-3') and a BamHI site in the reverse sequence (5'-TTAGGATCCGGCCGGTAAG-3') in order to subclone the NcoI-BamHI polymerase chain reaction product into the pQE60 expression vector. The translation product resulted in a decapeptide Gly-Ser-Arg-Ser-6'-His extension at the carboxyl terminus of Apa. This construct was introduced into the M15 host strain carrying the plasmid pREP-4, which constitutively expresses the lac repressor ensuring a tight regulation of protein expression. The construct included Apa signal peptide coding sequence, the recombinant species were detected among the periplasmic proteins by immunoblotting. A single colony of the transformant was used to inoculate 10 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. After overnight growth, 1 liter of LB broth with appropriate antibiotics was inoculated with the preceding culture and cells were further grown at 37 °C with vigorous shaking. When the OD₆⁰₀ reached 0.7, 2 mM isopropyl-β-D-thiogalactopyranoside was added and the culture incubated for 5 additional hours. Cells were harvested by centrifugation at 4000 × g and 4 °C for 10 min. The pellet suspended in 30 ml Tris-HCl, pH 8, 20% sucrose (80 ml/g wet weight) and 1 mM EDTA was incubated on ice for 10 min with gentle agitation. After centrifugation at 8000 × g and 4 °C for 20 min, the supernatant was discarded, and the pellet resuspended with the same volume of 5 mM imidazole. After centrifugation at 8000 × g for 20 min, the supernatant containing periplasmic proteins was collected. The sample was dialyzed against 10 mM Tris-HCl, pH 8.0, and loaded onto an Ni-NTA-agarose column equilibrated with the same buffer. The column was washed with 10 volumes of 10 mM Tris-HCl buffer + 10 mM imidazole and with 10 volumes of the same buffer + 20 mM imidazole. The recombinant antigen was eluted with 10 mM Tris-HCl + 200 mM imidazole. Samples were dialyzed against 20 mM Tris-HCl, pH 8.0, then applied onto a Source 15Q column (Amersham Pharmacia Biochem) equilibrated with the same buffer. The column was washed with 10 to 20 volumes of the buffer, then the recombinant antigen eluted with a 0 to 0.5 M NaCl gradient. Fractions containing recombinant antigen were pooled, dialyzed against deionized water, and lyophilized.

**Immunoblotting**

Protein fractions were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). Twin SDS-PAGE (12.5%) were run, one PVDF sheet being stained with Azure A (Amersham Pharmacia Biotech) to detect proteins, the other being prepared for immunoblot analysis. Membranes, treated with 5% nonfat dry milk in phosphate-buffered saline (PBS) at 37 °C for 1 h, were washed three times with PBS containing Tween 20 (0.2%), then further incubated for 1 h at 37 °C with either a rabbit immune serum directed against a crude M. tuberculosis culture filtrate, or a rabbit immune serum anti-Apa (1/3000 dilution) (10), or a monoclonal antibody supernatant (1/10–0.3) (1/1000 dilution) (12). Antibodies were diluted in PBS containing nonfat dry milk and Tween 20. After 3 washing steps in PBS/Tween, the sheets were incubated for 1 h at 37 °C with either an anti-rabbit or an anti-mouse IgG goat antibodies labeled with alkaline phosphatase (Bioys) diluted 1/3000 in PBS/nonfat milk/Tween 20. After incubation for 1.5 h at 37 °C, the PVDF sheets were washed three times in PBS/Tween 20 and revealed with a bromochlorindol phosphate-nitro blue tetrazolium substrate (13).

**Competitive Enzyme-linked Immunosorbent Assay**

A competitive enzyme-linked immunosorbent assay was used to detect and measure the concentration of Apa in the crude and partly purified samples as described previously (10). In brief, a potent polyclonal rabbit immune serum was obtained against the antigen of the Apa construct by using a classical immunization procedure involving dilution of 50 μg of the complex mixed with incomplete Freund adjuvant and an injection of 25 μg 1 month later. The purified antigen complex was immobilized on a plastic surface (100 μl at 1 μg/ml in carbonate buffer). The optimal dilution of rabbit serum (1/8000) was chosen in preliminary experiments as the last dilution of the plateau, just before the beginning of the decreasing slope. After incubation for 1 h at 37 °C with the fraction to be assayed, the remaining antibodies were measured on a plate coated with purified Apa complex. Known amounts of the Apa complex was included in each assay to determine the 50% value. Phosphatase-labeled antibodies directed against rabbit immunoglobulin G were used to determine the amounts of bound anti-Apa antibodies, resulting in a sensitivity of 2 ng/ml.

**Amino Acid Composition and Amino-terminal Sequence**

Amino acid composition was performed using a Beckman autoanalyzer 6300, a known amount of norleucine was included in each sample as an internal control. Amino-terminal sequence was performed by automatic Edman degradation using an Applied Biosystems 473A Sequencer. These chemical analysis were the basis for the determination of protein concentrations in the different immunological assays.

**Carbohydrate Analysis**

Carbohydrate composition analysis was performed on 20-μg samples of native or recombinant Apa molecules, hydrolyzed for 4 h in Teflon-capped vials in 100 μl of 4 M trifluoroacetic acid at 100 °C. The hydrolysates, dried under vacuum in a SpeedVac, were dissolved in 100 μl of deionized water. A sample of 10 μl was loaded onto a high pH anion exchange column (CarboPak PA1). The elution was run at 1 ml/min with a buffer and a Dienes Ninhydrin-chromatochromatography system equipped with a pulsed amperometric detector. 10-μl samples were injected in triplicate and mannosaccharides were identified in parallel runs by injection of samples with known amount of standard sugars.

**Mass Spectrometry Analysis**

ESI-MS—Mass spectrometry was performed with an API 365 triple-quadrupole mass spectrometer (Perkin-Elmer-Sciex, Thornhill, Canada). Samples (0.15 mg/ml) dissolved in water/methanol/formic acid (50:50:5, v/v/v) were introduced with a spray pump (5 μl/min) (Harvard Apparatus, South Natick, MA). The device was equipped with an atmospheric pressure ion source used to collect positive ions produced from a pneumatically assisted electrospray interface. The ion spray probe tip was held at 4.5 kV and the orifice voltage was set at 14 V. The mass...
spectrum was scanned continuously from m/z 900 to 1700 with a scan step of 0.1 and a dwell time per step of 2.0 s resulting in a scan duration of 16.0 s. Ten scans were averaged for each analysis. Mass calibration of the instrument was accomplished by matching ions of polypropylene glycol to known reference masses stored in the mass calibration table of the mass spectrometer. Data were collected on a Power Macintosh 8600/200 and processed through the Biotoolbox 2.2 software from Sciex.

MALDI-TOF—A Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) was used. The instrument was equipped with a nitrogen laser. All experiments were carried out using 300-nm time delay with a grid voltage of 92% of full accelerating voltage and an external calibration. The molecules were run in sinapinic acid solubilized in a mixture of water/acetonitrile (7:3) containing 0.1% trifluoroacetic acid.

**Immunization of Guinea Pigs and Measurement of DTH Reactions**

Groups of 10–12 out-bred guinea pigs (Hartley), weighing between 250 and 300 g at the beginning of experiments, were immunized intradermally with a single injection of 10⁷ living bacteria (BCG) in 0.2 ml of saline solution. One to six months after immunization, the guinea pigs were checked for their DTH reactivity. The DTH reactions were performed on the flanks of guinea pigs plucked the day before. Four different intradermal injections were performed on each flank. A standard PPD dose (0.25 μg corresponding to 10 tuberculin units (TU)) in 0.1 ml of PBS solution containing Tween 80 (0.05%) was injected intradermally in one site in order to measure the DTH reactivity level of each guinea pig toward an internal control. Dilutions of native proteins purified from *M. tuberculosis*, *M. bovis*, or BCG, or recombinant proteins purified from *M. smegmatis*:apa or *E. coli*:apa in 0.1 ml of saline/Tween solution were injected in the other sites. The areas of induration were measured 24 h later by two independent readers who measured 2 traverse diameters of induration. For each tested material a curve was drawn using classical regression analysis and compared with standard PPD values allowing conversion of the results into conventional TU/mg.

**Lymphocyte Proliferation Assay**

Four to five weeks after their immunization with living BCG, guinea pigs were euthanized by carbon dioxide breathing. The lymph nodes draining the sites of BCG injection were collected. Dissociated cells were adjusted at 10⁷ cells/ml in RPMI 1640 (Seromed), supplemented with glutamine, β₂-mercaptoethanol (5 × 10⁻⁵ M) and 10% heat-inactivated fetal calf serum. A volume of cell suspension (50 μl) was added to 50 μl of culture medium containing crude culture filtrate, native purified Apa, recombinant purified Apa, or control medium alone, in flat-bottomed 96-well plates (Corning). Plates were incubated at 37 °C in a humidified air/CO₂ incubator during 3 days, and 10 μl of [¹⁴C]thymidine solution in RPMI at 50 μCi/ml were added for an overnight period. The labeled cells were harvested onto glass fiber filters for liquid scintillation counting. Results were expressed as mean counts per minute from triplicate culture wells (mean ± 2 S.D.).

**RESULTS**

**Purification of the Apa from Mycobacterial Strains—**Mycobacterial Apa from culture filtrates was purified to homogeneity by conventional chromatography (Fig. 1). Gel permeation chromatography was used as the first step. Individual fractions were extensively washed, concentrated on a PM-10 membrane and freeze-dried. SDS-PAGE followed by transfer onto PVDF, allowed identification of antigen in each fraction by staining PVDF sheet with antibodies. The concentration of antigen in each fraction was also determined by a competitive enzyme-linked immunosorbent assay (E-ELISA).

**Figure 1.** Purification of Apa from *M. tuberculosis* culture filtrate by gel permeation (A), ion exchange (B), and reversed phase (C) chromatography. Absorbance at 220 nm is indicated by a continuous line, the gradient profiles in B and C is indicated by dashed lines. The concentration of 45/47-kDa molecules was determined by a competitive enzyme-linked immunosorbent assay (E-ELISA).

The same protocol was used to purify the native antigen from the culture filtrates of *M. bovis* or BCG. Chromatographic profiles were very similar with those obtained with *M. tuberculosis* (data not shown). The proteins were analyzed for purity by SDS-PAGE and staining with AuroDye or with antibodies after transfer on PVDF sheets (Fig. 2, lanes 1–3). Amino acid composition analysis and NH₂-terminal sequence (DEPEPAPPVPT) confirmed the identity of each sample. No contaminating sequences were found in the samples purified from *M. tuberculosis*, *M. bovis*, or BCG culture filtrates.

Recombinant molecules from *M. smegmatis* expressing the apa gene were purified by the methodology used for culture filtrates of *M. tuberculosis* complex (data not shown). SDS-PAGE analysis, immunoblotting, and the amino acid composition analysis showed that the recombinant proteins were similar to the species isolated from culture filtrates (Fig. 2, lanes 4). However, the NH₂-terminal sequence showed two species. The major one (65% of total) was identical to that of the native protein (DEPEPAPPVPT). The minor sequence (35% of total) missed the first Asp residue (PEPAPPVPT). Like in *M. tuberculosis*, *M. bovis*, or BCG the products of the single copy apa gene were found as two bands at 45 and 47 kDa. The slight difference observed in the migration of the 47-kDa molecules could be related to the difference in glycosylation pattern (see “Mass Spectrum Analysis”).

**Purification of Recombinant Apa Expressed in *E. coli*—**The recombinant antigen was also expressed in *E. coli* M15 (pREP4) strain as a fusion protein containing the Gly-Ser-Arg-
Ser-His$_6$ decapeptide at its COOH-terminal end, as a tag for affinity purification. The fusion protein was separated from most contaminants on a Ni-NTA column. Some minor contaminants, i.e., a band at 30 kDa, were observed on PVDF sheet after SDS-PAGE of the imidazole eluate. Ion exchange chromatography (Source$^{TM}$ 15Q Pharmacia) with NaCl gradient removed contaminating proteins and a single band of Apa was detected on PVDF sheet with AuroDye (Fig. 2, lane 5). Only the 47-kDa band was obtained, stained with the monoclonal antibody I10–0.3 (12). The 45-kDa molecules were present in the periplasmic crude extract. Their absence after the Ni-NTA affinity step was related to the loss of the COOH-terminal part, including the hexahistidine tag. The presence of the tag did not affect the relative mobility of the protein in SDS-PAGE as might be expected for molecules with a percentage of proline under 5–7% (Fig. 2).

The amino acid composition of the recombinant protein expressed in E. coli was in agreement with that deduced from the nucleotide sequence, which also included one Gly, one Arg, two Ser, and six His residues belonging to the tag. The NH$_2$-terminal sequence identified two species in equivalent quantities, namely DPEPAPPV and NADPEPAPPV. The origin of the two amino acids (Asn and Ala) at the NH$_2$ terminus of the second sequence was related to the presence of a second consensus site (ATA) recognized by the signal peptidase of E. coli (Table I).

**Table I**

**Sequence of Apa molecules**

| M. tuberculosis | M. bovis | E. coli |
|----------------|----------|--------|
| MHQVDPLNLTRRRGLAALAIAAMASLVLTVAVPATANA | DPEPAPPVPTTAAASPSTAAAPAPTPATVPAPPAAAN | |
| TPNQPGDPNAPPPFADPAPPPVIAFAPAFVRIDN | TPNAQPGDPNAPPPFADPAPPPVIAFAPAFVRIDN | |
| PVGGFPSALFAGQESDAEH | PVGGFPSALFAGQESDAEH (F/L) | |
| PWFGCPPPVNANDTVLGLRDLQKLYSAASEATDSKAAAR | PWFGCPPPVNANDTVLGLRDLQKLYSAASEATDSKAAAR | |
| LGSDMGEFMYPYPTGRINGEVTSDLANGVSASAYYE | LGSDMGEFMYPYPTGRINGEVTSDLANGVSASAYYE | |
| VKFSDPSKPLNQIWTGTVGSPAANPADGPPQRFVV | VKFSDPSKPLNQIWTGTVGSPAANPADGPPQRFVV | |
| WLGTANNPVDKAALAESIRLFLVPAPPPAPAPAE | WLGTANNPVDKAALAESIRLFLVPAPPPAPAPAE | |
| PAPAPAPAGEVAPPTPTTPQRTLPAGSRSHHHHH | PAPAPAPAGEVAPPTPTTPQRTLPAGSRSHHHHH | |

If the height of each molecular peak is related to its relative abundance the most frequent molecular species for the native antigen is that containing 7 mannose residues. The difference in molecular masses (34 Da) between M. tuberculosis and M. bovis or BCG (Fig. 3) results from the presence of a Phe$_{97}$ in the M. tuberculosis protein instead of a Leu residue in BCG.

The M. tuberculosis Apa glycoprotein structure and their glycoforms were supported by MALDI-TOP mass spectrometry analysis. The mass spectrum (Fig. 4) shows three sets of peaks in the mass range between 15 and 60 kDa assigned to double and single charged monomeric and dimeric molecular ions of the 45- and 47-kDa Apa proteins. The analysis of the double (Fig. 4A) and single charged (Fig. 4B) of the 47-kDa molecular ions has revealed the presence of 9 glycoforms which differ by one hexose residue. In addition, this analysis supported the absence of pentose residue covalently bound to the proteins. In agreement with the ESI-MS data, the glycoforms containing 6, 7, or 8 Man residues were the most abundant.

According to the NH$_2$-terminal sequence the recombinant Apa expressed in M. smegmatis should contain two distinct families of glycosylated species differing by 115 mass units. ESI-MS showed that it was the case. The visual inspection of the individual peaks observed by ESI-MS indicated that the
glycosylation pattern of the recombinant species expressed in M. smegmatis was different from that observed with the molecules purified from M. tuberculosis, M. bovis, or BCG. Most of the M. smegmatis recombinant protein contained 8 or 9 mannose residues/chain. Low level glycosylation (1 to 5) was less abundant (Fig. 5).

The recombinant antigen expressed in E. coli contains two distinct protein species in agreement with the NH2-terminal sequence analysis. The measured masses of these two proteins were 29,993 and 30,178 Da, respectively. The mass difference of 185 units accounted for the extra Asn-Ala dipeptide found at the NH2 terminus of the recombinant antigen. The contribution of the tag decapeptide moiety (1,210 Da) subtracted from the measured mass of the recombinant species gave the mass of the tag coding sequence has been added at the 3'-end cleavage. The appreciable differences between exact mass measurement. No post-translational modification, particularly glycosylation, was detected in the sample of recombinant protein expressed in E. coli (Fig. 6).

**DTH Reactions and T-cell Proliferation Assay—**Different amounts of the purified native or recombinant antigens were injected intradermally in sensitized guinea pigs. The reactions were read 24 h later and the transversal diameters of the induration were measured. A dose of PPD (0.25 μg corresponding to 10 TU) was injected to the same guinea pigs to evaluate the relative sensitization of each animal and to calculate the potency of each preparation in TU/mg (Fig. 7). The Apa isolated from M. tuberculosis, M. bovis, or from BCG culture filtrates elicited similar DTH reactions, while the recombinant protein expressed in M. smegmatis exhibited a 4-fold lower specific activity. The recombinant protein purified from E. coli:apa elicited only marginal DTH reactions in BCG-sensitized guinea pigs.

T lymphocytes, known to play a major role in the immune defense against mycobacterial infection (14), are the immune cells initiating the DTH reaction. Lymphocytes were obtained from the draining lymph nodes of guinea pigs immunized intradermally with living BCG 1 month before. The *in vitro* T-cell responses to the native and recombinant Apa were compared.

All crude or purified preparations exhibited a marked capacity to stimulate *in vitro* T lymphocytes. The stimulations observed with purified molecules were in the range of those obtained with the complex crude culture filtrates. The response was supported by CD4+ T lymphocytes as demonstrated by the total inhibition observed in the presence of anti-CD4+ immune serum (data not shown).

The 45/47-kDa recombinant antigen expressed in M. smegmatis required 10-fold higher concentrations to induce the same effect in *vitro* on sensitized T lymphocytes, whereas, the antigen expressed in E. coli induced only a marginal proliferation of sensitized T lymphocytes (Fig. 8).

**DISCUSSION**

Glycosylation of proteins was considered to be restricted to the eukaryotic kingdom until glycoproteins were found on the surface layer of the archaeabacterium *Halobacterium salinarium* (15) and served to differentiate Archaea and Eubacteria. Soon after, the existence of glycoproteins in Eubacteria was firmly established. The structure of the glycan chain of the crystalline surface layer of *Thermoanaerobacter thermohydrodsulfuricus* (16), and the structure of the glycosyl residues linked to the pilin of *Neisseria meningitidis* (17) were reported. Secreted proteins of *Flavobacterium meningosepticum* were also found to be glycosylated, and the detailed structural analysis of the O-linked glycan reported (18).

The Apa (45/47-kDa antigen complex) of *M. tuberculosis* was suggested to be glycosylated on the basis of concanavalin A binding assay (19). A more direct proof was obtained from the analysis of the NH2-terminal glycopeptide obtained by proteolysis which was shown to be *O*-glycosylated on Thr10 with 2 Man residues (20). Thereafter, other glycosylation sites and the structure of oligosaccharide residues were reported. The 45/47-kDa molecules (286 aa) were reported to be *O*-glycosylated on Thr10 and Thr18 with mannobiose (α-D-Manp(1→2)α-D-Manp), on Thr27 with a single Man(α-D-Manp), and on Thr277 with a mannose, a mannobiase, or a mannotriose (α-D-Manp(1→2)α-D-Manp(1→2)α-D-Manp) (21).

The three major peaks observed by mass spectrometry of the Apa molecules purified from *M. tuberculosis* were assigned to the protein moieties (28,780 Da) to which 6, 7, or 8 mannose residues (162 mass units/residue) have been covalently bound. These relatively frequent species (22, 24, and 17%, respectively, of the total if we refer to the peak height as a quantitative indicator) corresponded to the molecules which have been previously reported (21). Minor species, undetected until now, were revealed by ESI-MS. Although quantitation by peak height analysis remains subject of caution, we estimated that significant amounts of antigen correspond to those containing 3 mannoses (~5%), 4 mannoses (~9%), and 5 mannoses (~14%). Those species containing 1, 2, or 9 mannoses are less than 3% each, whereas unglycosylated protein represents ~1% of total. Such estimations were supported by the results obtained from the MALDI-TOF analysis known to preserve native structure diversity (22). The presence of arabinose in the chemical analysis was related to a low amount of contaminating polysaccharides like arabinomannan in the purified samples.

We assigned the 45-kDa band on SDS-PAGE as the source of three peaks on ESI-MS with masses of 27,616, 27,778, and 27,941 Da. As they differed by 162 and 163 mass units, respectively, it was reasonable to speculate that they belong to the same polypeptide chain with various degrees of glycosylation. Moreover, it is tempting to propose that the 27,616 Da peak corresponds to the COOH-terminal truncated peptide (1–275), resulting from the proteolytic cleavage between Pro275 and Thr276. The calculated mass of the fragment 1–275 devoid of mannose is 27,616 Da. The hypothesis of the COOH-terminal truncation of the native protein was in agreement with our results using the recombinant antigen expressed in E. coli. The tag coding sequence has been added at the 3’ end of the *apa* gene. The presence of the signal sequence directed the recombinant species to the periplasmic space of transformed bacteria. SDS-PAGE and immunoblot analysis of the crude preparation of the periplasmic proteins obtained from transformed E. coli revealed two bands at 45 and 47 kDa (9). However, in the present genetic setting only the 47-kDa molecules loading the His tag at the COOH-terminal were retained and further purified by Ni-NTA chromatography. The molecules lacking the tag were found in the flow-through fraction of the Ni-NTA step (data not shown), supporting the proposal for a COOH-terminal cleavage. The appreciable differences between exact mass determinations performed with ESI-MS or MALDI and the SDS-PAGE method were certainly related to the important percentage in proline (20%) of the Apa molecules as it has been
demonstrated for other proline-rich molecules such as collagen (23).

Mass spectrometry analysis of the recombinant molecules purified from *M. smegmatis::apa* confirmed the presence of the two peptide sequences found by NH₂-terminal sequencing. A major protein (65%) bearing the DPEVAPPVPT... sequence was found associated with a minor protein (35%) bearing the PEVAPPVPT... sequence. The major peak, measured at 30,080 Da by mass spectrometry, was attributed to the protein core (28,780 Da) on which 8 Man residues (1,296 Da) have been added. Some molecules at 29,918 and 30,242 Da bore 7 or 9 Man residues and some molecules bearing 3, 4, 5, or 6 Man were detected. The molecules with 0, 1, or 2 Man were not detected in the present purified batch. The minor important peak at 29,965 Da was attributed to the peptide sequence lacking the NH₂-terminal Asp (28,665) on which 8 Man residues (1,296 Da) have been added. The pattern of glycosylation appeared similar to the preceding one.

The mass spectrometry analysis of the recombinant molecules purified from *E. coli* confirmed the difference observed in the NH₂-terminal sequence when the NH₂-terminal sequencing was performed on the purified sample. The two peaks were equivalent, supporting the 50/50 evaluation of NH₂-terminal sequencing. The difference in their masses (185 Da) was in

![Fig. 3. ESI-MS analysis of Apa molecules, 45/47 kDa, isolated from culture filtrates of *M. tuberculosis*, *M. bovis*, and BCG. All spectra are deconvoluted into the corresponding molecular weights through Biotoolbox 2.2. The number (in bold) on each peak corresponds to the degree of glycosylation.](image)

![Fig. 4. Positive MALDI-TOF mass spectrum of Apa purified from *M. tuberculosis*. The three set of peaks were assigned to double (M⁺2), single (M⁺) charged, and dimeric (2M⁺) molecular ions of the 45-kDa (●) and the 47-kDa (□) Apa proteins. The insets of A and B show the single and double charged molecular ions typifying the glycoforms. The number (in bold faced type) on each peak corresponds to the degree of glycosylation.](image)

![Fig. 5. ESI-MS analysis of the recombinant Apa expressed in *M. smegmatis*. Two NH₂-terminal sequences were determined (DPEVAPPVPT... and PEVAPPVPT...) corresponding to the 115 mass units difference. The molecular masses of complete molecules were indicated in normal type and italic for the molecules lacking the NH₂-terminal aspartic acid. The number (in bold) on each peak corresponds to the degree of glycosylation. All spectra are deconvoluted into the corresponding molecular weights through Biotoolbox 2.2.](image)
E. coli.

Two NH2-terminal sequences were determined (DPEPAPPV)

Biotoolbox 2.2
deconvoluted into the corresponding molecular weights through

protein (28,780 Da) in the limits (±3 Da) of measures. All spectra are deconvoluted into the corresponding molecular weights through Biotoolbox 2.2.

The contribution of the tag decapeptide moiety (1,210 Da) subtracted from the measured mass (29,993 Da) gave the mass calculated for the

peptide (29,783 Da) corresponding to the 185 mass units difference.

purified from M. smegmatis::apa

DTH reactivity level of each guinea pig. Dilutions of native Apa proteins

standard PPD values allowing conversion of the results into conven-

later. A

dose (0.25

nized with living BCG.

FIG. 6. ESI-MS analysis of the recombinant Apa expressed in E. coli. Two NH2-terminal sequences were determined (DPEPAPPV and NADPEPAPPV) corresponding to the 185 mass units difference. The contribution of the tag decapeptide moiety (1,210 Da) subtracted from the measured mass (29,993 Da) gave the mass calculated for the protein (28,780 Da) in the limits (±3 Da) of measures. All spectra are deconvoluted into the corresponding molecular weights through Biotoolbox 2.2.

have been previously performed (25). In these models, the glycopeptide-specific T lymphocyte responses implicated that both glycans and peptides make contact with the T lymphocyte receptor-binding site (reviewed in Ref. 26).

In parallel with this possibility for a modification on the binding capacity of the peptides by their glycosylation, an important role certainly exists for the glycosylation of the bacterial proteins by mannose. In a recent review, Stahl and Ezekowitz (27) emphasized the role of carbohydrates that decorate the surface and cell walls of infectious agents. According to their views, the macrophages, which play a key role in both innate and adaptive immunity, are able to handle a wide range of molecules, and/or pathogens through their mannose receptors. Similarly, the dendritic cells, which appear unique in their capacity to initiate primary antigen-specific immune response (28), are also able to pick up mannosylated molecules with a higher efficiency than non-mannosylated ones. The mannosylation of protein antigen and peptides resulted in a 200/10,000-fold enhanced potency to stimulate class II-restricted peptide-specific T cell clones compared with non-mannosylated peptides (29).

In accordance with these views, the fully mannosylated Apa molecules purified from M. tuberculosis, M. bovis, or BCG displayed a high capacity to stimulate specifically T lymphocytes collected from BCG-immunized guinea pigs. The molecules were found to be “immunodominant” antigens when their capacity to stimulate the cells was compared with the molecules present in crude culture filtrates.

Recombinant molecules obtained from M. smegmatis were also mannosylated. But their capacity to stimulate T lymphocytes was found decreased when compared with native purified molecules. It is tempting to speculate that changes in mannosylation sites and extend could influence the responses. The mass spectrometry analysis indicated a slight change in the global extent of mannosylation. Only more detailed studies comparing the masses of peptides generated by appropriated proteolytic enzymes acting on the Apa obtained from M. tuberculosis and from recombinant M. smegmatis could give unambiguous responses on the site(s) and extent of mannosylation. The slight decrease in the capacity of Apa purified from M. smegmatis to elicit DTH reactions also supported that mole-
cules were slightly different than the native ones for the immune responses.

The recombinant molecules purified from *E. coli* were highly impaired in their capacity to stimulate sensitized T lymphocytes *in vivo* and *in vitro*. A larger production of recombinant molecules produced in *E. coli* will permit an analysis of larger amounts of molecules in order to evaluate more precisely the decreased capacity of non-mannosylated molecules to stimulate T lymphocytes *in vitro* and *in vivo*. The availability of larger quantities of recombinant molecules will permit an analysis on which cell type (macrophages, dendritic cells, or T lymphocytes) the major effect is acting.

In parallel work Apa molecules purified from *M. tuberculosis* were demannosylated by trifluoromethane sulfonic acid or by α-mannosidase treatment. The assays of these demannosylated molecules showed a profound decrease of their capacity to stimulate T lymphocytes *in vitro* and *in vivo* (30). Such results emphasized the role of mannoseylation of bacterial proteins to increase their capacity to be recognized by the cells of the immune system.

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