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Purification and Characterization of a Low-Molecular-Mass T-Cell Antigen Secreted by *Mycobacterium tuberculosis*

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A novel immunogenic antigen, the 6-kDa early secretory antigenic target (ESAT-6), from short-term culture filtrates of *Mycobacterium tuberculosis* was purified by hydrophobic interaction chromatography and anion-exchange chromatography by use of fast protein liquid chromatography. The antigen focused at two different pIs of 4.0 and 4.5 during isoelectric focusing, and each of these components separated into three spots ranging from 4 to 6 kDa during two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The apparent differences in molecular masses or pIs of these isoforms were not due to posttranslational glycosylation. The molecular weight of the purified native protein was determined by applying gel filtration and nondenaturing polyacrylamide gel electrophoresis and found to be 24 kDa. ESAT-6 is recognized by the murine monoclonal antibody HYB 76-8, which was used to screen a recombinant λgt11 *M. tuberculosis* DNA library. A phage expressing a gene product recognized by HYB 76-8 was isolated, and a 1.7-kbp fragment of the mycobacterial DNA insert was sequenced. The structural gene of ESAT-6 was identified as the sequence encoding a polypeptide of 95 amino acids. The N terminus of the deduced sequence could be aligned with the 10 amino-terminal amino acids derived from sequence analyses of the native protein. N-terminal sequence analysis showed that the purified antigen was essentially free from contaminants, and the amino acid analysis of the antigen was in good agreement with the DNA sequence-deduced amino acid composition. Thus, the heterogeneities observed in the pI and molecular weight of the purified antigen do not derive from contaminating proteins but are most likely due to heterogeneity of the antigen itself. Native and recombinant ESAT-6 are immunologically active in that both elicited a high release of gamma interferon from T cells isolated from memory-immune mice challenged with *M. tuberculosis*. Analyses of subcellular fractions of *M. tuberculosis* showed the presence of ESAT-6 in cytosol- and cell wall-containing fractions. Interspecies analyses showed the presence of ESAT-6 in filtrates from *M. tuberculosis* complex species. Among filtrates from mycobacteria not belonging to the *M. tuberculosis* complex, reactivity was observed in *Mycobacterium kansasii*, *Mycobacterium szulgai*, and *Mycobacterium marinum*.

The efficacy of the live attenuated strain of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which is presently the only available antituberculosis vaccine, varies considerably from one population to another. In a recent meta-analysis of 14 prospective trials and 12 case-control studies, the BCG vaccine was found on average to reduce the risk of tuberculosis by only 50% (15). In view of this and the current alarming increase in the incidence of tuberculosis all over the world, there is now, more than ever, an urgent need for research in improved methods of immunoprophylaxis against tuberculosis.

Previous reports from this (6, 8, 10) and other laboratories (29, 30) have suggested that surface proteins and proteins actively secreted by *Mycobacterium tuberculosis* are important targets for the immune system during the early phase of an infection. Such antigens are, therefore, candidates to be included in a future vaccine which could be in the form of a live vaccine, e.g., a genetically modified BCG, or a nonliving, subunit vaccine. Studies of memory immunity to tuberculosis have demonstrated the specificity of immune, gamma interferon (IFN-γ)-producing T lymphocytes in mice to be directed against two fractions of secreted proteins ranging in molecular mass from 3 to 9 kDa and from 25 to 31 kDa (10). In a recent study, the key targets for this early recognition of infecting *M. tuberculosis* were identified to be the MPT59 component of the fibronectin-binding protein complex, often designated the antigen 85 complex (26, 27), and a protein with an apparent molecular mass of 6 kDa designated ESAT-6 (6-kDa early secretory antigenic target) (7). In this report, we describe the purification and biochemical characterization of ESAT-6 from *M. tuberculosis* culture filtrates as well as the cloning, expression, and sequence of the structural gene.

MATERIALS AND METHODS

Antigen preparation. Short-term culture filtrate (ST-CF), which is highly enriched in proteins actively secreted by *M. tuberculosis*, was produced with minor modifications as described previously (9). *M. tuberculosis* H37Rv was grown on an orbital shaker at 37°C in modified Sauton medium. The bacteria were removed from the cultures by filtration (Milliguard housing system [Millipore]) after 7 days of growth, and the culture supernatant was concentrated (×100) on YM3 membrane (Amicon, Danvers, Mass.), resulting in protein concentrations ranging from 5 to 20 mg/ml.

Three- to 5-week-old culture filtrates from stationary cultures of *M. tuberculosis* H37Rv were prepared as described previously (27). Culture filtrates from other mycobacterial species were prepared as described before (3).

MAbs. The monoclonal antibodies (MAbs) reacting with low-molecular-mass components in ST-CF used in this study were ST-3 and HYB 76-8. ST-3 was generated by immunization of C57BL/6J mice with low-molecular-mass components from ST-CF as described previously (7). HYB 76-8, a MAb isolated from purified protein derivative-immunized mice (23), was kindly provided by J. Klausen, Statens Seruminstitut, Copenhagen, Denmark.

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The following MAbs, also used in this study, have been described previously: L24.b5, which reacts with the secreted protein MPT64 (3); HBT12, which reacts with the phosphate-binding membrane protein PstS (2, 25); and HAT5, which reacts with the cytoplasmic 65-kDa heat shock protein GroEL (4) (all produced at the Statens Seruminstitut).

Purification of ESAT-6. Hydrophobic interaction chromatography was used as the first step of protein fractionation of ST-CF. Ammonium sulfate (3 M concentration) in 50 mM phosphate buffer (pH 8.5) was added to concentrated ST-CF to obtain a final concentration of 1 M ammonium sulfate. The resulting precipitate was removed by centrifugation. The supernatant was applied to a phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated previously with 50 mM phosphate buffer (pH 8.5) containing 1 M ammonium sulfate. Filtrate components bound to the column were eluted by decreasing the concentration of ammonium sulfate along a linear gradient from 1 to 0.5 M and further in steps from 0.5 to 0.4 M, 0.4 to 0.2 M, and 0.2 to 0 M. Following elution with phosphate buffer, the column matrix was washed with water and then with a 70% ethanol in water solution. Fractions containing the HYB 76-8-reactive antigen were pooled and concentrated, and the buffer was exchanged for a 10 mM Tris buffer (pH 8.0) on an Amicon YM3 membrane. Further fractionation was performed by anion-exchange chromatography with a Mono Q column (Pharmacia) and 10 mM Tris buffer (pH 8.0) including 3 M urea. The proteins were eluted by applying a linear gradient of 0 to 0.5 M NaCl. Following purification, the purified antigen was concentrated and the buffer was exchanged for phosphate-buffered saline (PBS) on an Amicon YM3 membrane.

When ESAT-6 was purified from 3- to 5-week-old culture filtrates, the first step applied was anion-exchange chromatography, as described previously (27). In brief, concentrated culture filtrate was loaded onto a DEAE-Sepharose CL-6B (Pharmacia) column preequilibrated with 30 mM Tris buffer (pH 8.7) containing 3% Methylcellosolve (ethyleneglycol-3-methoxyether [Wako Chemicals, Osaka, Japan]), and proteins bound to the matrix were eluted by applying a linear gradient of sodium chloride in the Tris buffer. Methylcellosolve is a mild hydrophobic solvent which improves separation of the culture filtrate components. Fractions containing large amounts of antigen reacting with HYB 76-8 were collected and concentrated, the buffer was exchanged, and the fractions were separated further by the same steps as those described here for the purification of ESAT-6 from ST-CF.

SDS-PAGE and immunoblotting. Antigen samples from fractions obtained during the purification procedures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 12.5% or a 10 to 20% polyacrylamide separation gel (24). Low-molecular-mass protein standards, ranging from 200 kDa (myosin) to 14.4 kDa (lysozyme), obtained from Bio-Rad Laboratories (Richmond, Calif.), and myoglobin molecular mass markers, ranging from 16,949 to 2,512 kDa, obtained from LKB (Bromma, Sweden), were used in this study. The components in the fractions were visualized by silver staining of the fixed gels (13). To identify antibody reactivities, the separated proteins from the SDS-polyacrylamide gels were transferred with 20 mM Tris-HCl buffer (pH 8.5) containing 192 mM glycine and 20% ethanol to nitrocellulose membranes with a semidry electroblotter. The membranes were incubated with MAbs and then with peroxidase-labeled rabbit anti-mouse immunoglobulin (Dakopatts). The antibody reactivity was detected by staining with 3-3′,5,5′-tetramethylbenzidine (0.6 mg/ml) in the presence of hydrogen peroxide (0.05%).

Molecular mass determination. Molecular sieving on a Superdex 75 column (Pharmacia) in the presence of 3 M urea and 0.5 M NaCl in 10 mM Tris-HCl buffer was used. The standard proteins were bovine serum albumin (67 kDa), ovalbumin (43 kDa), RNase A (13.7 kDa; Pharmacia) and aprotinin (6.5 kDa; Sigma).

The native molecular mass of the HYB 76-8-reactive antigen was also determined by PAGE as described previously (31). This method involves electrophoresis under nondenaturing and nonreducing conditions in a series of gels with various concentrations of polyacrylamide.

Two-dimensional gel electrophoresis (2D-E). Samples of ST-CF and purified ESAT-6 were submitted to isoelectric focusing by the method described by Hochstrasser et al. (21). The pI range of the ampholines included in the first dimension was from 3 to 10 (Biolyt 3/10 and Biolyt 4/6; Bio-Rad). Following first-dimension focusing, samples were separated by electrophoresis in an SDS–12.5% polyacrylamide gel with diacrylylpiperazine as the cross-linker. Gels were either blotted onto nitrocellulose membranes and subsequently analyzed for antibody reactivities or silver stained.

Glycoprotein analysis. A glycan-protein double-binding kit (Boehringer Mannheim) was used to detect and differentiate between glycoproteins and nonglycosylated proteins on SDS-PAGE-separated preparations of either ST-CF or purified HYB 76-8-reactive antigen blotted onto polyvinylidene difluoride membranes. A nonglycosylated control protein (creatinase) and a control glycoprotein (fetuin) were included. The membranes were washed with PBS (pH 6.5) prior to analysis. Sugar hydroxyl groups were oxidized to aldehydes with sodium metaperiodate at 10 mM in 100 mM sodium acetate buffer (pH 5.5). After a washing with PBS, the membranes were incubated in acetate buffer with digoxigenin-3-*O*-succinyl-ε-aminocaproic acid hydrazide, thus permitting the covalent coupling of digoxigenin to the generated aldehyde groups. The amino groups of the proteins present were labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester in 50 mM potassium phosphate buffer (pH 8.5). The membranes were washed in Tris-buffered saline (TBS; pH 7.5), blocked for 30 min in the blocking reagent of the kit dissolved in TBS, washed, and incubated with anti-digoxigenin horseradish peroxidase-conjugated antibody and anti-fluorescein alkaline phosphatase-conjugated antibody in TBS for 1 h. Finally, the membranes were washed and stained with 5-bromo-4-chloro-3-indolylphosphate, 2-(-4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride, 4-triethylenetrioxy-1-naphthol, and H_2O_2 in TBS. Protein staining appears reddish brown, and glycan staining appears blue.

SDS-PAGE and electroblotting to polyvinylidene difluoride membranes for N-terminal sequencing. Gels (20%) were subjected to preelectrophoresis overnight with resolving gel buffer and 20 mM mercaptopropionic acid as a scavenger as described by Houen (22). Subsequently, samples were boiled in sample buffer and subjected to electrophoresis as described for SDS-PAGE. Gels were then used for electrotransfer of proteins to polyvinylidene difluoride membranes as described previously (22).

Protein sequencing. Amino acid sequence analysis of purified protein and of electroblotted samples was done on a model 477A sequenator with on-line analysis of phenylthiohydantoin amino acids with chemicals and programs supplied by the manufacturer (Applied Biosystems, Foster City, Calif.).

Amino acid analysis. Samples were hydrolyzed in 6 M HCl containing 0.5% phenol for 24 h at 110°C. After drying, the samples were analyzed on an ion-exchange column with a Waters high-performance liquid chromatography system with postcolumn ortho phthal aldehyde (OPA) quantitation of amino acids (12).

Cellular assays. The biological activity of purified ESAT-6 and the fusion proteins recognized by HYB 76-8 and ST-3 were monitored by a combination of two parameters: T-cell proliferation and T-cell production of IFN-γ. Cells were obtained from the spleens of memory-immune C57BL/6J mice, generated as described in detail by Andersen et al. (7, 10). Briefly, mice received a primary infection with 10^4 CFU of *M. tuberculosis* and were treated with antibiotics in their drinking water to clear the infection, after which they were rested for 4 to 6 months. The mice were reinfected at a time point at which no recall response to mycobacterial antigens by the splenic lymphocytes could be detected and were therefore regarded as having a resting recirculating memory T-cell pool at the time of challenge. Spleen lymphocytes were isolated 6 days after the challenge, and the test antigens were added in various concentrations to the lymphocyte cultures as described previously (10). After 48 h of incubation, supernatants from one set of cultures were harvested and quantitated for IFN-γ by an enzyme-linked immunosorbent assay (Holland Biotechnology, Leiden, The Netherlands). The other set of cultures was pulsed with radiolabeled thymidine (1 μCi per well) for 22 h, harvested, and subjected to liquid scintillation counting (LKB beta counter), by which the level of cell proliferation was monitored.

Subcellular fractionation of *M. tuberculosis*. Seven-day-old cultures of *M. tuberculosis* H37Rv were harvested by centrifugation and washed twice in cold PBS. The wet pellet was weighed and resuspended at a ratio of 1 ml per g of bacteria (in this case, 23 g of wet, drained pellet) in a lysis buffer consisting of PBS supplemented with the protease inhibitor phenylmethylsulfonyl fluoride (Boehringer Mannheim) and EDTA to a final concentration of 1 mM. The bacteria were lysed with a French pressure cell (American Instrument Company, Silver Spring, Md.), contained in an MSCIII safety cabinet, at a pressure of 12,000 lb/in^2. This procedure was repeated three times. Bacterial cells resisting this treatment were recovered by low-speed centrifugation (1,000 × *g*) × 2. The supernatant was centrifuged for 20 min at 20,000 × *g*. The pellet from this centrifugation was assumed to consist mainly of cell walls. The supernatant (in this case, 36 ml) represented a mixture of cytoplasmic components and cell membranes. RNA was degraded by RNase treatment for 1 h at 4°C (0.45 mg of RNase A [Boehringer Mannheim] per ml), after which the sample was subjected to ultracentrifugation at 45,000 rpm for 3 h at 5°C in four tubes in a fixed-angle Sorvall rotor (TH1270; DuPont Medical Products, Newtown, Conn.). The pellets, containing the cell membranes, were washed in fresh lysis buffer and recentrifuged. For SDS-PAGE analyses, the fractions were solubilized in sample buffer as described above.

Cloning of genes encoding low-molecular-weight proteins. The recombinant lambda gt11 *M. tuberculosis* DNA library constructed by Young et al. (33) and obtained through the World Health Organization IMMTUB program (WHO. 0032.wibr) was screened for phages expressing gene products which would bind HYB 76-8 and ST-3, MAbs known to recognize low-molecular-mass components of the ST-CF. The screening and recloning procedure for the phages have been described previously (5).

Production of fusion proteins. *Escherichia coli* Y1089 was grown at 37°C in Luria-Bertani medium supplemented with 100 μg of ampicillin per ml, $MgCl_2$ to a final concentration of 10 mM, and 0.2% maltose to an optical density at 460 nm of 0.6 when infected with either lambda AA226 or lambda AA227. Isopropyl-β-D-thiogalactopyranoside was added at this time point to a final concentration of 5 mM, and the cultures were incubated for an additional 2 h. The bacteria were harvested by centrifugation, and the recombinant material was released by resuspending the bacterial pellet in PBS (pH 7.4) with 5 mM EDTA and subjecting the material to three passages through a French pressure cell (American Instrument Company) at 12,000 lb/in^2. The viscosity was reduced by DNase and RNase treatment in the presence of 10 mM $MgCl_2$. The fusion proteins were recovered from the pellets obtained after centrifugation at 12,000 × *g*, solubilized in SDS sample buffer, and purified to homogeneity on a 5% preparative SDS-PAGE column (PREP-CELL; Bio-Rad). The buffer used in isolated recombinant protein preparations was exchanged for PBS, and residual SDS was removed by

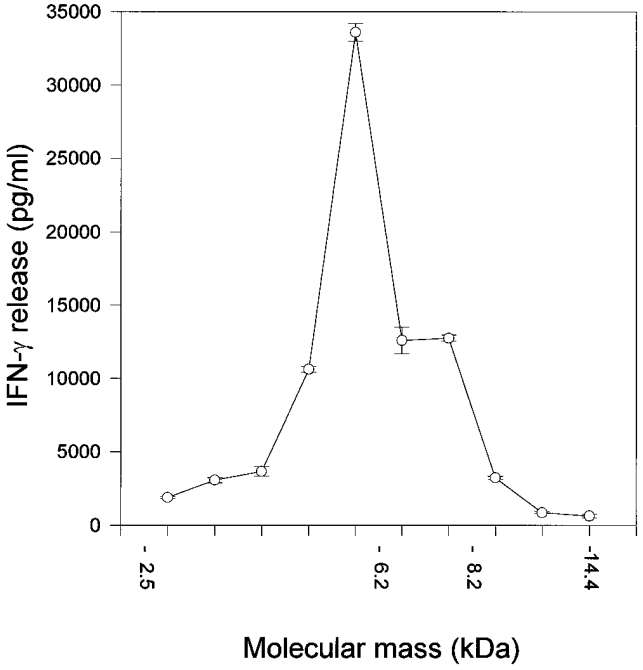

FIG. 1. IFN-γ release from memory effector cells in response to narrow-molecular-mass fractions of ST-CF. Each symbol represents the mean of triplicate values ± standard error of the mean, and the values are from cells pooled from five mice.

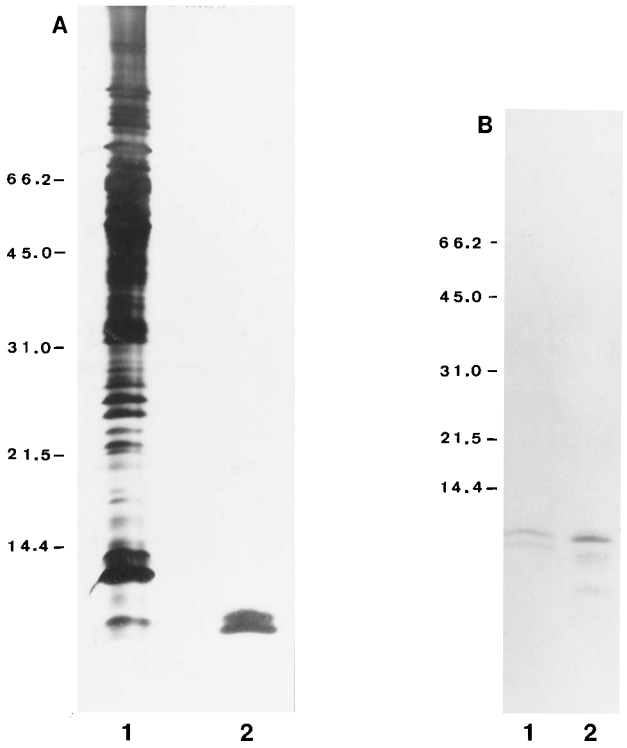

FIG. 2. SDS-PAGE analyses of ESAT-6. Separation of *M. tuberculosis* filtrate components by conventional chromatography led to the purification of ESAT-6. ST-CF and ESAT-6 were subjected to SDS-PAGE and either silver stained (A) or immunoblotted with the MAb HYB 76-8 (B). Lanes: 1, ST-CF; 2, ESAT-6. Molecular masses are indicated in kilodaltons to the left of each panel.

passage through detergent-binding affinity columns (Pierce Europe, Oud-Beijerland, The Netherlands) as described previously (10). The recovery of the fusion proteins was quantitated by the bicinchoninic acid method (Pierce Europe). The fusion proteins were stored at −80°C until used in cell cultures.

DNA sequence analyses. To obtain the nucleotide sequence of the gene encoding the HYB 76-8-binding protein, the 1.7-kbp *M. tuberculosis*-derived *Eco*RI-*Bam*HI fragment from lambda AA227 was subcloned in pBluescriptSK+ (Stratagene, La Jolla, Calif.) and used to transform *E. coli* XL-1Blue (Stratagene). The DNA sequence was obtained by the dideoxy sequencing method adapted for supercoiled DNA by use of the Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and by cycle sequencing using the Dye Terminator system in combination with an automated gel reader (model 373A; Applied Biosystems). The sequence data were analyzed by use of the Sequence Analysis Software Package version 7.1 from the Genetics Computer Group associated with the University of Wisconsin (17).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X79562.

RESULTS

Purification of ESAT-6. The recall of memory immunity to *M. tuberculosis* is expressed as an accelerated recruitment of potent memory effector cells to the infected organs (7, 28). The molecules recognized by these cells were mapped recently by applying narrow-molecular-mass fractions of ST-CF to purified memory effector cells. This study demonstrated a 6-kDa fraction reacting with the MAb HYB 76-8 to be a key antigen target recognized by IFN-γ-secreting effector cells during the recall of memory immunity (Fig. 1) (7). To further characterize the responsible protein, a purification procedure was established. Previously, we have been able to purify several mycobacterial antigens by the use of immunoaffinity chromatographic procedures with MAbs covalently coupled to Sepharose (32). Several attempts to exploit HYB 76-8 for this purpose were not successful, and we therefore developed a protocol for purification of ESAT-6 based on conventional biochemical purification procedures. The initial purification step was based on the observation that the addition of ammonium sulfate to ST-CF resulted in a precipitate which did not contain the HYB 76-8 antigen. By use of hydrophobic interaction chromatography, most of the components and all of the HYB 76-8 antigen in the supernatant bound to a phenyl-Sepharose column. Components were eluted from the column by reducing the ammonium sulfate concentration along a linear gradient from 1 to 0.5 M and further in a stepwise fashion as described in Materials and Methods. During the gradient elution, most of the heat shock protein GroES along with a few other components was removed from the column. After elution of components with phosphate buffer without ammonium sulfate, the column was first washed with water and finally with 70% ethanol. ESAT-6 was collected at 400 to 200 mM ammonium sulfate in fractions devoid of many other ST-CF components. Very little HYB 76-8 reactivity was observed in fractions from 200 to 0 mM ammonium sulfate and the water step. No HYB 76-8-reactive antigen was retrieved when the column was subjected to 70% ethanol elution. The antigen was therefore effectively concentrated and separated from many other ST-CF components during this first step of chromatography. After thorough buffer exchange and concentration of the ESAT-6-containing fractions, further separation was performed by anion-exchange chromatography with a Mono Q column. Tris buffer was used alone during the first attempts to purify the antigen. Unfortunately, several contaminating components were consistently observed along with the HYB 76-8-reactive antigen. We therefore included urea in the buffers, and by this approach, highly pure preparations of ESAT-6 appeared at NaCl concentrations ranging from 100 to 150 mM (Fig. 2).

The use of 3- to 5-week-old culture filtrates was included in this study to scale up the production of ESAT-6 (from micro-

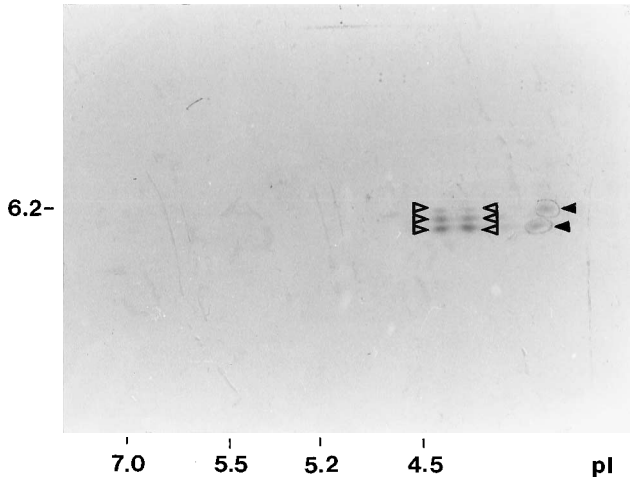

FIG. 3. 2D-E analyses of ST-CF. Samples of ST-CF were subjected to 2D-E. Following the 2D-E, the proteins were blotted onto nitrocellulose and analyzed for reactivities with HYB 76-8 (open triangles) and ST-3 (closed triangles). Molecular mass is indicated in kilodaltons to the left of the figure.

gram to milligram quantities). Again, a highly pure HYB 76-8-reactive product was obtained, this time by use of a DEAE-Sepharose-separated culture filtrate fraction enriched for ESAT-6 as the starting material. After this first step of protein separation, the same purification steps as those outlined for antigen purification from ST-CF were applied.

Characterization of purified native ESAT-6: 2D-E. ST-CF was submitted to 2D-E, and the gel was electroblotted onto a polyvinylidene difluoride membrane. The membrane was screened sequentially for reactivity to HYB 76-8 and to ST-3, which reacts with another low-molecular-mass component in ST-CF. As may be observed in Fig. 3, the HYB 76-8-binding protein focused at two pIs. Furthermore, the second-dimension electrophoresis separated the protein into three discrete spots. The same pattern of HYB 76-8 reactivity was observed when purified ESAT-6 was subjected to 2D-E and immunoblotting (results not shown). The MAb ST-3 recognized antigen focusing at two positions distinct from antigen recognized by HYB 76-8.

Is ESAT-6 glycosylated? Molecular heterogeneity may be due to differences in glycosylation, and whether ESAT-6 appeared in a glycosylated form was therefore investigated. Results obtained from this study using oxidation, labeling, and antibody detection of glycoproteins present in ST-CF showed glycosylation of a protein with a molecular mass of 19 kDa and of at least three proteins in the region from 35 to 40 kDa. In the low-molecular-mass region (GroES and below), however, no sign of glycosylation was evident. When purified ESAT-6 (5 μg) was tested by this assay, the result was also negative (results not shown).

Mass determination of the purified native protein. The molecular mass of ESAT-6 was determined by comparing the migration of the purified protein with molecular mass standards composed of myoglobin fragments in SDS-PAGE. The major band migrated to a position equivalent to the 6-kDa standard, and two other faint bands occurred between 6 and 4 kDa. ESAT-6 migrated to the same position relative to the molecular weight markers under reducing and nonreducing conditions. The native molecular mass of ESAT-6, however, was determined by both gel filtration and by PAGE under nondenaturing conditions to be 24 kDa.

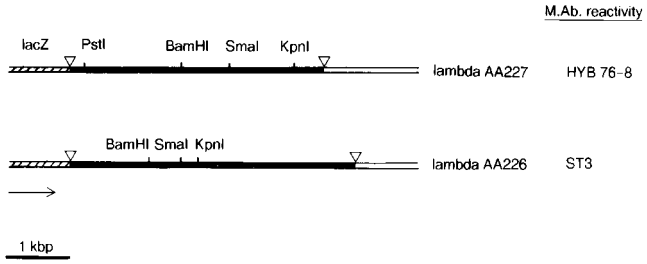

FIG. 4. Physical map of recombinant lambda phages expressing gene products reactive with the MAbs recognizing the low-molecular-mass components from ST-CF, HYB 76-8, and ST-3. Symbols: solid bar, *M. tuberculosis* DNA; open bar, lambda gt11 DNA; open triangles, *Eco*RI cloning site of lambda gt11; arrow, direction of transcription.

N-terminal amino acid sequencing of ESAT-6. The amino-terminal 10 amino acids of native ESAT-6 purified from *M. tuberculosis* ST-CF were determined to be Thr-Glu-Gln-Gln-Trp-Asn-Phe-Ala-Gly-Ile-.

Cloning of genes expressing low-molecular-weight proteins. Two phage clones were isolated from the recombinant *M. tuberculosis* lambda gt11 DNA library (33) by screening with two MAbs recognizing low-molecular-mass components. The physical map of these two phages, lambda AA226 and lambda AA227, is shown in Fig. 4. Western blot (immunoblot) analyses of *E. coli* Y1089 lysogenized with lambda AA226 and lambda AA227 showed that the HYB 76-8- and ST-3-binding proteins are expressed as fusion proteins fused to β-galactosidase (Fig. 5). In both cases, the fusion proteins are subject to some degree of proteolytic degradation in *E. coli*. Despite the fact that these strains are *lon* mutants, this phenomenon has been observed previously when mycobacterial proteins are overexpressed in *E. coli* (5).

With the purpose of further characterizing the structural gene encoding the HYB 76-8-binding protein, we subcloned the 1.7-kbp *Eco*RI-*Bam*HI fragment located immediately downstream of the *lacZ* gene of lambda AA227 into a plasmid vector and determined the nucleotide sequence (Fig. 6). An open reading frame extending from a putative ATG start

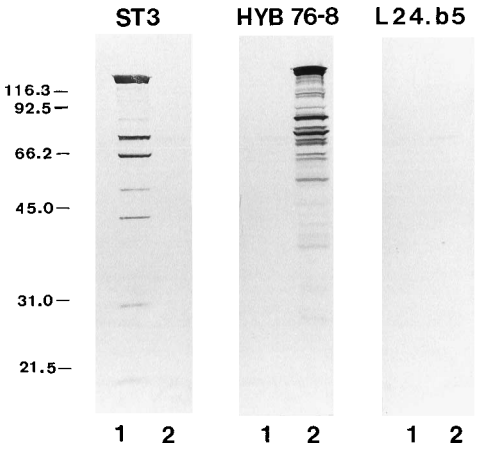

FIG. 5. Western blot analysis demonstrating recombinant expression of the low-molecular-mass components reacting with either ST-3 or HYB 76-8. Lysates of *E. coli* Y1089 lysogenized with lambda AA226 or lambda AA227 were analyzed in Western blotting experiments after SDS-PAGE. Lanes: 1, lambda AA226; 2, lambda AA227. The MAbs are indicated at the top of each panel. L24.b4 is an anti-MPT64-reactive antibody. Molecular masses are indicated in kilodaltons to the left of the figure.

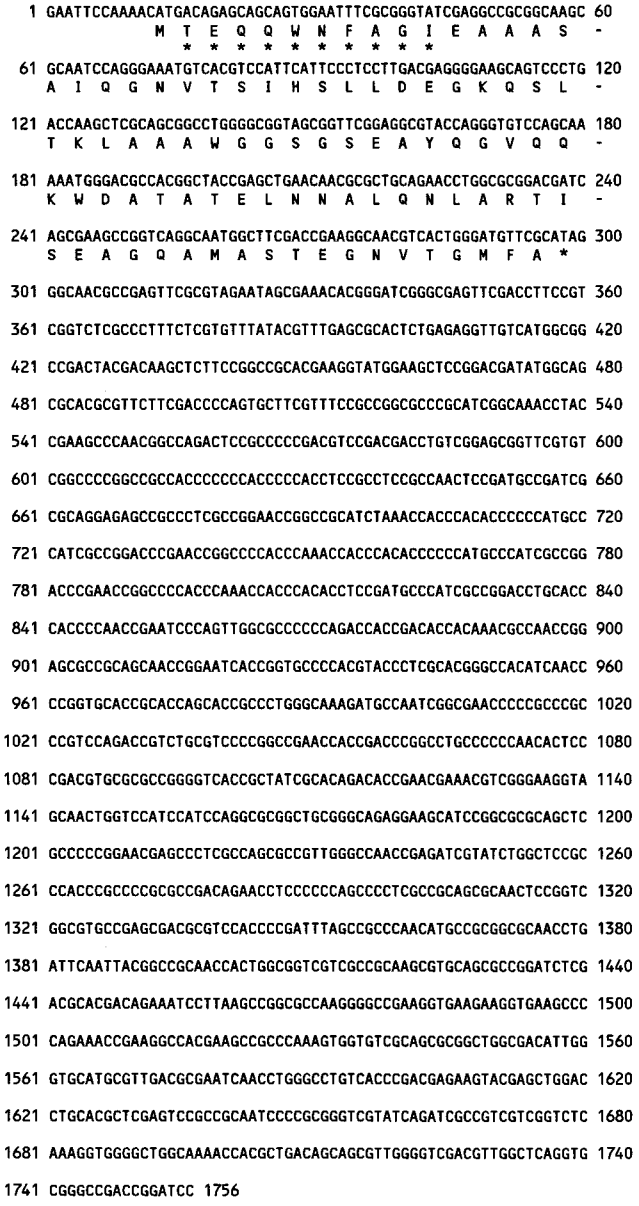

FIG. 6. Nucleotide sequence of the cloned 1.7-kbp *Eco*RI-*Bam*HI fragment. The deduced amino acid sequence of ESAT-6 is given in a one-letter code below the nucleotide sequence. *, amino acid residues confirmed by N-terminal sequence analysis of native ESAT-6.

codon at position 13 to 16 to a stop codon at position 298 to 300 was identified. This reading frame is in frame with that of β-galactosidase and leads to the inclusion of four extra amino acid residues in the fusion protein (Glu-Phe-Gln-Asn). The deduced amino acid sequence of the putative protein is shown in Fig. 6. The molecular weight of the deduced gene product is 9,975, and the pI is 4.5. The sequence of the N-terminal 10 amino acid residues of the native protein was determined and could be aligned to the deduced amino acid sequence as shown in Fig. 6. The total amino acid composition of the native, biochemically purified ESAT-6 was also determined and compared with the composition of the protein deduced from the DNA sequence (Table 1). No significant homologies to proteins deposited in available databases were observed.

TABLE 1. Comparison of deduced and observed amino acid compositions of ESAT-6

Amino acid(s)	Comparison of amino acid with leucine[a]	
	Observed	Deduced from DNA sequence
Asp + Asn	1.05	1.16
Thr	0.98	1.1
Ser	1.00	1.1
Glu + Gln	2.57	2.3
Pro	0.02	0.14
Gly	1.43	1.4
Ala	2.22	2.4
Val	0.43	0.6
Met	0.18[b]	0.43
Ile	0.53	0.6
Leu	1.00	1.00
Phe	0.26	0.3
His	0.16	0.14
Lys	0.38	0.43
Arg	0.14	0.14
Cys	0.00	0

[a] The amino acids are compared with leucine, which was assigned the value of 1.

[b] Determined as methionine sulfene.

Biological activity of ESAT-6. To confirm the identity and the immunological relevance of ESAT-6, memory effector cells were isolated from memory-immune animals 6 days after challenge. These cells were stimulated in vitro with different concentrations of purified recombinant and native ESAT-6 (Table 2), and the cellular responses were compared with the response to ST-CF. Both preparations of ESAT-6 were found to elicit a dose-dependent release of IFN-γ detectable in the lymphocyte culture supernatants. The native preparation was highly active and induced almost the same level of IFN-γ as that of total ST-CF. The recombinant preparation also evoked a marked IFN-γ release. A recombinant preparation identified by the MAb ST-3 was not recognized by the memory effector cells and served as a negative control in this experiment.

Interspecies analyses. To examine the species distribution of ESAT-6, we analyzed a panel of culture filtrates from various mycobacterial species in Western blot experiments using HYB 76-8 as the probe. As can be seen in Fig. 7, the expression of HYB 76-8-reactive antigen is confined to *M. tuberculosis* complex strains and a few other pathogenic strains like *Mycobac-*

TABLE 2. Biological activity of purified native and recombinant produced ESAT-6[a]

Antigen	IFN-γ release (pg [10^3]/ml)[b]
ST-CF, 1 μg/ml	17.10 ± 2.08
Native ESAT-6	
2 μg/ml	9.87 ± 0.48
0.5 μg/ml	4.93 ± 0.35
0.1 μg/ml	1.93 ± 0.18
Recombinant ESAT-6	
2 μg/ml	3.47 ± 0.27
0.5 μg/ml	1.20 ± 0.23
0.1 μg/ml	0.73 ± 0.07

[a] Biological activity was monitored with memory effector T cells isolated 4 days after challenge.

[b] IFN-γ release is expressed as mean values of triplicate analysis (± standard errors of the mean) performed with cells pooled from five mice. No IFN-γ release was detected when recombinant MAb ST-3-reacting protein was added to the cultures.

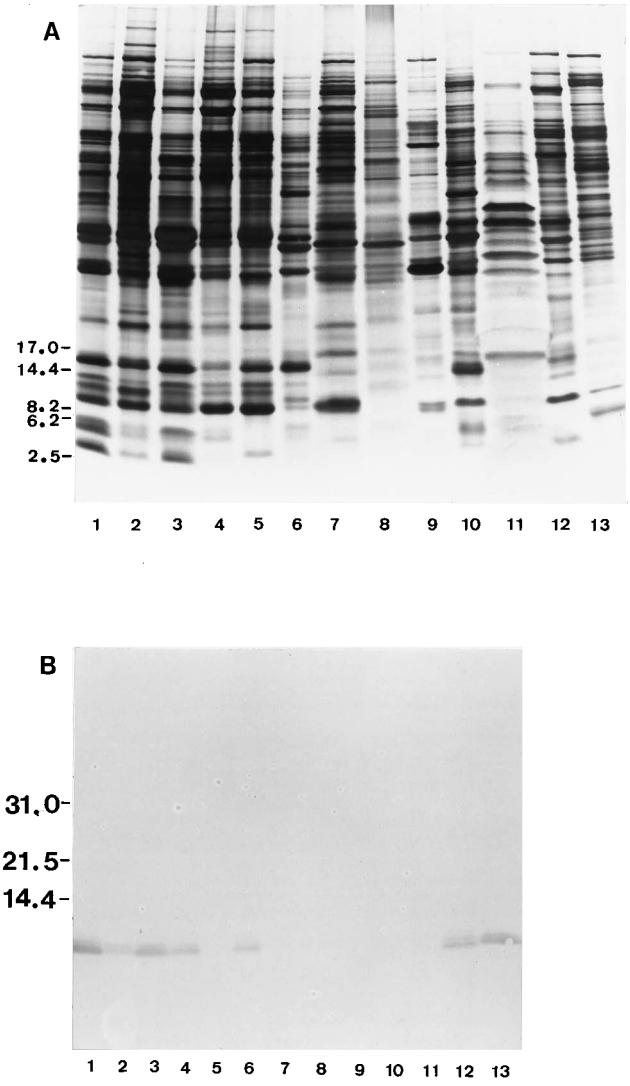

FIG. 7. Interspecies analyses of the HYB 76-8-reactive antigen. Culture filtrates from a panel of mycobacteria were separated by SDS-PAGE and analyzed by either silver staining (A) or by immunoblotting (B) for the presence of the HYB 76-8 antigen. Lanes: 1, *M. tuberculosis* R1609 (clinical isolate); 2, *M. tuberculosis* H37Rv; 3, *Mycobacterium africanum*; 4, *M. tuberculosis* H37Ra; 5, *M. bovis* BCG Copenhagen; 6, *M. kansasii*; 7, *Mycobacterium avium*; 8, *Mycobacterium intracellulare*; 9, *Mycobacterium fortuitum*; 10, *Mycobacterium scrofulaceum*; 11, *Mycobacterium xenopi*; 12, *M. szulgai*; 13, *M. marinum*. Molecular masses are indicated in kilodaltons to the left of each panel.

terium kansasii, *Mycobacterium szulgai*, and *Mycobacterium marinum*. Interestingly, BCG Copenhagen did not express the HYB 76-8 antigen.

Analyses of subcellular fractions of *M. tuberculosis*. Components contained in ST-CF and in the prepared subcellular fractions were analyzed by SDS-PAGE and immunoblotting (Table 3 and Fig. 8). The fractionation procedure appeared to have been successful as evaluated by the predominantly cytoplasmic occurrence of GroEL (also designated the 65-kDa protein reacting with MAb HAT 5). The PstS homolog, i.e., the 38-kDa antigen recognized by the MAb HBT 12, was, as expected, highly enriched in the cell wall and membrane fractions. The ST-CF was composed mainly of secretory products as illustrated by the strong reaction of L24.b5, a MAb reacting with the secreted antigen MPB/T64. In particular, it was noted

TABLE 3. MAb reactivities with ST-CF and subcellular fractions of *M. tuberculosis*

ST-CF or subcellular fraction	MAb reactivity[a]			
	HAT 5	HBT 12	L24.b5	HYB 76-8
ST-CF	−	+	+++	++
Whole cells	+++	+++	++	++
Cell walls	+	+++	+	+
Cytosol	+++	−	−	++
Membranes	−	+++	−	−

[a] Values are reported as follows: −, no reactivity; +, weak reactivity; ++, intermediate reactivity, +++, strong reactivity.

that the ST-CF did not contain detectable levels of GroEL, indicating that no significant contamination by autolytic products had occurred. ESAT-6 could be detected in all of the fractions apart from the membrane fraction, as assessed by HYB 76-8 reactivity (Fig. 8D).

DISCUSSION

In this study, a novel protein designated ESAT-6 from culture filtrates of virulent *M. tuberculosis* was purified and char-

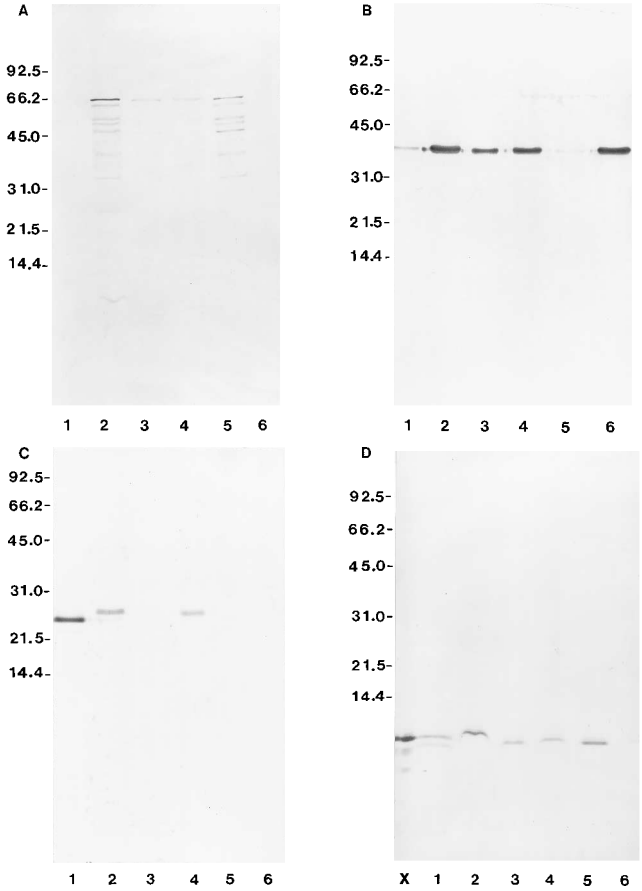

FIG. 8. MAb analyses of subcellular fractions of *M. tuberculosis*. ST-CF and subcellular fractions were subjected to SDS-PAGE and analyzed by immunoblotting for the presence of various antigens. (A) GroEL with MAb HAT 5; (B) the 38-kDa antigen with MAb HBT 12; (C) MPT64 with MAb L24.b5; (D) ESAT-6 with MAb HYB 76-8. Lanes: X, purified ESAT-6 protein; 1, ST-CF; 2, whole-cell fraction; 3, cytosol and membrane fraction; 4, cell wall fraction; 5, cytosol fraction; 6, membrane fraction. Molecular masses are indicated in kilodaltons to the left of each panel.

acterized biochemically. This antigen has been demonstrated recently to be one of the major targets for memory effector cells during the recall of memory immunity in a mouse model of tuberculosis (7). The gene encoding this protein was isolated, and data obtained from DNA sequencing studies were compared with results from amino acid analyses of the native protein.

Initially, we attempted to purify ESAT-6 by immunoaffinity chromatography, a simple one-step procedure, with an available MAb, HYB 76-8, with specificity for this protein. Unfortunately, this approach was unsuccessful, and conventional and biochemical purification procedures were chosen instead. For the purification of ESAT-6 from ST-CF, hydrophobic interaction chromatography followed by anion-exchange chromatography was applied. The use of urea in the buffer during the final step of purification improved separation considerably, a finding consistent with results obtained recently from studies concerned with the purification of other mycobacterial antigens (27). The biological activity of purified native ESAT-6 and recombinant ESAT-6 was monitored by measuring the ability of the proteins to stimulate memory effector cells isolated during the recall of immunity. The antigens were strongly recognized and elicited dose-dependent IFN-γ responses comparable to that of ST-CF, thus confirming the identity of the isolated native protein and verifying the successful cloning and expression of this protein. ESAT-6 occurs in two different pI forms within the range of 4 to 4.5, as demonstrated by 2D-E of ST-CF or of the purified protein followed by immunoblotting. A number of other mycobacterial proteins such as MPB70 have been reported to have more than one isoelectric form (18–20). Modification of the protein such as by deamidation of amide side chains or oxidation or phosphorylation could explain this observation.

Our results show that the occurrence of charge or size differences is not likely to be explained by glycosylation. Acetylation is another possibility which has been described for other mycobacterial proteins, especially in cases where the protein is anchored by the lipid moiety in the cell wall. We did not observe ESAT-6 to have a preference for the cell wall or membrane fractions. Therefore, we do not expect lipidation of this molecule, although this clearly is a subject for further investigation.

A recombinant lambda gt11 *M. tuberculosis* DNA library was screened by use of two MAbs, HYB 76-8 and ST-3, both of which react with low-molecular-mass components contained in ST-CF, resulting in the isolation of two phages. The mycobacterial DNA insert encoding the HYB 76-8-reacting protein was sequenced, and the structural gene of ESAT-6 was identified as a DNA sequence encoding a polypeptide of 95 amino acids. The N terminus of the deduced sequence could be aligned with the 10 amino-terminal amino acids derived from sequence analyses of the native protein, thus confirming the identity of the isolated gene. Furthermore, we observed a high degree of consistency between the DNA-deduced amino acid composition of ESAT-6 and the analyzed amino acid composition of the purified native protein. The pI of the DNA-deduced gene product of 4.5 is also in accordance with the observed pI.

The presence of a consensus signal sequence is a characteristic shared by several previously cloned mycobacterial secretory proteins, for instance, the members of the antigen 85 complex, MPT45, MPT44, and MPT59 (14, 16, 26). A conventional leader sequence is absent from the gene encoding ESAT-6. Other mycobacterial proteins such as GroES, superoxide dismutase, and alanine dehydrogenase, all of which occur very early in the culture medium and are thus considered to be secretory proteins (1, 11, 34), are translocated across the bacterial membrane by signal sequence-independent mechanisms. How ESAT-6 is exported to the surrounding medium is currently under investigation. It was noted that the cytosol preparation contained significant amounts of ESAT-6 in contrast to MPT64, which was undetectable. The efficacy of translocation of polypeptides lacking a consensus signal sequence may thus be less efficient.

The molecular mass of native ESAT-6 was determined to be below 10 kDa and therefore in agreement with the deduced molecular mass. However, under nondenaturing conditions during either size chromatography or PAGE, ESAT-6 was found to have a molecular mass of 24 kDa, indicating that the native protein may exist as a polymer.

The physiological role of ESAT-6 in *M. tuberculosis* still remains to be established since no significant sequence homology to sequences of other *M. tuberculosis* antigens or to sequences of other proteins or enzymes was found. Site-specific gene inactivation would clearly be of great value in answering this question.

A panel of culture filtrates from various mycobacterial species were analyzed for the presence of ESAT-6, and our results show that the antigen is present in strains belonging to the *M. tuberculosis* complex, with the exception of BCG Copenhagen. The culture filtrates derived from three other pathogenic mycobacterial strains, *M. kansasii*, *M. szulgai*, and *M. marinum*, also contained the HYB 76-8 antigen. Whether the gene encoding ESAT-6 occurs in mycobacteria not expressing HYB 76-8-reactive antigen or whether the gene product is present but in quantities not detectable by the HYB 76-8 MAb is currently under investigation. If the expression of ESAT-6 in BCG is low in general, or the encoding gene is not present, the efficacy of this existing vaccine may be improved by gene manipulation resulting in overexpression of ESAT-6. Investigations in this area of research are now under way. Recent results from our laboratory have shown that an experimental vaccine composed of ST-CF in combination with the adjuvant dimethyldioctadecylammonium chloride induced protective immunity comparable to that induced by BCG vaccination in a mouse infection model (6). Application of ESAT-6 as a single-component vaccine or as a member of a multiple-component vaccine in conjunction with the appropriate adjuvant is presently being investigated. Another feasible possibility to be considered is that of engineering a DNA vaccine that includes the ESAT-6-encoding gene. The diagnostic value of purified protein derivative, the skin test reagent used currently, is low because it does not always, in each individual case, clearly differentiate between BCG vaccination and infection with *M. tuberculosis*. Because Western blot analyses of a variety of mycobacterial culture filtrates showed the presence of the HYB 76-8-reactive antigen in filtrates from *M. tuberculosis*, but not from BCG Copenhagen, we are going to study ESAT-6 as an alternative skin test reagent capable of differentiating between BCG vaccination with this particular strain of BCG and disease caused by infection with *M. tuberculosis*.

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