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Evidence for Occurrence of the ESAT-6 Protein in *Mycobacterium tuberculosis* and Virulent *Mycobacterium bovis* and for Its Absence in *Mycobacterium bovis* BCG

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ESAT-6 is a secreted protein present in the short-term culture filtrate of *Mycobacterium tuberculosis* after growth on a synthetic Sauton medium. ESAT-6 has recently been demonstrated to induce strong T-cell responses in a mouse model of memory immunity after infection with *M. tuberculosis*. In Western blotting (immunoblotting), the monoclonal antibody HYB76-8, reacting with ESAT-6, gave a 6-kDa band in culture filtrates from *M. tuberculosis* and virulent *Mycobacterium bovis*. A distinct band in the 24-kDa region was observed in filtrates from four of eight substrains of *M. bovis* BCG that produced high levels of MPB64, while no band occurred in the 6-kDa region with any of these BCG substrains. Southern blotting and PCR experiments with genomic mycobacterial DNA showed the presence of the *esat-6* gene in reference strains and clinical isolates of *M. tuberculosis* as well as in virulent *M. bovis*. The *esat-6* gene could not be demonstrated in any of the eight substrains of *M. bovis* BCG tested by these techniques. Two gene deletions that distinguish *M. bovis* BCG from virulent *M. bovis* have thus now been demonstrated. Deletion of *mpb64* affects four of the eight substrains tested; deletion of *esat-6* affects all of them. The reaction of HYB76-8 at 26 kDa with four of the BCG substrains was demonstrated to result from cross-reactivity with MPB64. HYB76-8 was also shown to cross-react with the A, B, and C components of the antigen 85 complex and MPT51.

It remains essential to establish whether particular antigens are of major importance for the development of protective immunity after infection with *Mycobacterium tuberculosis*. Various techniques are in current use for their identification, and we have lately studied the specificity of T-cell responses in a mouse model of memory immunity after infection with *M. tuberculosis* (4, 5, 8). C57BL/6J mice were infected with *M. tuberculosis* for 1 month and then treated with isoniazid and rifampin for 2 months to clear the infection, as described previously (16, 26). We have focused our studies on long-lived memory immunity and have therefore allowed the mice to rest for 4 to 6 months before reinfection.

Following reinfection with *M. tuberculosis*, an intense T-cell response showing a distinct pattern of specificity rapidly evolves. By testing individual fractions of a short-term culture filtrate which is highly enriched in proteins actively secreted by *M. tuberculosis* (7), two fractions were shown to have high-level activity with regard to induction of proliferation as well as gamma interferon production during in vitro culture (8). In the first fraction, containing 25- to 31-kDa proteins, the 85B component of the fibronectin-binding antigen 85 complex (25, 28, 32, 34, 38) was shown to possess the main activity (5, 6). In the second fraction, containing proteins ranging in molecular mass from 3 to 9 kDa, a protein with an apparent molecular mass of 6 kDa designated ESAT-6 (for 6-kDa early secretory antigenic target) was shown to possess the main activity (5). The gene encoding ESAT-6 (5, 30) contains no signal sequence, and the protein is released from the mycobacterial cell by a signal peptide-independent process.

The purpose of the present work was to extend our studies of ESAT-6 to different mycobacteria, including virulent *Mycobacterium bovis* and various substrains of *M. bovis* BCG, at the protein and gene levels.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The clinical isolates of *M. tuberculosis* from 10 Danish and 10 Tanzanian tuberculosis patients and of *M. bovis* from 10 Danish patients with *M. bovis* infection were identified by standard diagnostic methods at the Mycobacteria Department, Statens Seruminstitut.

Bacterial cultures and antigen preparations. Short-term culture filtrate, which is highly enriched in proteins actively secreted by *M. tuberculosis* H37Rv, was produced as described previously (7). Three- to 5-week-old culture filtrates from stationary-phase cultures of *M. tuberculosis* H37Rv were prepared as described previously (23, 35).

Clinical isolates of *M. tuberculosis*, *M. bovis*, and other mycobacteria were initially grown on Ogawa slants or Løwenstein-Jensen medium and then transferred for further cultivation in liquid Sauton medium.

Isolation of proteins. Isolation of proteins was performed as described in detail previously and was followed by testing for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunological techniques. MPT32, MPT59 (85B), MPT51, MPT64, MPT63, and MPT46 were purified from *M. tuberculosis* culture filtrate (23). The term MPB was introduced by Nagai et al. (22) for the designation of a protein purified from *M. bovis* BCG; it is followed by a number denoting the relative mobility in 7.7% PAGE at a running pH of 9.5. The corresponding term MPT is used to denote a protein isolated from *M. tuberculosis*. 85A and 85C were purified from culture filtrate of the *M. bovis* BCG Danish 1331 substrain (37), and MPB70 was purified from BCG Tokyo culture filtrate (22, 23). ESAT-6 was purified from 3- to 5-week-old culture filtrate of *M. tuberculosis* H37Rv (30).

Antibodies. The monoclonal antibody (MAb) HYB76-8, reacting with ESAT-6, was obtained from purified protein derivative-immunized mice (15). The monoclonality of this reagent was ascertained by repeated limiting-dilution cloning (three cycles), after which the clone was found to have a stable reactivity pattern. L24b4, reacting with MPT64, was obtained from mice immunized with *M. tuberculosis* culture filtrate and selected on the basis of an enzyme-linked immunosorbent assay (ELISA) differential screening system showing stronger activity with *M. tuberculosis* than with BCG Danish 1331 culture filtrate (3).

Monospecific, polyclonal rabbit antibodies were obtained by immunization with purified proteins according to standard procedures (9, 10), with tests for specificity of the same kind and sensitivity as in the subsequent assay.

TABLE 1. Mycobacterial strains used in this study

Species and strain(s)	Source
M. tuberculosis	
H37Rv (ATCC 27294)	ATCC[a]
Erdman	Obtained from A. Lazlo, Ottawa, Canada
H37Ra (ATCC 25177)	ATCC
M. bovis	
BCG substrain:	
Danish 1331	SSI[b]
Tokyo	WHO[c]
Moreau	SSI[d]
Russia	SSI[d]
Glaxo	SSI[d]
Pasteur	SSI[d]
Canadian	SSI[d]
Tice	SSI[d]
MNC 27	SSI[d]
M. africanum	Isolated from a Danish patient
Mycobacterium leprae (armadillo derived)	Obtained from J. M. Colston, London, United Kingdom
Mycobacterium avium ATCC 15769	ATCC
M. kansasii ATCC 12478	ATCC
M. marinum ATCC 927	ATCC
Mycobacterium scrofulaceum ATCC 19275	ATCC
Mycobacterium intracellulare ATCC 15985	ATCC
Mycobacterium fortuitum ATCC 6841	ATCC
Mycobacterium xenopi	Isolated from a Danish patient
M. flavescens	Isolated from a Danish patient
M. szulgai	Isolated from a Danish patient
Escherichia coli	SSI[e]
Staphylococcus aureus	SSI[e]
M. tuberculosis	
10 Danish clinical isolates	Isolated from Danish patients (isolates D13, D14, D15, D16, D19, D22, D26, D34, D38, and D42)
10 Tanzanian clinical isolates	Isolated from Tanzanian patients (isolates T260, T267, T269, T271, T278, T282, T295, T306, T307, and T310)
M. bovis (10 Danish clinical isolates)	Isolated from Danish patients (isolates R2957, R3000, R3072, R3112, R3174, R3225, R3290, R3312, R3320, and R3360)

[a] American Type Culture Collection, Rockville, Md.
[b] Statens Seruminstitut, Copenhagen, Denmark.
[c] WHO International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark.
[d] Our collection, Mycobacteria Department, Statens Seruminstitut, Copenhagen, Denmark.
[e] Department of Clinical Microbiology, Statens Seruminstitut, Copenhagen, Denmark.

SDS-PAGE and two-dimensional electrophoresis with immunoblotting. SDS-PAGE was performed with the Pharmacia system for horizontal electrophoresis in a Multifor II electrophoresis unit 2117 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with precast polyacrylamide gels, ExcelGel SDS gradient 8-18. Ten microliters of the various culture filtrates containing 10 μg of total protein was applied in each lane. Two-dimensional electrophoresis combining isoelectric focusing (12) and SDS-PAGE was performed in the same apparatus. The first-dimension isoelectric focusing step was done with reswelled 18-cm Immobiline dry strips pH 4-7 in Immobiline strip tray 18-1004-31 and was followed by PAGE as described above. Semidry Western blotting (immunoblotting) was performed with Novablot electrophoretic transfer kit 2117-250 (LKB, Bromma, Sweden) onto 0.2-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After electrophoretic transfer, the membranes were subjected to blocking in phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA) and 1% gelatin for 1 h. Antigen bands and dots were detected by using monoclonal antibodies as indicated in Results and peroxidase-labelled $F(ab')_2$ sheep anti-mouse immunoglobulin (Amersham International plc, Amersham, United Kingdom) diluted 1:2,000 in PBS containing 0.2% Tween 20 and 0.2% BSA. Diaminobenzidine (Sigma) in 0.1 M sodium acetate buffer (pH 4.0) was used as the substrate. Between steps the membrane was washed four times for 10 min each in PBS with 0.1% Tween 20 on a rotary shaker. Rainbow protein molecular weight markers (Amersham) were used as standards for SDS-PAGE.

Double-antibody ELISA. The double-antibody ELISA was set up as described in detail previously (36). Briefly, Immunoplate MaxiSorp (no. 442404; Nunc, Roskilde, Denmark) 96-well plates were coated with 100 μl of purified MAb HYB76-8 diluted 1:1,000. Control experiments established that blocking was not required in the system. The second layer was purified mycobacterial protein (1 μg per well) as indicated in Results. The third layer was polyclonal rabbit antibody to the antigen provided in the second layer. The indicator system was horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham) (1:1,000). The substrate was 2,2′-azino-di(ethylbenzthiazolinesulfate) (ABTS). Washing with PBS containing 0.1% Tween 20 was done four times between steps. All reaction mixtures were set up in triplicate, with the median value being used for recording and calculations. Results were read on a Dynatech MR 7000 ELISA reader (Dynatech Laboratories Inc., Chantilly, Va.).

Preparation of DNA. Genomic DNA was prepared from mycobacterial cells as described previously (1). Standard procedures were used for further preparation and handling of DNA, as described by Sambrook et al. (29).

Synthesis and design of probes. Oligonucleotide primers were synthesized automatically on a DNA synthesizer (Applied Biosystems, Foster City, Calif.; ABI-391, PCR mode), deblocked, and purified by ethanol precipitation.

Four oligonucleotides were synthesized on the basis of the nucleotide sequence from *esat-6* (30). The sense primers were A (CAT GAC AGA GCA GCA GTG) and B (CAA GCT CGC AGC GGC CTG GG), corresponding to positions 12 to 29 and 123 to 142, respectively. The antisense primers were C (GTT GTT CAG CTC GGT AGC CG) and D (GCC CTA TGC GAA CAT CCC), corresponding to positions 213 to 195 and 303 to 286, respectively. The four possible combinations of these primers from the ESAT-6-encoding gene are shown in Fig. 1.

PCR technology. PCRs were carried out as described in detail previously (24). In brief, the amplifications were carried out in a thermal reactor (Hybaid, Teddington, United Kingdom) by incubation of 100 ng of chromosomal DNA brought to a final volume of 37 μl with Milli Q water at 70°C for 5 min and then cooled on wet ice for 10 min. Thirteen microliters of PCR master mix was added. The PCR master mix contained 192 mM KCl, 38.5 mM Tris-HCl (pH 8.3), 5.8 mM $MgCl_2$, 0.77 mM each deoxynucleoside triphosphate, and 3.8 μM each oligonucleotide primer. The reaction mixture was overlaid with 100 μl of mineral oil. Denaturation of the DNA was carried out at 94°C for 5 min. The reaction mixture was brought to the annealing temperature (60°C), and 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added to the master mix. The amplifications were performed for 30 cycles of 72°C for 3 min, 94°C for 1 min 20 s, and 60°C for 2 min.

Ten microliters of the PCR product was fractionated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. Negative controls containing all PCR reagents except template DNA were run in parallel with the samples.

Southern blotting. The Southern blotting was carried out as described elsewhere (24). In brief, 4 μg of genomic DNA was digested with *Pvu*II, electrophoresed in an 0.8% agarose gel, and transferred onto GeneScreen Plus membranes (NEN Research Products, Boston, Mass.). A 292-bp *esat-6* fragment was amplified by PCR from the plasmid pAA227 (27) by using primers A and D (Fig. 1). The PCR fragment was nick translated by using a kit from Boehringer Mann-

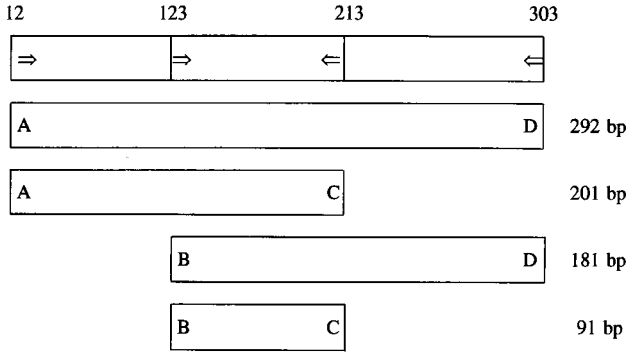

FIG. 1. Positions of oligonucleotides used for amplification of the gene encoding ESAT-6. Numbers at the top indicate the 5′ ends of the oligonucleotides according to the numbering of Sørensen et al. (30). The arrows indicate the directions of the primers. The length of each PCR product is shown at the right.

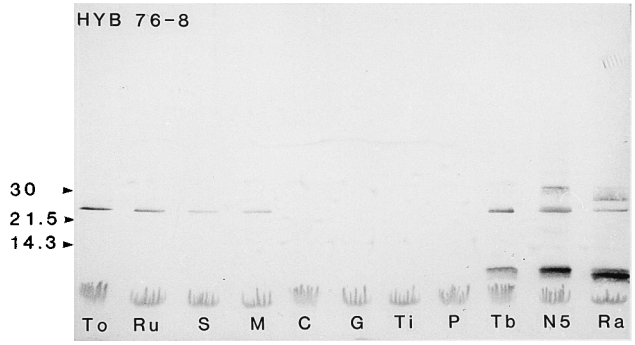

FIG. 2. Reactivity of MAb HYB76-8 with culture filtrates of substrains of BCG, *M. tuberculosis*, and virulent *M. bovis* in SDS-PAGE and immunoblotting. Substrains of BCG: To, Tokyo; Ru, Russia; S, Sweden; M, Moreau; C, Copenhagen (Danish 1331); G, Glaxo; Ti, Tice; P, Pasteur. Other strains: Tb, *M. tuberculosis* H37Rv; N5, *M. bovis* AN5; Ra, *M. bovis* Ravenel. Numbers at the left indicate molecular masses in kilodaltons.

heim (Mannheim, Germany) and used as a probe. Hybridization was performed at 65°C in an aqueous solution containing 1% SDS, 1 M NaCl, 10% dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, and an [α-^{32}P]dCTP-labelled nick-translated *esat-6* probe, according to the instructions provided. Washing of the membrane was performed as described by the manufacturer.

Molecular mass determination. Purified ESAT-6 was prepared for mass analysis by reversed-phase liquid chromatography on a Vydac (Hesperia, Calif.) 214TP52 C_4 column (2.1 by 25 mm) equilibrated with 9.5% isopropanol in 0.1% trifluoroacetic acid. Elution was performed with a 9.5 to 76% linear gradient of isopropanol in 0.1% trifluoroacetic acid. The collected peak material was analyzed by SDS-PAGE and subjected to laser desorption mass spectrometry (19, 31).

RESULTS

Distribution of HYB76-8-reactive material in different mycobacterial strains. The presence of ESAT-6 in culture filtrates from 11 different mycobacterial strains was investigated with Western blots developed with MAb HYB76-8 (Fig. 2). There is a strong band at about 6 kDa in *M. tuberculosis* H37Rv and the two strains of virulent *M. bovis*, AN5 and Ravenel. In these three culture filtrates a distinct band is also seen at about 24 kDa, which corresponds in position to a single band observed with four of the substrains of BCG, i.e., Tokyo, Russia, Sweden, and Moreau. In the other four substrains of BCG tested, no band was observed at this position. No band was seen in the lower-molecular-mass region, around 6 kDa, in any of the BCG culture filtrates.

Presence of *esat-6* in different mycobacterial species. In order to determine the distribution of the *esat-6* gene in species belonging to the *M. tuberculosis* complex and in other mycobacteria, the 292-bp *esat-6* PCR fragment was used as a probe in Southern blot experiments. An example is shown in Fig. 3, and the results are summarized in Table 2. The probe hybridized to a *Pvu*II fragment of approximately 6 kb in the *M. tuberculosis* substrains H37Rv and Erdman and in *M. bovis* MNC 27, but the probe did not hybridize to any *Pvu*II fragment from the BCG substrains examined (Fig. 3). Furthermore, the probe hybridized to *Pvu*II fragments from *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium szulgai*, and *Mycobacterium flavescens* (Table 2).

In order to extend the study of the *esat-6* gene distribution within other mycobacteria, PCR analyses were used. The results of PCR experiments are summarized in Table 2. The analysis confirmed and extended the Southern blot analysis concerning the *M. tuberculosis* complex and *M. kansasii*. However, the primer sets employed (Fig. 1) did not allow demonstration of the *esat-6* gene on chromosomal DNA from *M. marinum* and *M. szulgai* (Table 2). Thus, it is concluded that the gene encoding ESAT-6 is confined to *M. tuberculosis* complex strains, *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. flavescens*. None of the *M. bovis* BCG vaccine substrains revealed the gene encoding the ESAT-6 antigen.

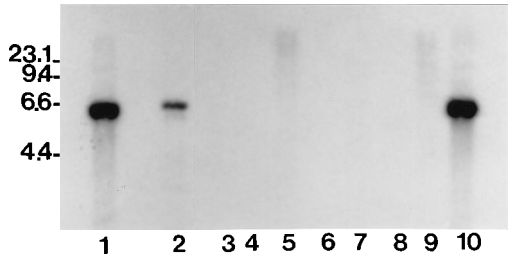

FIG. 3. Pattern of Southern hybridization of the nick-translated *esat-6* probe to *Pvu*II-digested chromosomal DNAs from various mycobacterial species. Lanes: 1, *M. tuberculosis* H37Rv; 2, *M. tuberculosis* Erdman; 3, *M. bovis* BCG Danish 1331; 4, *M. bovis* BCG Tokyo; 5, *M. bovis* BCG Glaxo; 6, *M. bovis* BCG Pasteur; 7, *M. bovis* BCG Tice; 8, *M. bovis* BCG Moreau; 9, *M. bovis* BCG Russia; 10, virulent *M. bovis* MNC 27. The numbers at the left indicate sizes of standard DNA fragments in kilobase pairs.

Presence of *esat-6* in *M. tuberculosis* strains isolated from tuberculosis patients. To investigate whether *esat-6* was present in different clinical isolates of *M. tuberculosis*, the PCR technology described previously (24) was used. We tested 20 patient samples by using the primers shown in Fig. 1. All 20 samples (from 10 Tanzanian and 10 Danish patients) were

TABLE 2. Comparison of Southern blotting and PCR analyses for the *esat-6* gene with mycobacterial genomic DNA

Species and strain(s)	Result[a] of:	
	Southern blotting	PCR
M. tuberculosis		
H37Rv	+	+
Erdman	+	+
H37Ra	ND	+
M. bovis		
BCG substrain:		
Danish 1331	−	−
Tokyo	−	−
Glaxo	−	−
Pasteur	−	−
Tice	−	−
Moreau	−	−
Russia	−	−
MNC 27	+	+
M. africanum	ND	+
M. leprae	−	−
M. avium	−	−
M. kansasii	+	+
M. marinum	+	−
M. scrofulaceum	−	−
M. intracellulare	−	−
M. fortuitum	−	−
M. xenopi	−	−
M. flavescens	+	+
M. szulgai	+	−
E. coli	−	−
S. aureus	−	−
M. tuberculosis		
10 Danish clinical isolates	ND	+
10 Tanzanian clinical isolates	ND	+
M. bovis, 10 Danish clinical isolates	ND	+

[a] +, positive reaction; −, no reaction; ND, not determined.

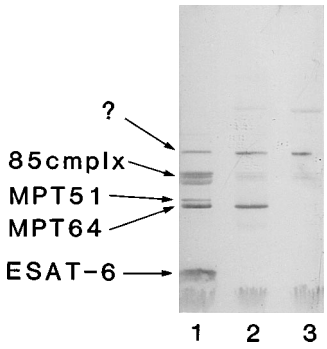

FIG. 4. Western blot for demonstration of cross-reactivity of MAb HYB76-8. Lanes: 1, *M. tuberculosis*; 2, BCG Tokyo; 3, BCG Copenhagen (Danish 1331). 85cmplx, antigen 85 complex.

found to be positive, and no nonspecific reactions were observed (Table 2).

This result shows that the *M. tuberculosis* strains isolated from Danish and Tanzanian tuberculosis patients all contained *esat-6*. Therefore, the gene encoding ESAT-6 may be consistently present in *M. tuberculosis* strains giving rise to clinical disease. *esat-6* was also demonstrated in all of the 13 virulent *M. bovis* strains tested.

Test for cross-reactivity. The BCG substrains tested by the Western blotting experiments illustrated in Fig. 2 gave no HYB76-8-reactive band in the 6-kDa region. This finding corresponded to the results obtained by Southern blotting and PCR, as the gene encoding ESAT-6 was not demonstrable in any of the BCG substrains tested. However, the nature of the 24-kDa band observed in four of the eight substrains remained to be explained.

Different substrains of BCG vary markedly in production and secretion of distinct marker proteins. The first clear distinction was established for MPB70 (10, 21), with BCG Tokyo and BCG Danish 1331 being reference strains producing high and low levels, respectively. In BCG substrains the same division between strains producing high and low levels was observed with regard to MPB64 (11, 17). Since the occurrence or lack of HYB76-8-reactive material illustrated in Fig. 2 corresponds to the distinction based on secretion of MPB64, which has a similar molecular mass, experiments were performed to see whether cross-reactivity with MPB64 could explain the observations made.

Figure 4 shows the results of Western blotting with HYB76-8 and a reference MPT/MPB64-reactive MAb, L24b4 (3). In these experiments an increased concentration of HYB76-8 was used to obtain a more sensitive system for demonstration of cross-reactivity. Reactivity with *M. tuberculosis* culture filtrate is shown in Fig. 4, lane 1. The presence of a strong band in the 6-kDa region and no reactivity with the two substrains of BCG in lanes 2 and 3 in this area is confirmed. In *M. tuberculosis*, distinct bands corresponding to the known molecular masses of MPT64, MPT51, and the antigen 85 complex are observed. An additional distinct band is seen at 38 kDa in the *M. tuberculosis* as well as the two BCG substrain culture filtrates. The nature of this 38-kDa cross-reacting protein has not yet been established. It is not the well-known 38-kDa lipoprotein of *M. tuberculosis* (2), which is demonstrable, but only in a far lower concentration in BCG (13, 33).

To exclude the possibility that the reactivity of HYB76-8 in the 24-kDa area was with another protein with the same molecular mass as MPT64, two-dimensional electrophoresis with blotting was performed as illustrated in Fig. 5. *M. tuberculosis* culture filtrate was separated by isoelectric focusing in the first dimension and then subjected to SDS-PAGE in the second dimension in two plates run in parallel in the same apparatus. For alignment, a trace amount of ^{125}I-labelled purified 85B was added to the culture fluid prior to electrophoresis, and autoradiography of the developed nitrocellulose membrane was performed to identify the position of the protein in each membrane. Figure 5A shows the Western blot developed with HYB76-8. Distinct spots corresponding to the known positions of ESAT-6, MPT64, 85B, and 85C were seen, confirming cross-reactivity. Development with L24b4 (Fig. 5B) gave a single spot, confirming the position of MPT64 in Fig. 5A.

After SDS-PAGE and blotting, isolated MPT64 did not react with HYB76-8. Cross-reactivity in mycobacterial, as well as several other, protein systems is critically dependent on protein conformation, which may be altered during isolation procedures. Catching, double-antibody ELISA is often more sensitive for demonstration of cross-reactivity. The findings of such tests are summarized in Table 3. There was distinct cross-reactivity of HYB76-8 with MPT64, MPT51, and the three constituent components of the antigen 85 complex and a lack of reactivity with four other proteins isolated from culture filtrates.

Molecular mass determination. The mobility of ESAT-6 in SDS-PAGE corresponds to a polypeptide with a molecular mass of 6 kDa. By gel filtration and nondenaturing PAGE of *M. tuberculosis* culture filtrate, the mass of ESAT-6 was estimated to be 24 kDa, and the presence of polymers could explain this result (30). In order to obtain an accurate determination, laser desorption mass spectrometry was performed on ESAT-6, and a molecular mass of 9,890 ± 10 Da was observed, which is in good agreement with the deduced mo-

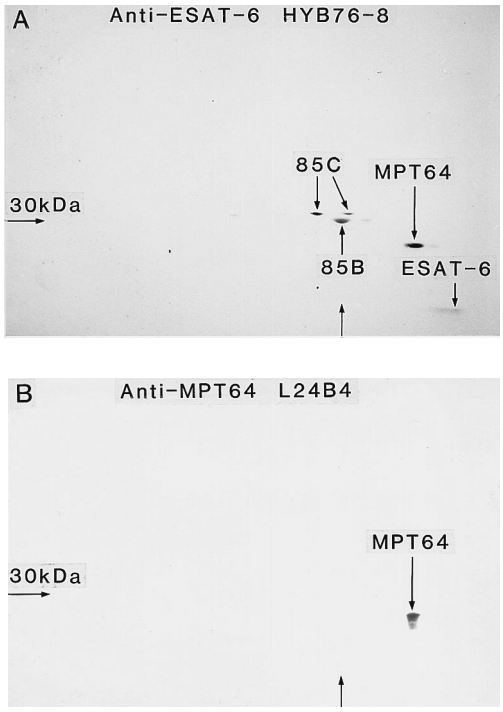

FIG. 5. Two-dimensional electrophoresis of *M. tuberculosis* culture fluid and blotting. (A) Development with MAb HYB76-8; (B) development with MAb L24b4. The positions of ^{125}I-labelled 85B determined by autoradiography of both membranes after first- and second-dimension electrophoresis and blotting are indicated with unmarked vertical arrows and horizontal arrows, respectively.

TABLE 3. Double-antibody ELISA with MAb HYB76-8 as catching antibody in the first layer

Protein in second layer			ELISA	
Designation	Molecular mass (kDa)[a]	N-terminal amino acid sequence	Polyclonal antibody in third layer	Signal[b]
MPT32	41	DPEPA PPVPP	K180	− (0.036)
85C[c] (MPB45)	31.5	FSRPG LPVEY	K21	+++ (0.452)
85A[d] (MPB44)	31	FSRPG LPVEY	K18	+++ (0.343)
85B[e] (MPT59)	30	FSRPG LPVEY	K93	+++ (0.307)
MPT51	27	APYEN LMVPS	K14	+++ (0.805)
MPT64[f]	26	APKTY CEELK	K95	++ (0.166)
MPB70	22	GDLVG PGCAE	K34	− (0.021)
MPT63	18	AYPIT GKLGS	K64	− (0.098)
MPT46	14	TDSEK SATIK	K961	− (0.042)

[a] From Nagai et al. (23).
[b] +, ++, and +++ represent increasing signal strength. The values in parentheses indicate optical density values above the background value in controls in the same plate without antigen in the second layer, with values of <0.100 being insignificant (−).
[c] Identified by A in position 16 (38).
[d] Identified by A and L in positions 31 and 36, respectively (38).
[e] Identical to the deduced sequence from the cloned alpha gene (20).
[f] There was an error in previous publications (14, 23) showing the N-terminal sequence of its signal peptide.

lecular mass of 9,904 Da for the *esat-6* gene product. If ESAT-6 forms polymers, only weak associations are involved, as no dimers or trimers were detected by mass spectrometry.

DISCUSSION

A mouse model of memory immunity after infection with *M. tuberculosis* has been studied extensively, but only recently has information on the specificity of the T-cell responses generated in this model been obtained (5, 8). ESAT-6 in this model has been identified as a major target molecule for gamma interferon-producing Th1 cells (5, 30). The present investigation confirms and extends previous observations on this molecule.

In the previous investigation of the species distribution of ESAT-6, a panel of culture filtrates from various mycobacterial species was studied in Western blot experiments using HYB76-8 as the probe. Expression of an HYB76-8-reactive antigen was confined to *M. tuberculosis*, *Mycobacterium africanum*, and a few other pathogenic strains, such as *M. kansasii*, *M. szulgai*, and *M. marinum* strains. Among *M. tuberculosis* complex strains, *M. bovis* BCG Danish 1331 did not express the HYB76-8 antigen (30).

The present study extends the findings of Western blotting, particularly regarding *M. bovis*. This is illustrated in Fig. 2, which shows HYB76-8-reactive material in the 6-kDa region with two virulent strains of *M. bovis*, AN5 and Ravenel, but no reaction in this region with any of the eight *M. bovis* BCG substrains tested.

Corresponding findings were made for the gene level. Southern blot experiments extended by PCR analysis gave positive reactions for the *esat-6* gene in the *M. tuberculosis* isolates tested, in virulent *M. bovis*, and in *M. africanum*, whereas all of the *M. bovis* BCG substrains examined gave negative reactions indicating the lack of the gene (Fig. 3 and Table 2). Furthermore, the *esat-6* gene was demonstrated in genomic DNAs from *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. flavescens.*

The results of Southern blotting corresponded to the PCR findings except for *M. marinum* and *M. szulgai*, both of which reacted positively in Southern blotting but negatively in PCRs. Differences in the nucleotide sequences of the *esat-6* genes of *M. tuberculosis* and the other two species may account for these discrepancies, since the PCR technique is more sensitive to subtle changes in nucleotide sequence than Southern blotting, in which a probe corresponding to the entire *esat-6* gene was employed.

Additional reactivity was observed in the 24-kDa region after SDS-PAGE and Western blotting with the MAb HYB76-8 in *M. tuberculosis* and virulent *M. bovis*. Four of the eight BCG substrains also showed a distinct reaction in this area (Fig. 2). In the BCG substrains this reactivity showed the same distribution as MPB64 at the protein level (11, 17) as well as at the gene level (17, 24). These observations raised the question of whether MAb HYB76-8 was cross-reacting with MPB/MPT64, being of similar size. Figure 4 demonstrates HYB76-8-reactive material in the 24-kDa region, corresponding to the mobility of MPT64 after SDS-PAGE of *M. tuberculosis* culture filtrate and blotting. The cross-reactivity of HYB76-8 with MPT64 was confirmed by two-dimensional electrophoresis of *M. tuberculosis* culture filtrate and blotting (Fig. 5). This may explain the previous demonstration of HYB76-8 reactivity in the 24-kDa region (30).

However, isolated MPT64 did not react with HYB76-8 in similar tests. Cross-reactivity between proteins is critically dependent on protein conformation, which may be altered during isolation procedures. Catching ELISA is often more sensitive for the demonstration of cross-reactivity. This was confirmed, as shown in Table 3, which also documents additional cross-reactivity with proteins in the antigen 85 complex and MPT51. These findings are similar to the previous demonstration of cross-reactivity between MPB64 and proteins in the antigen 85 complex (37).

The polyclonal antibodies used previously (37) and in the present investigation were prepared by immunization with 85A and 85C isolated from BCG Danish 1331 culture filtrate, which does not contain the MPB64 protein since the *mpb64* gene is missing in this BCG substrain (17, 24). A possible error due to the simultaneous presence of small amounts of antibodies to MPB64 raised against contaminating material in the antigen preparations should therefore be excluded.

While extensive homology between 85A, 85B, 85C, and MPB/MPT51 at the protein and gene levels has been demonstrated (25, 28, 32, 34, 38), similar homology at the gene level has not been demonstrated with *mpb64*, nor does the *esat-6* gene show homology with *mpb64*. At the protein level, similarities between MPB64 and 85B have been reported, particularly in the N-terminal region (37), although several gaps had to be introduced. The HYT27 MAb showed cross-reactivity (37). A tendency to similarity in the N-terminal region is probably a feature of molecules actively secreted from the mycobacterial cell, indicated in the primary amino acid sequence, as for MPB64 and the antigen 85 complex, or possibly arising through folding of the polypeptide chain. The latter is more probable in the case of ESAT-6, since examination of the primary sequence of this molecule revealed no close similarity with MPB64 or the components of the 85 complex. Here a particular feature of ESAT-6 should be noted: although the gene contains no signal sequence (30), the protein appears in short-term culture filtrate, probably as a result of a signal peptide-independent secretion process.

A point of particular interest is how and when the *M. bovis* BCG substrains have lost the gene encoding ESAT-6. All of the substrains originate in one way or another from the same ancestor, virulent *M. bovis*. The deletion of a gene in BCG coding for a well-known protein was first demonstrated in the case of *mpb64* by Li et al. (17) and later confirmed (24). This deletion occurred in only four of the eight BCG substrains

tested. By contrast, the deletion of *esat-6* demonstrated in the present work affected all of the eight substrains tested, indicating an earlier occurrence, prior to 1925, which is the latest time at which the presently examined substrains are known to have a common origin (27). Since the *esat-6* gene was consistently demonstrated in the 11 virulent strains of *M. bovis* tested, the deletion involving *esat-6* may have been of essential importance in the initial attenuation of *M. bovis* leading to establishment of the BCG vaccine. Furthermore, the hybridization studies illustrated in Fig. 3 showed no differences in the restriction enzyme pattern within species belonging to the *M. tuberculosis* complex, indicating that the localizations of *esat-6* on the chromosome were identical. The function of the gene product is as yet unknown. It is apparently a dispensable one for in vitro growth, but the lack of the *esat-6* gene in BCG makes it tempting to speculate on a role in the virulence of the pathogenic strains. Fully compatible with our findings and views is a recent report describing three deletions in the genome of an *M. bovis* BCG vaccine strain compared with that of a virulent *M. bovis* strain. One of the deletions includes the *esat-6* gene (18).

ESAT-6 is a major target molecule for the memory T-cell response in mice (5, 30). The molecule seems to be a broadly recognized target consistently responded to in genetically different strains of mice (unpublished results). We have previously suggested a role for ESAT-6 in protective immunity and as a possible candidate molecule for a future subunit vaccine (5). In the present study, analyses at both the genetic and protein levels have demonstrated the lack of ESAT-6 in vaccine strains of BCG and in most of the mycobacteria other than those of the *M. tuberculosis* complex tested. By contrast, the gene was consistently found in clinical isolates of *M. tuberculosis* and *M. bovis*. This result points to the possibility of using ESAT-6 as a diagnostic reagent for infection with *M. tuberculosis*. The diagnostic value of tuberculin purified protein derivative is limited, as this reagent does not clearly differentiate between sensitization after BCG vaccination, exposure to a broad range of environmental mycobacteria, and infection with virulent *M. tuberculosis* at the level of the individual. Ongoing studies with cattle have provided very promising results indicating the potential of ESAT-6 as a new specific reagent for the diagnosis of bovine tuberculosis (unpublished results). Similar studies are under way to clarify the relevance of this reagent for the diagnosis of tuberculosis in humans.

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