Res. Microbiol.
1998, 149, 265-275

Identification and molecular cloning of a novel secretion antigen from *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG

G. Freer (*), W. Florio, B. Dalla Casa, D. Bottai, G. Batoni, G. Maisetta, S. Senesi and M. Campa

Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, Universitá degli Studi di Pisa, Via S. Zeno 35-39, 56127 Pisa (Italy)

SUMMARY

A novel protein called SA-5K was identified in *Mycobacterium bovis* BCG (BCG) short-term culture filtrates (CFs) by means of a recently described monoclonal antibody (mAb), L8D8. This protein had an apparent molecular mass (MM) of 5 kDa, as judged by Western blotting after sodium dodecyl sulphate-polyacrylamide gel electrophoresis in reducing conditions, and did not seem to contain any sugar or lipid substituents.

In the present work, SA-5K was purified from BCG CFs by affinity chromatography. A protein that could be detected in Western blot but not by standard protein staining techniques was obtained. When SA-5K was subjected to aminoterminal sequencing, the 10 amino acids (aa) found matched the first 10-aa sequence deduced from an open reading frame (ORF) of *M. tuberculosis*. The ORF encodes a polypeptide, likely to include a signal for secretion, with an estimated MM of 8.3 kDa after signal peptide cleavage. The secretory nature of SA-5K was confirmed by the fact that it could only be detected in CFs, but not in other BCG subcellular fractions. After size exclusion chromatography, reactivity with mAb L8D8 was found to peak in the 45-50- and 14-16-kDa fractions. The latter MM was close to that estimated from the ORF of *M. tuberculosis*, implying that the 5-kDa antigen detected initially by Western blot in reducing conditions was a portion of SA-5K released after reduction of a disulphide bridge.

The presence of the gene for SA-5K in BCG and its identity were confirmed by PCR (polymerase chain reaction) with specific primers and restriction analysis: the PCR product was slightly shorter in BCG than in *M. tuberculosis*. The gene coding for SA-5K was cloned by PCR from BCG and *M. tuberculosis* DNA and was expressed in *Escherichia coli*.

Key-words: Mycobacterium tuberculosis, BCG, Protein SA-5K; Secretion antigens, Molecular cloning.

Submitted October 22, 1997, accepted January 16, 1998.

(*) Corresponding author.

INTRODUCTION

Mycobacterium bovis BCG (BCG) is an attenuated *M. bovis* strain widely used as a vaccine against tuberculosis. Its efficacy as a human vaccine varies greatly in different trials. One reason for such variability seems to depend on the ability of BCG to replicate in the host and therefore to produce sufficient quantities of antigens able to elicit a protective response (Daugelat *et al.*, 1995; Fine, 1989; Milstien and Gibson, 1990). Because tuberculosis remains a worldwide health problem, much effort is being devoted to the identification of individual mycobacterial antigens that may be used to develop improved diagnostic methods for tuberculosis and/or a subunit vaccine alternative to BCG. Monoclonal antibodies (mAbs) and polyclonal sera have been widely used as tools to identify and isolate mycobacterial proteins and/or their genes in different studies (Ohara *et al.*, 1995; Young *et al.*, 1992).

A number of reports have provided evidence for the presence of prominent T-cell antigens for both animal models and humans among proteins released by actively growing tubercle bacilli (Andersen and Heron, 1993; Andersen, 1994; Boesen *et al.*, 1995; Orme, 1988; Pal and Horwitz, 1992). Only a few of the proteins present in culture filtrates (CFs) have been identified (Andersen and Hansen, 1989; Ashbridge *et al.*, 1989; Bigi *et al.*, 1995; Manca *et al.*, 1997; Ohara *et al.*, 1995; Sørensen *et al.*, 1995; Wiker and Harboe, 1992; Yamaguchi *et al.*, 1989; Zhang *et al.*, 1992). At least two mycobacterial antigens proved to be effective in inducing protection against tuberculosis when injected as DNA vaccines in animal models (Tascon *et al.*, 1996; Huygen *et al.*, 1996). It is likely that mycobacterial CFs still include unknown major T-cell antigens that might be useful to develop a multiple-antigen human vaccine as an alternative to BCG (Andersen and Brennan, 1994; Young *et al.*, 1992).

A panel of mAbs directed against BCG CF components was recently characterized (Freer *et al.*, 1998). One of these mAbs, L8D8, was able to react with a protein (SA-5K) present only in CFs and not in other subcellular fractions. In the present study, mAb L8D8 was used to identify SA-5K, a novel secretion protein of roughly 8.3 kDa. The gene encoding such an antigen was cloned by PCR (polymerase chain reaction) from BCG and *M. tuberculosis* DNA and expressed in *Escherichia coli*. The features of the antigen found experimentally and deduced from its DNA sequence are discussed.

MATERIALS AND METHODS

Bacterial strains

BCG, strain Pasteur, was originally supplied by Pasteur Merieux (Lyon, France).

Preparation of CFs

Two × 10^9 CFUs (colony-forming units) of BCG were inoculated in 200 ml of Sauton's modified medium without Tween 80 (Florio *et al.*, 1997). After 8 days, unless otherwise stated, cultures were filtered through 0.22-μm filters and concentrated by ultrafiltration through "Amicon YM3" filters with a cutoff of 3 kDa (Amicon, Danvers, MA). Dialysis was performed by further ultrafiltration with 2 changes of sterile distilled water. CFs were stored at −20°C until use. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

aa = amino acid.
BCG = bacillus Calmette Guerin (attenuated *Mycobacterium bovis* strain).
CF = culture filtrate.
CFU = colony-forming unit.
DTT = dithiothreitol.
mAb = monoclonal antibody.
MM = molecular mass.
ORF = open reading frame.
PAGE = polyacrylamide gel electrophoresis.
PCR = polymerase chain reaction.
SDS = sodium dodecyl sulphate.
Taq = *Thermus aquaticus*.

Monoclonal antibodies

mAb L8D8 was generated from C57BL/6 mice immunized intraperitoneally with BCG CFs in incomplete Freund's adjuvant, as previously described (Freer *et al.*, 1998). It recognized a protein of 5 kDa in BCG CFs and not in other subcellular fractions. The mAb isotype was IgG1.

Production of ascitic fluid

CB6F1 (BALB/c × C57BL/6) (Charles River, Calco, Italy) mice were injected intraperitoneally with 0.5 ml pristane and, 7 days later, with 2×10^6 hybridoma L8D8 cells in 0.5 ml PBS and processed as described (Harlow and Lane, 1988).

Purification of SA-5K

Immunoaffinity chromatography was used to purify antigen SA-5K from 12-day-old BCG CFs. mAb L8D8 was added as ascitic fluid to "Sepharose-Protein A" (Sigma Chemical Co, St. Louis, MO) at 5 mg/ml of gel slurry. Sepharose-Protein A was then covalently bound to mAb L8D8 by use of dimethyl pimelimidate, following standard procedures (Harlow and Lane, 1988). CFs were added to the gel slurry at 1 mg/ml in phosphate-buffered saline (PBS) and incubated overnight. After washing the gel with 10 mM glycine, pH 2.5, the antigen bound to the column was eluted with 100 M Na_3PO_4, pH 12. The eluate was precipitated with 90% ammonium sulphate and dialysed against 3 changes of PBS.

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Gels at 15% acrylamide-bisacrylamide in 0.37 M Tris Cl, pH 8.6, 0.1% SDS were used to separate antigens. Samples were diluted in Laemmli sample buffer, with DTT (dithiothreitol) and loaded after boiling 5 min unless otherwise stated. Each well was loaded with 15 μg of CFs or 1-2 μg of purified SA-5K. Broad MM protein standards ranging from 6.5 to 175 kDa were purchased from New England Biolabs (Beverly, MA).

Western blotting

After the SDS-PAGE run, antigens were electrophoretically transferred onto nitrocellulose paper (Amersham, UK) by the "Transblot" apparatus (Bio-Rad Laboratories, Richmond, CA) in 0.7 M glycine, 25 mM Tris.Cl, 20% ethanol and the filters were blocked in Tris-buffered saline (20 mM Tris.Cl, pH 8.8, 0.5 M NaCl, pH 7.5) (TBS), 0.5% Tween 20. mAb supernatants were used at a 1/15 dilution in TBS, 0.5% Tween 20. Secondary peroxidase-labelled goat anti-mouse IgG and IgM sera (Sigma Chemical Co., St. Louis, MO) were used at a 1/1,000 dilution. Blots were developed with 3,3'-diaminobenzidine as a substrate.

Glycoprotein analysis

A glycan-protein detection kit (Boehringer Mannheim, Germany) was used to assess whether antigen SA-5K was a glycoprotein. Purified SA-5K, a non-glycosylated control protein (creatinase) and a control glycoprotein (transferrin) were separated by SDS-PAGE and analysed by glycoprotein oxidation, labelling and antibody detection according to the manufacturers' instructions.

Protein sequencing

Purified SA-5K was loaded onto a 15% SDS-PAGE gel in reducing conditions and electrophoresis was carried out as above. Electrotransfer was performed as described by Houen (1993). Western blot with mAb L8D8 was performed on a strip of the blotted membrane to locate the antigen, and the band corresponding to SA-5K was cut. Aminoterminal sequencing was performed by Edman's degradation at Primm srl, Milan, Italy. To determine sequence homology, searches of the GenBank and SWISS-PROT data bases were conducted by BLAST.

Gel filtration chromatography

A "HiLoad 16/60 Sephacryl S-100" high resolution column (Phamarcia Biotech, Uppsala, Sweden) was equilibrated in 50 mM Tris Cl, pH 7.2. An apparent MM standard curve was determined by monitoring absorbance at 280 nm of eluted cytochrome *C*, chymotrypsinogen A, ovalbumin and aldolase as MM markers and "Blue Dextran 2000" for determination of void volume. Twelve-day-old CFs, 1 mg, in 50 mM Tris Cl, pH 7.2, were applied to the column. Fractions of 1.5 ml were collected and concentrated to 50 μl under a vacuum; then, 5 μl of individual fractions were separated by SDS-PAGE in reducing conditions and tested by Western blot using mAb L8D8.

Polymerase chain reaction

Genomic DNA was obtained from BCG as described (Van Soolingen *et al.*, 1991). DNA from

M. tuberculosis $H_{37}Rv$ was a kind gift of Dr. J. Belisle, Colorado State University (USA). The upstream primer used for analysis was UPL8: 5'-ACTCATTTCTCGGGC-3' and the downstream primer was LOL8: 5'-ATGGTTTCGAACACG-3'. Each reaction was made up of 10 ng of BCG DNA, 5 µl of 10 × *Taq* polymerase buffer (Pharmacia), 100 µM dNTP, 0.4 µM primer UPL8 and LOL8, and 2 U *Taq* polymerase to a final volume of 50 µl. The reactions were subjected to 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min in a "GeneAmp PCR system 9600" thermal cycler (Perkin-Elmer, Germany). Restriction analysis was performed as follows: 8 PCR reactions, each with BCG and *M. tuberculosis* DNA as templates, were set up, pooled and precipitated with ethanol. The pellets were resuspended in 20 µl of water, 3 µl of which were digested with 10 U of either *Apa*I, *Bam*HI or *Kpn*I for 1 h at 37°C. The digestion mix was loaded onto a 1% agarose gel and analysed.

Cloning and expression of the SA-5K gene

The gene encoding SA-5K was cloned by PCR from genomic DNA of BCG and *M. tuberculosis* $H_{37}Rv$. The upstream primer used was UP3: 5'-GAGATCTGGAGAGTGAACTGCG-3' and the downstream primer was LO3: 5'-TGGCCGGTTCCGAAGACG-3'. Each reaction was made up of 100 ng of DNA, 5 µl of 10 × Deep Vent™ polymerase buffer (New England Biolabs, Beverly, MA), 200 µM dNTP, 0.4 µM primer UP3 and LO3, and 0.5 U Deep Vent™ polymerase to a final volume of 50 µl. The reactions were subjected to 30 cycles at 95°C 1 min, 62°C 1 min, 72°C 1 min. The PCR amplification products were ligated in two directions into the "pCR-Blunt" vector (Invitrogen, the Netherlands) following the manufacturers' instructions. The plasmids obtained were transformed into *E. coli* TOP10 cells. Screening and determination of the insert orientation were performed by restriction analysis.

To check for expression of SA-5K, overnight cultures of recombinant *E. coli* TOP10 cells in Luria-Bertani broth, 1.5 ml, were centrifuged at 12,000 *g* for 5 min. The cell pellets were resuspended in 200 µl of water and cells were disrupted by freeze-thawing. Debris was removed by centrifugation and supernatants were concentrated to 15 µl under vacuum. Laemmli sample buffer with DTT was added and samples were loaded onto a 15% SDS-PAGE gel to perform Western blot.

Nucleotide sequence accession number

The nucleotide sequence of cosmid Y348 was found in the MycDB database and has the accession number AD000020. The Sanger sequence accession number is TB-652. The Tigr sequence is tb_09109.

RESULTS

Purification of SA-5K from BCG CFs

Recently, we described a mAb, L8D8, which is able to detect a protein of an apparent MM of 5 kDa in BCG CFs, as judged by Western blot after SDS-PAGE of BCG CF proteins in reducing conditions. mAb L8D8 did not exhibit any reactivity against different subcellular fractions of BCG (total cell lysate, cytoplasm-, cell wall- and membrane-enriched) (Freer *et al.*, 1998). Because no other antigen of such apparent MM has yet been described, SA-5K was purified by affinity chromatography in order to characterize it. The purified protein was analysed for purity by SDS-PAGE and silver staining. Surprisingly, although the purified antigen could be detected as a strong 5-kDa band by Western blot, no 5-kDa band could be seen on SDS-PAGE gels after Coomassie blue or silver nitrate staining (data not shown, see discussion). Thus, SA-5K could only be visualized by immunoblot.

Glycoprotein analysis

At least one other mycobacterial protein has been shown not to stain by conventional techniques (Dobos *et al.*, 1995). Because such a feature has been hypothesized to relate to glycosylation (Dobos *et al.*, 1995), attempts were made to assess whether SA-5K was glycosylated (data not shown). Failure of SA-5K to be detected by the method described in "Materials and methods" indicates that this protein is not glycosylated.

Determination of the native MM of SA-5K

During initial characterization of mAb L8D8, it was noticed that recognition of SA-5K by such a mAb did not occur if Western blot was performed after SDS-PAGE in non-reducing conditions. This feature was analysed by running whole BCG CFs in reducing and non-reducing conditions on 15% SDS-PAGE gels, after either boiling the samples or not (fig. 1). As can be seen, the presence of DTT in Laemmli loading buffer was essential for mAb L8D8 to react with

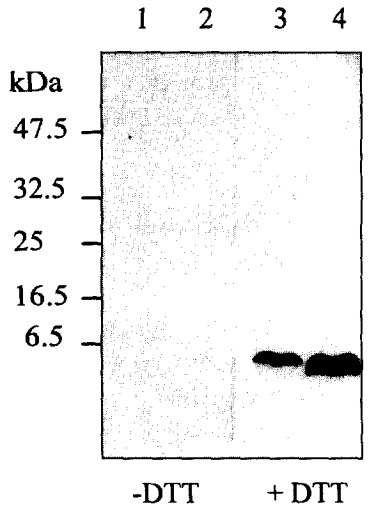

Fig. 1. Effect of reducing conditions on reactivity of mAb L8D8 with SA-5K in Western blot.

CFs, 15 μg, were separated by SDS-PAGE in non-reducing conditions without boiling (lane 1) or after boiling (lane 2) or in reducing conditions without boiling (lane 3) or after boiling (lane 4). MM markers are indicated on the left.

its 5-kDa antigen, while boiling the samples only seemed to enhance the effect of DTT. Thus, the epitope binding to mAb L8D8 was only exposed after reduction of the antigen.

Because it was not possible to stain the protein in SDS-PAGE gels, nor was it possible to detect it by Western blot after electrophoresis in non-denaturing conditions, native SA-5K MM was determined by size exclusion gel filtration: BCG CFs were fractionated through a "Sephacryl S-100" column. Fractions of different MM were analysed for mAb L8D8 recognition by Western blot in reducing conditions. Western blot analysis of equal volumes of the fractions eluted is shown in figure 2: two major mAb L8D8 reactivity peaks were obtained in the fractions corresponding to approximately 48 and 14-16 kDa, while no reactivity peak could be seen in fractions corresponding to 5 kDa (not shown). An attempt was made to confirm the native MM found for SA-5K by running BCG CFs and purified SA-5K by

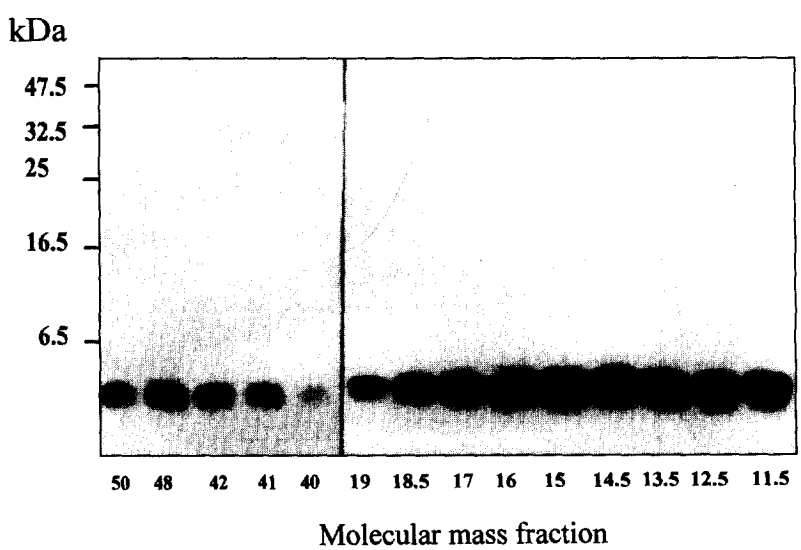

Fig. 2. mAb L8D8 reactivity in different apparent MM fractions.

Twelve-day-old CFs (1 mg) were fractionated by size exclusion chromatography on a Sephacryl S-100 column in 50 mM Tris Cl, pH 7.2. Western blot analysis was performed on equal volumes of fractions. Mean MMs calculated for each fraction on the basis of the standard curve are shown below the figure, while SDS-PAGE MM markers are indicated on the left.

non-reducing SDS-PAGE and reducing the gel after the run by soaking it in 50 mM DTT for 10 min and performing Western blot. Unexpectedly, a band with an apparent MM of roughly 6 kDa could be detected in the lanes run in non-reducing conditions, both with the purified antigen and with that present in CFs (fig. 3).

Aminoterminal sequencing of SA-5K

The N-terminal sequence of purified SA-5K was determined by Edman's degradation method. The peptide sequence found was DPVDAVINTT. A search in the Sanger and Tigr databases yielded a sequence of aa completely matching the first 10 of SA-5K. The whole open reading frame (ORF) encoding the DPVDAVINTT peptide is shown in figure 4. The ORF is made up of 330 nucleotides, extending from a putative ATG start site and ending at a TAA stop codon. A possible Shine-Dalgarno ribosome-binding sequence is located in position –9 and a possible Pribnow box is located in position –44. The sequence encodes 110 aa with an estimated MM of 10.9 kDa.

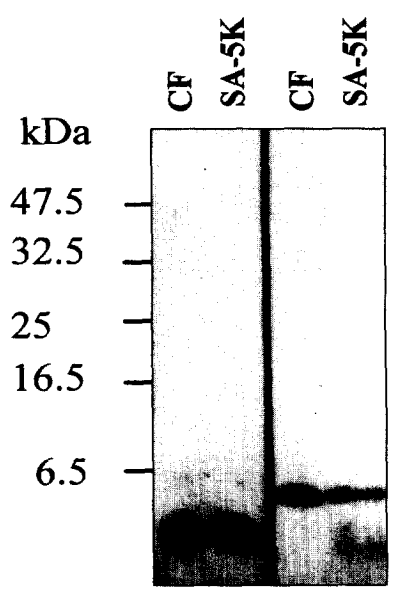

Fig. 3. Western blot analysis of BCG CFs and purified SA-5K with mAb L8D8.

BCG CFs, 15 µg, or purified SA-5K, 1-2 µg, were run by SDS-PAGE in reducing (left) or non-reducing (right) conditions. Before transfer to nitrocellulose, the gel was soaked in 50 mM DTT for 10 min. MM markers are indicated on the left.

PCR analysis of BCG and *M. tuberculosis* $H_{37}Rv$ genomic DNA

To analyse whether the sequence described for *M. tuberculosis* could also be found in BCG, a PCR primer pair was designed based on the sequence in the Sanger database. Genomic DNA from both BCG and *M. tuberculosis* $H_{37}Rv$ were subjected to PCR amplification. As can be seen in figure 5, the expected amplification products of about 800 bp could be detected when DNAs from both species were amplified, although the product from BCG DNA was calculated to be approximately 50 bp shorter. The PCR products obtained were also subjected to restriction analysis to confirm amplification of the expected DNA sequence. Three natural unique restriction sites present in the *M. tuberculosis* 811-bp sequence expected were exploited (see figure 4): a *Kpn*I site, dividing the *M. tuberculosis* PCR fragment obtained into a 114- and a 697-bp fragment, a *Bam*HI site, yielding a 353- and a 458-bp fragment and an *Apa*I site, dividing the product into a 541- and a 270-bp fragment. Indeed, this was the restriction pattern observed after digestion of the PCR product obtained by amplifying *M. tuberculosis* DNA with these 3 enzymes (fig. 5). In agreement with the fact that amplification of BCG DNA yielded a slightly shorter product, all restriction fragments of amplified BCG DNA were shorter than expected when they contained the 3' terminus.

Cloning of the gene for SA-5K from BCG and *M. tuberculosis* DNA

In order to clone the gene for SA-5K from *M. tuberculosis* and BCG, a PCR primer pair was designed based on the SA-5K DNA sequence that was expected to yield a 943-bp PCR product from *M. tuberculosis* DNA including the putative promoter region. To prevent amplification errors,

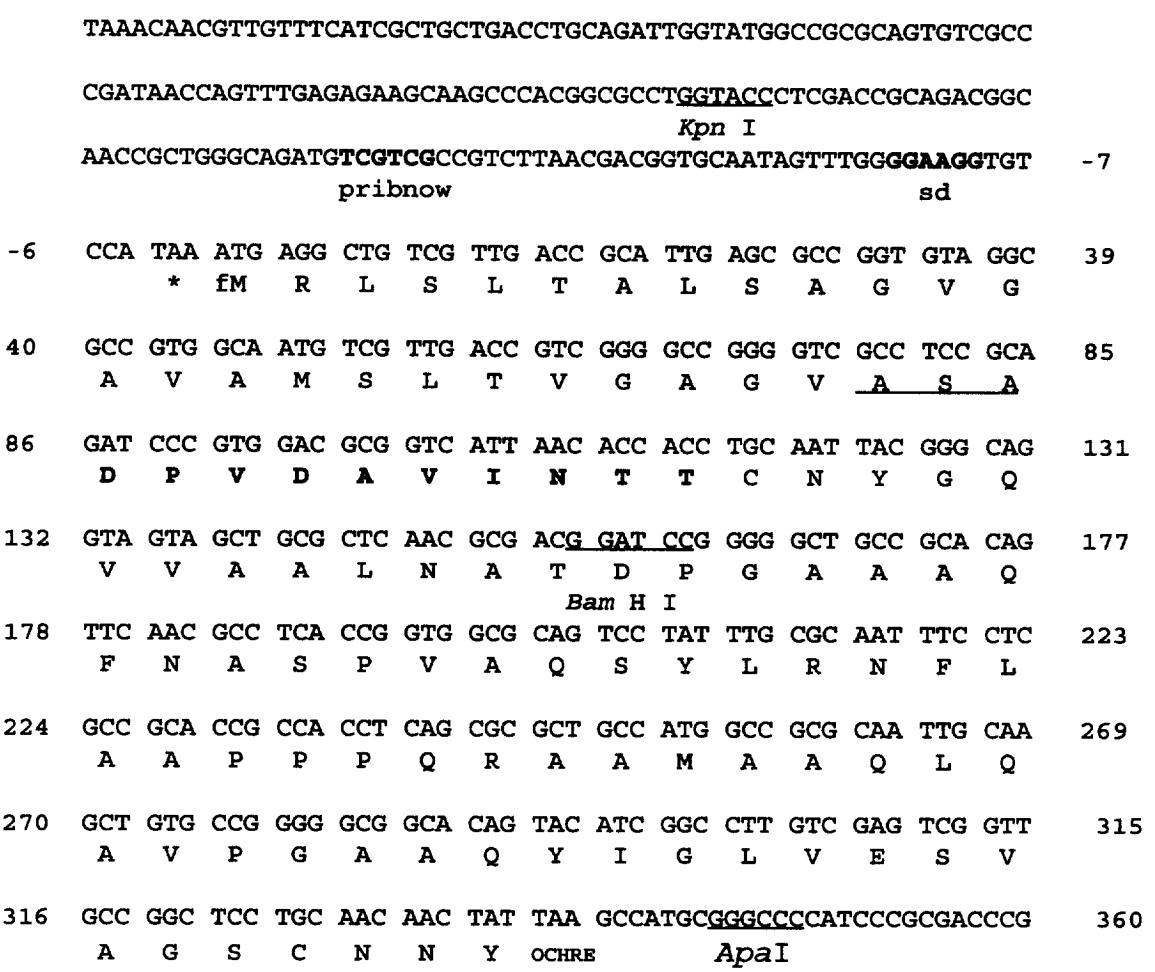

Fig. 4. Nucleotide and deduced aa sequence of the *M. tuberculosis* SA-5K gene.

The aminoterminal sequence found for SA-5K is in boldface. The putative Shine-Dalgarno sequence (sd), a Pribnow box in position −49 and the putative TATA box are in boldface. A signal peptidase consensus region in position 76 is underlined. Asterisks indicate stop codons.

proofreading DNA polymerase was used. As expected, the product obtained from BCG was slightly shorter than that from *M. tuberculosis*. Both products were cloned into the "pCR Blunt" vector, so that the SA-5K gene could be driven by the *lacZ* or the T7 promoter. Four kinds of plasmids were therefore obtained: pCR-lacZ-B, with the BCG SA-5K gene driven by the *lacZ* promoter, pCR-T7-B, with the gene driven by the T7 promoter, pCR-lacZ-H, containing the *M. tuberculosis* SA-5K gene driven by the *lacZ* promoter, and pCR-T7-H, containing the gene driven by the T7 promoter. To assess whether the plasmids were expressed in *E. coli* TOP10 cells, which lack $lacI^q$, resulting in constitutive expression of the *lacZ* promoter, cell lysates were analysed by immunoblotting with mAb L8D8. The results obtained are shown in figure 6: *E. coli* cells containing both the BCG and the *M. tuberculosis* gene driven from the *lacZ* promoter expressed the

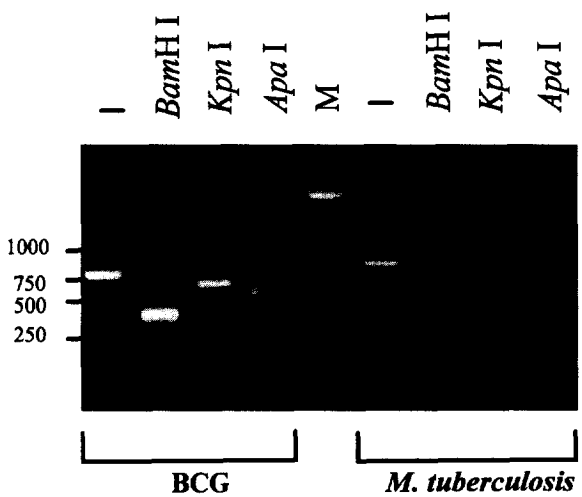

Fig. 5. PCR amplification of the putative SA-5K gene from BCG and *M. tuberculosis* $H_{37}Rv$ genomic DNA and restriction analysis of the product.

BCG (left) and *M. tuberculosis* $H_{37}Rv$ (right) DNA, 10 ng, were subjected to PCR amplification as described in "Materials and Methods" by use of oligonucleotide primers complementary to the flanking regions of the *M. tuberculosis* ORF encoding SA-5K. The PCR products were digested with *Bam*HI, *Kpn*I or *Apa*I. MM markers are indicated on the left.

Dashes signify "undigested".

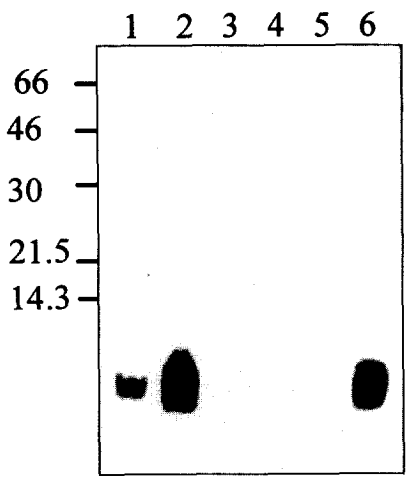

Fig. 6. Western blot analysis of recombinant SA-5K produced by "pCR Blunt" in *E. coli* cells.

BCG CF, 10 μg (lane 1) or lysates of 1.5 ml of overnight cultures of *E. coli* cells containing pCR-lacZ-B (lane 2), pCR-T7-B (lane 3), pCR vector alone (lane 4), pCR-T7-H (lane 5) or pCR-lacZ-H (lane 6) were separated by SDS-PAGE in reducing conditions and transferred onto a nitrocellulose membrane and reacted with mAb L8D8. MM markers are indicated on the left.

gene, detected as a 5-kDa product, while *E. coli* cells with the genes driven from the T7 promoter did not express SA-5K, since the T7 promoter is not recognized by *E. coli* RNA polymerase.

DISCUSSION

In this study, a novel secretion protein reacting with a mAb, L8D8, was studied in detail (Freer *et al.*, 1997). mAb L8D8 recognized its epitope only if reduced prior to SDS-PAGE (fig. 1). This seems to indicate that reduction of a disulphide bridge is required for optimal binding of mAb L8D8 to the antigen. Similar behaviour was also observed during immunochemical characterization of another mycobacterial antigen, MPB70/80 (Wiker, personal communication).

The immunoaffinity-purified protein was subjected to aminoterminal sequencing, yielding a peptide identical to the aa sequence deduced from an ORF of the *M. tuberculosis* genome. Interestingly, the N-terminal peptide is located right after a sequence encoding Ala-Ser-Ala, which may be a signal peptidase consensus region. Strong evidence suggests that the 28 aa preceding the peptide sequenced make up a signal for secretion: i) the length of signal sequences ranges between 15 and 30 aa; ii) a consensus region for secretion signal peptidase (Ala-X-Ala) lies adjacent to the N-terminal peptide sequenced on the ORF (Perlman and Halvorson, 1983); iii) signal sequences usually contain at least one positively charged aa at the N-terminus, such as the Arg residue at the N-terminus of the aa sequence encoded by the ORF. The presence of a signal sequence explains why the DPVDAVINTT sequence lies internally to the ORF in figure 4 and is also in agreement with the fact that mAb L8D8 reactivity could only be found in CFs of BCG (Freer *et al.*, 1998). There is a discrepancy in the sequence of cosmid Y348 and in the Sanger and Tigr sequences: the G residue in position 276 is missing in cosmid Y348, and this leads to a difference in the ORFs found. Figure 4 is based on the Sanger and Tigr sequences.

Purified SA-5K did not stain in SDS-PAGE gels by conventional protein staining techniques,

such as Coomassie blue, silver nitrate or $CuCl_2$. This behaviour has been described for at least one other mycobacterial antigen, the 45-kDa protein (Dobos *et al.*, 1995). It does not seem to depend on the sensitivity of the methods used for staining, since the amount of protein required for aminoterminal sequencing of proteins on PVDF is much higher than the sensitivity limit of any of them; while it was not possible to stain SA-5K with Coomassie after transfer on PVDF, it was possible to sequence the protein on the same. Therefore, the purified antigen was only visualized as a 5-kDa band after SDS-PAGE in reducing conditions and Western blot with mAb L8D8.

The MM deduced from the gene for SA-5K was close to that determined for the native antigen present in BCG CFs by size exclusion gel filtration. mAb L8D8 reactivity was found to peak in fractions corresponding to 14-16 and 45-50 kDa, while no reactivity peak could be detected in the fractions around 5 kDa. This strongly suggests that SA-5K is a protein of around 10 kDa, which might assemble into polymers in BCG CF, either as an artifact or as a result of its quaternary structure. It is tempting to speculate that SA-5K is translated as a 10.8-kDa molecule and then cleaved both after the signal for secretion yielding a protein of 8.3 kDa and at a site lying between two Cys residues that are linked by a disulphide bridge, which would hold the 2 parts together after cleavage. Reduction of the bridge would cause complete exposure of the epitope for mAb L8D8 and the separation of the two protein fragments making up SA-5K, only one of which (5 kDa) would be visualized by Western blot.

Separating CF proteins and purified SA-5K by non-reducing SDS-PAGE (with DTT reduction after the gel run) led to the detection of a 6-kDa band by Western blot: it is shown that certain proteins may migrate faster in SDS-PAGE gels when unreduced, since intact disulphide bridges do not allow binding the optimum amount of SDS, and cause different hydrodynamic volumes (See and Jackowski, 1990).

Previous work showed that antigen SA-5K is not acylated (Freer *et al.*, 1998). In the present work, no sugar residues could be detected on the molecule. The 5-kDa antigen detected by mAb L8D8 was also shown to be resistant to trypsin/papain digestion, which is in agreement with the observation that no cutting sites (Arg-X/Lys-X) for such proteases are to be found in the first 5 kDa of the ORF in figure 4 (Freer *et al.*, 1998).

The presence of the SA-5K gene in BCG was confirmed by PCR and restriction analysis: the PCR product is slightly longer in *M. tuberculosis* than in BCG, which seems to miss around 50 nucleotides towards the 3' terminus. However, the component reacting with mAb L8D8 is of 5 kDa both in BCG and in *M. tuberculosis* (Freer *et al.*, 1998). To compare SA-5K protein in BCG and *M. tuberculosis*, we tried: a) to develop a polyclonal serum in the mouse that could detect SA-5K without the need to denature the protein, but the serum had the same features as mAb L8D8; b) to compare the proteins by the technique used in figure 3, but no difference was seen. Therefore, comparison of the SA-5K protein from BCG and *M. tuberculosis* could not be carried out.

The gene for SA-5K was cloned from BCG and *M. tuberculosis* and expressed in *E. coli*. The gene and its putative promoter region were inserted in two directions into the "pCR Blunt" vector. Only plasmids containing inserts driven by the *lacZ* promoter were expressed by *E. coli* cells, showing that the mycobacterial promoter was not responsible for transcription of the gene.

No similarity with any other known proteins was found; the function of the antigen in mycobacterial physiology remains obscure. Experiments are in progress to evaluate whether SA-5K is able to induce protective immunity in experimental animal models and to assess whether it can be useful as a diagnostic tool for tuberculosis.

Acknowledgments

This work was supported by the National Tuberculosis Project (Istituto Superiore di Sanità-Ministero della Sanità)", Rome, grant n° 96/D/T18 and by the EU BIOMED 2 Programme, contract BMH4-CT97-2671.

We are grateful to Harald Wiker for valuable suggestions.

Identification et clonage moléculaire d'un nouvel antigène de sécrétion de *Mycobacterium tuberculosis* et de *Mycobacterium bovis* BCG

Une nouvelle protéine nommée SA-5K a été identifiée dans des filtrats de culture à court terme (CFs) de *Mycobacterium bovis* BCG, au moyen de l'anticorps monoclonal L8D8 récemment décrit. Evaluée par Western blot après SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) en conditions de réduction, cette protéine a une masse moléculaire de 5 kDa et ne semble pas contenir de substituants osidiques ou lipidiques.

Nous avons présentement purifié cette protéine par chromatographie d'affinité. Une protéine pouvant être détectée par Western blot, mais non par des techniques standards de coloration protéinique, a été obtenue. Le séquençage amino-terminal de SA-5K permet de voir que les 10 aa (acides aminés) concernés sont semblables à la première séquence des 10 aa déduite d'une ORF (open reading frame) de *M. tuberculosis*. Cette ORF code un polypeptide, incluant probablement un signal de sécrétion, dont la masse moléculaire est estimée à 8.3 kDa après le signal de clivage du peptide. La nature secrétoire de SA-5K a été confirmée par le fait que la protéine est détectée seulement dans les CFs à l'exclusion des autres fractions subcellulaires du BCG. Après chromatographie par exclusion de taille, ce sont les fractions de 45-50 et de 14-16 kDa qui réagissent le plus avec l'anticorps monoclonal L8D8. La fraction 14-16 kDa est proche de celle estimée à partir de l'ORF de *M. tuberculosis*, ce qui implique que l'antigène de 5 kDa détecté initialement par Western blot en conditions de réduction est une fraction de SA-5K libérée après réduction d'un pont disulfure.

La présence du gène de la SA-5K dans le BCG et son identité ont été confirmées par PCR (polymerase chain reaction) à l'aide d'amorces spécifiques et par analyse de restriction: le produit de la PCR est légèrement plus court dans le BCG que chez *M. tuberculosis*. Le gène codant la SA-5K a été cloné par PCR à partir de l'ADN de *M. tuberculosis* et du BCG et a été exprimé chez *Escherichia coli*.

Mots-clés: BCG, *Mycobacterium tuberculosis*, Protéine SA-5K; Antigènes de sécrétion, Clonage moléculaire du gène.

References

Andersen, A.B. & Brennan P.J. (1994), Pathogenesis, protection and control *in* "Tuberculosis". (B.B. Bloom). ASM Press, Washington DC.

Andersen, B.A. & Hansen, E.B. (1989), Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. *Infect. Immun.*, 57, 2481-2488.

Andersen, P. (1994), Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. *Infect. Immun.*, 62, 2536-2544.

Andersen, P. & Heron I. (1993), Specificity of a protective memory immune response against *Mycobacterium tuberculosis*. *Infect. Immun.*, 61, 844-851.

Ashbridge, K.R.A., Booth, R.J., Watson, J.D. & Lathigra, R.B. (1989), Nucleotide sequence of the 19 kDa antigen gene from *Mycobacterium tuberculosis*. *Nucleic Acids Res.*, 17, 1249-1253.

Bigi, F., Alito, A., Fisanotti, J.C., Romano, M.I. & Cataldi, A. (1995), Characterization of a novel *Mycobacterium bovis* secreted antigen containing PGLTS repeats. *Infect. Immun.*, 63, 2581-2586.

Boesen, H., Jensen, B.N., Wilcke, T. & Andersen, P. (1995), Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. *Infect. Immun.*, 63, 1491-1497.

Bradford, M.M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254.

Daugelat, S., Ladel, C.H. & Kaufmann, S.H.E. (1995), Influence of mouse strain and vaccine viability on T-cell responses induced by *Mycobacterium bovis* bacillus Calmette-Guerin. *Infect. Immun.*, 63, 2033-2040.

Dobos, K.M., Swiderek, K., Khoo, K.-H., Brennan, P.J. & Belisle, J.T. (1995), Evidence of glycosylation sites on the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. *Infect. Immun.*, 63, 2856-2853.

Fine, P.E.M. (1989), The BCG story: lessons from the past and implications for the future. *Rev. Infect. Dis.*, 12, 353-359.

Florio, W., Freer, G., Dalla Casa, B., Batoni, G., Maisetta, G., Senesi, S. & Campa, M. (1997), Comparative analysis of subcellular distribution of protein antigens in *Mycobacterium bovis* bacillus Calmette-Guérin. *Can. J. Microbiol.*, 43, 744-750.

Freer, G., Florio, W., Dalla Casa, B., Castagna, B., Maisetta, G., Batoni, G., Corsini, V., Senesi, S. & Campa, M. (1998), Subcellular localization of antigens recognized by new monoclonal antibodies against proteins of *Mycobacterium bovis* bacillus Calmette-Guérin. *FEMS Immunol. Med. Microbiol.*, 20, 129-138.

Harlow, E. & Lane, D. (1988), Antibodies: A laboratory manual. Cold Spring Harbor laboratory, New York.

Houen, G. (1993), Microsequencing of polypeptides after electrophoresis in modified Laemmli gels and recovery of polypeptides from polyvinyl difluoride membranes. *Methods Mol. Cell. Biol.*, 4, 105-114.

Huygen, K., Content, J., Denis, O., Montgomery, D.L., Yawman, A.M., Deck, R.R., DeWitt, C.M., Orme, I.M., Baldwin, S., D'Souza, C., Drowart, A., Lozes, E., Vandenbussche, P., Van Vooren, J.-P., Liu, M.A. & Ulmer, J.B. (1996), Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nature Med.*, 2, 893-898.

Laemmli, U.K. (1970), Cleavage of structural proteins

during assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Manca, C., Lyashchenko, K., Wiker, H.G., Usai, D., Colangeli, R. & Gennaro, M.L. (1997), Molecular cloning, purification and serological characterization of MPT63, a novel antigen secreted by *Mycobacterium tuberculosis. Infect. Immun.*, 65, 16-23.

Milstien, J.B. & Gibson, J.J. (1990), Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety. *Bull. W. H. O.*, 68 (1), 93-108.

Ohara, N., Kitaura, H., Hotokezaka, H., Nishiyama, T., Wada, N., Matsumoto, S., Matsuo, T., Naito, M. & Yamada, T. (1995), Characterization of the gene encoding the MPB51, one of the major secreted protein antigens of *Mycobacterium bovis* BCG, and identification of the secreted protein closely related to fibronectin binding 85 complex. *Scand. J. Immunol.*, 41, 433-442.

Orme, I.M. (1988), Induction of nonspecific acquired resistance and delayed type hypersensitivity, but not specific acquired resistance, in mice inoculated with killed mycobacterial vaccines. *Infect. Immun.*, 56, 3310-3312.

Pal, P.G. & Horwitz, M.A. (1992), Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. *Infect. Immun.*, 60, 4781-4792.

Perlman, D. & Halvorson, H.O. (1983), A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.*, 167, 391-409.

See, Y.P. & Jackowski, G. (1990), *in* "Protein structure: a practical approach", T.E. Creighton (ed.), (pp. 1-2). IRL Press at Oxford University Press, Oxford, England.

Sørensen, A.L., Nagai, S., Houen, G., Andersen, P. & Andersen, A.B. (1995), Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis. Infect. Immun.*, 63, 1710-1717.

Tascon, R.E., Colston, M.J., Ragno, S., Stavropoulos, E., Gregory, D. & Lowrie, D.B. (1996), Vaccination against tuberculosis by DNA injection. *Nature Med.*, 2, 888-892.

Van Soolingen, D., Hermans, P.W.M., de Haas, P.E.W., Soll, D.R. & van Embden, J.D.A. (1991), The occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains; evolution of IS-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol.*, 29, 2578-2586.

Wiker, H.G. & Harboe, M. (1992), The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis. Microbiol. Rev.*, 56, 648-661.

Yamaguchi, R., Matsuo, K., Yamazaaki, A., Abe, C., Nagai, S., Teresaka, K. & Yamada, T. (1989), Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. *Infect. Immun.*, 57, 283-288.

Young, D.B., Kaufmann, S.H.E., Hermans, P.W.M. & Thole, J.E.R. (1992), Mycobacterial protein antigens: a compilation. *Mol. Microbiol.*, 6, 133-145.

Zhang, Y., Lathigra, T., Garbe, T., Catty, D. & Young, D. (1992), The catalase-peroxidase gene and isoniazide resistance of *Mycobacterium tuberculosis. Nature*, 358, 591-593.