Secretion of the Mycobacterial 19-Kilodalton Protein by *Escherichia coli*, a Novel Method for the Purification of Recombinant Mycobacterial Antigens

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We have reported previously (R. G. Hewinson and W. P. Russell, J. Gen. Microbiol. 139:1253–1259, 1993) the secretion by *Escherichia coli* of a recombinant form of the immunogenic protein MPB70 of *Mycobacterium bovis* when the protein is translated from its native initiation codon. N-terminal sequence analysis of the purified protein revealed that the signal peptide of MPB70 was cleaved by an endopeptidase of *E. coli* at the same cleavage site as reported for the protein in *M. bovis*. Since both the B- and T-cell antigenicities of the purified recombinant protein were similar to that of the native protein, the 19-kDa antigen of *M. bovis* was used as a model to test whether the signal peptide of MPB70 could direct the secretion of heterologous proteins in *E. coli* and whether antigen produced in this way retained antigenicity superior to that of recombinant protein produced as a fusion to glutathione-S-transferase. A chimeric protein was produced in which the signal peptide of MPB70 was fused to the 19-kDa antigen of *M. bovis* at amino acid residue 23. This chimeric protein was found to be secreted into the periplasm and culture medium of recombinant *E. coli*, and the signal peptide was cleaved by an endopeptidase of *E. coli* during secretion. Secretion of the 19-kDa antigen facilitated purification of the antigen by two-stage preparative electrophoresis which gave yields of 2.5 mg of purified, soluble 19-kDa antigen from 2.5 g (wet weight) of *E. coli*. Antigen purified in this way retained both B- and T-cell antigenicities. Moreover, the nonspecific mitogenic activity of the purified 19-kDa antigen was low, while the magnitude of the T-cell response induced by the purified antigen was considerably higher than that observed with purified antigen produced as a fusion protein with glutathione-S-transferase.

The tuberculin purified protein derivatives currently used for the diagnosis of tuberculosis in cattle and humans contain a complex mixture of proteins derived from *Mycobacterium bovis* and *Mycobacterium tuberculosis*, respectively (6, 12, 35). Within this heterogeneous mixture of proteins are both species-specific epitopes and antigenic determinants common to many mycobacterial species (for a recent review, see reference 46). The antigenic cross-reactivity of purified protein derivatives is a major problem for disease diagnosis. Improvement of immunodiagnostic screening for *M. bovis* and *M. tuberculosis* infection therefore relies on the identification and purification of antigens exhibiting a high degree of specificity (4, 39, 40). To date a number of promising candidate antigens have been identified (8, 46). However, the use of isolated antigens in immunodiagnostic assays is hindered by difficulties in growing the organism and purifying the antigens. Recombinant proteins may provide a realistic alternative.

Previous reports have indicated that bulk purification of recombinant mycobacterial proteins in *Escherichia coli* may be achieved by fusion of the protein of interest to a second protein for which column purification procedures are available. However, this often results in the accumulation of the fusion protein as an insoluble mass within the bacterial cell. For example, although the 19-kDa antigen of *M. tuberculosis* has been over-expressed as a recombinant fusion protein in *E. coli* (1, 17) and also in the rapidly growing mycobacterial species *Mycobacterium smegmatis* (11), it has proven to be difficult to purify the

protein in quantities sufficient for immunological evaluation (11, 15).

Homologous 19-kDa lipoproteins which have been identified in a range of mycobacterial species possess a highly conserved amino-terminal consensus sequence for lipidation located adjacent to the hydrophobic signal peptide (2, 3, 5, 27). Biochemical studies indicate that the 19-kDa protein is glycosylated and that its signal peptide undergoes posttranslational acylation prior to secretion of the mature protein across the cell membrane (11, 27, 45). DNA sequence analysis has demonstrated that the 19-kDa lipoproteins of *M. bovis* and *M. tuberculosis* are identical (2, 5) and share approximately 75% amino acid identity with the homologous antigens from *Mycobacterium avium* subsp. *avium* and *M. avium* subsp. *intracellulare* (3, 27). The localization of T- and B-cell epitopes within the 19-kDa protein has been determined by using synthetic peptides, and despite sharing extensive amino acid sequence homology, species-specific B- and T-cell epitopes have been identified (15, 16, 24).

Recently, we described the production of a plasmid construct which generated a recombinant form of MPB70, an immunodominant secreted protein of *M. bovis* (8, 10, 13, 14, 30, 38), that was secreted across the cytoplasmic membrane of *E. coli* (19). The signal peptide of the recombinant protein was cleaved by an endopeptidase of *E. coli* resulting in the accumulation of a soluble, mature form of the protein in the periplasm and growth medium which facilitated purification of the antigen by two-step preparative electrophoresis (43). Antigen produced in this way was recognized by all monoclonal and polyclonal antibodies directed against MPB70 that were

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tested and elicited a T-lymphocyte response in *M. bovis*-infected cattle comparable to that observed with the purified native antigen (43). Here we report the ability of the signal peptide of MPB70 to direct the secretion of the 19-kDa antigen of *M. bovis* in a similar fashion, thereby facilitating the purification of the antigen which has until now proved refractory to high-level expression and purification in *E. coli* (1, 11, 15, 17). Moreover, we report that antigen produced in this way possesses improved T-cell antigenicity compared with that of antigen produced as a fusion protein with glutathione-S-transferase.

It is hoped that this novel methodology may prove to be widely applicable to the expression and purification of other mycobacterial proteins and provide a source of antigens of potential importance to the development of new vaccines and diagnostic reagents for mycobacterial diseases.

MATERIALS AND METHODS

Bacterial strains and vectors. *E. coli* DH5 α [$F^{-}\phi 80lacZ\Delta M15 \Delta (lacZYA-argF)U169 recA1 endA1 hsdR17 (r_k^{-} m_k^{+}) supE44 \lambda^{-} thi-1 gyrA relA1$] was purchased as Max efficiency competent cells (Gibco BRL, Paisley, Scotland) and was used as the host for the plasmid vectors pBluescript II KS $^{+}$ (Stratagene, Cambridge, United Kingdom) and pVW500 (19). Cells were grown at 37°C at 200 rpm in Luria-Bertani broth to which 0.1% (wt/vol) glucose was added (LBG [33]) or on Luria-Bertani agar (33) in the presence of 100 μ g of ampicillin ml $^{-1}$ to maintain plasmid selection. *E. coli* MC1061 [*araD139 \Delta(argF-lac)U169 trp(Am) malB(Am) rpsL relA thi supF \Phi (araBA'-lacZ^{+})101 [\lambda p1(209)]*] was used for the expression of the 19-kDa fusion protein encoded by a derivative of pGEX3X as described previously (17). *M. bovis* AN5 (Central Veterinary Laboratory, Weybridge, United Kingdom) was grown at 37°C on Lowenstein-Jensen medium containing 0.4% (wt/vol) pyruvate and was harvested as described previously (26).

Immunological reagents. *M. bovis* sonicated extract was prepared by the method of Morris et al. (26). Rabbits were immunized subcutaneously at multiple sites with *M. bovis* sonicated extract (100 μ g) in Freund's incomplete adjuvant. The rabbits were boosted twice at 14-day intervals. Murine anti-19-kDa antigen monoclonal antibody, CMA134.1, has been described previously (5) and was kindly provided by Coralabs Research.

Polyclonal antisera reactive with peptides p2 (residues 11 to 30) and p15 (residues 140 to 159) from the *M. tuberculosis* 19-kDa protein were produced by injecting C57BL/10 mice (groups of 3) intraperitoneally with 100 μ g of synthetic peptide emulsified in incomplete Freund's adjuvant. Mice were boosted intraperitoneally at weekly intervals with 50 μ g of peptide suspended in phosphate-buffered saline (PBS), and pooled serum was collected after the fourth boost. Monoclonal antibody TB-23 was kindly provided by Juraj Ivanyi, Medical Research Council Tuberculosis Unit (Hammersmith Hospital, London, United Kingdom). Recombinant *M. tuberculosis* 19-kDa fusion protein (rGST-19) was prepared from cell lysates of overexpressing *E. coli* MC1061 cells as described previously (17).

PCR. DNA was extracted from *M. bovis* AN5 by the method of Whipple et al. (44). PCR was performed in 50- μ l reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.01% (wt/vol) gelatin, 3 mM MgCl $_2$, 200 μ M (each) deoxynucleoside triphosphate, 2.5 U of thermostable *Taq* DNA polymerase (Perkin-Elmer, Applied Biosystems Ltd., Cheshire, United Kingdom), and 20 pmol of each oligonucleotide primer. The DNA sequence of the oligonucleotide primers used for this study were as follows (the restriction endonuclease sites used for cloning the PCR product are underlined): 19-F, 5'-TTTGTAAACATCAAGCAACAAGTCGACTACAG-3'; 19-R, 5'-TTAGGATCCCGCATCGACACGCTTTAGG-3'. The reaction mixture was overlaid with 50 μ l of mineral oil, and DNA samples (10 ng) were pipetted through the oil into the mixture. Thermal cycling was performed with a TR1 thermal cycler (Hybaid, Middlesex, United Kingdom). The parameters for amplification were denaturation at 94°C for 3 min for one cycle and then 30 cycles at 94°C for 1.5 min, 56°C for 1.5 min, and 72°C for 2 min. A final extension at 72°C for 10 min was also included. A reagent blank (negative control) was run as described above, but without the addition of DNA sample.

Sequence analysis. DNA sequencing of double-stranded template was performed by the primer-extension dideoxy chain-termination method (34) with α - 35 S-adenosine triphosphate and T7 polymerase (Sequenase; United States Biochemicals Corp. as recommended by the manufacturer). The products of the sequencing reaction were resolved by electrophoresis through 6 and 8% polyacrylamide gels containing 7 M urea by using an S2 sequencing rig (Gibco BRL). After electrophoresis, the gels were dried under vacuum and were exposed to X-ray film for 24 to 48 h at room temperature.

Other genetic procedures. The restriction endonucleases and other enzymes were used according to the manufacturers' instructions. Other genetic manipulations were as described by Sambrook et al. (33).

Osmotic shock procedure. The method used to obtain periplasmic and cytoplasmic cell fractions was a modification of that described by Koshland and Botstein (22) and is described in detail elsewhere (19).

Determination of protein concentration. Protein concentrations were determined by using the bicinchoninic acid protein assay (Pierce and Warriner, Chester, United Kingdom) according to the manufacturer's instructions, with bovine serum albumin used as the reference standard.

PAGE and Western blotting. The method used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) has been described previously (19). Rainbow (Amersham International) or Bio-Rad (Hemel Hempstead, United Kingdom) protein markers were run as molecular mass standards. The proteins resolved by SDS-PAGE were transferred to nitrocellulose by electroblotting as described by Matsudaira (25) and were Western blotted (immunoblotted) as described previously (19). For two-dimensional PAGE, 40 μ g of purified protein extracts was treated with sample buffer as described by Hochstrasser et al. (20) and was separated by isoelectric focusing in tube gels as described by O'Farrell (28) with 4% Ampholines (pH 3.5 to 10; Pharmacia LKB, Uppsala, Sweden). SDS-PAGE in the second dimension was then carried out as described above. The proteins were detected by silver staining (Bio-Rad) according to the manufacturer's instructions.

Antigen purification by preparative two-stage electrophoresis. A 1-liter overnight culture of bacterial cells was grown at 37°C and 200 rpm in LBG broth containing 100 μ g of ampicillin ml $^{-1}$, and the cells were harvested by centrifugation at 7,000 $\times$ g for 15 min at 20°C. The supernatant was discarded and the cell pellet was subjected to osmotic shock as described below. Cells were resuspended in 90 ml of ice-cold 20% (wt/vol) sucrose–10 mM Tris-HCl (pH 7.5)–3 ml of 0.5 M EDTA solution (pH 8). The cells were then incubated on ice for 10 min, recovered by centrifugation (13,000 $\times$ g, 10 min, 4°C), and rapidly resuspended by vigorous agitation in 20 ml of ice-cold distilled water. The resuspended cells were incubated on ice for 10 min and were then centrifuged at 13,000 $\times$ g for 10 min at 4°C. The supernatant containing the periplasmic proteins was recovered and was diluted with deionized water to a final volume of 50 ml. Bio-lyte Ampholines (pH range 3 to 10; Bio-Rad) were then added to a final concentration of 2% (vol/vol), and the sample was subjected to preparative isoelectric focusing at 12 W and 4°C by using a Rotofor (Bio-Rad). Isoelectric focusing was achieved after approximately 5 h, after which the fractions were collected, run on SDS-polyacrylamide gels, and analyzed by silver staining and Western blotting against monoclonal antibody CMA134.1. The fractions containing recombinant antigen were separated again by preparative isoelectric focusing and were concentrated to 1.5 ml by ultrafiltration (Centricon 10; Amicon, Stonehouse, United Kingdom). The concentrate was then mixed with an equal volume of loading buffer (75 mM Tris-HCl [pH 6.8], 5% [vol/vol] 2-mercaptoethanol, 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.002% [wt/vol] bromophenol blue), and the mixture was heated in a boiling water bath for 5 min. The protein fractions contained in the sample were resolved through a 15% (wt/vol) SDS-polyacrylamide gel (10 cm high, 2.8 cm in diameter) at 40 mA for 10 h by using a Prep Cell apparatus (Bio-Rad) and the discontinuous buffer system of Laemmli (23). The elution chamber outlet of the Prep Cell was connected to a fraction collector, and following elution of the bromophenol blue, approximately 120 fractions (each one 2.5 ml) were collected. Fractions containing recombinant antigen which gave only one band following silver staining of SDS-polyacrylamide gels were pooled, concentrated to 1 ml by ultrafiltration with Centricon 10 columns, and dialyzed against 0.01 M PBS (pH 7.2). Antigen purified in this way was stored at -20°C .

Synthetic peptides. Peptides p2 (residues 11 to 30), p7 (residues 61 to 80), and p15 (residues 140 to 159) were synthesized by a simultaneous (solid-phase) multiple peptide synthesis procedure as described in detail elsewhere (17). Peptides were purified by Sephadex G-15 gel filtration and preparative high-performance liquid chromatography. The amino acid composition was verified by Edman degradation (Biopolymer Synthesis and Analysis Unit, University of Nottingham, Nottingham, United Kingdom).

Amino acid sequence. The sequences of the amino-terminal amino acids of the recombinant proteins were determined by automated Edman degradation with the Applied Biosystems 470 A upgraded liquid-phase sequenator (Biochemistry Department, Royal Holloway and Bedford College, London, United Kingdom).

Immunization procedure and T-cell proliferation assay. C57BL/10 mice (groups of three mice each) were immunized subcutaneously in both hind footpads with 25 μ g of recombinant antigen or PBS emulsified in incomplete Freund's adjuvant, and 8 to 10 days later, draining popliteal lymph nodes of the three mice were removed and pooled and single-cell suspensions were prepared in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL), 5×10^{-5} M β -mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin ml $^{-1}$, and 100 μ g of streptomycin sulfate ml $^{-1}$. Cells (4×10^5 cells per well) were then added in triplicate to 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) containing antigen at different concentrations ranging from 0 to 100 μ g ml $^{-1}$. After 3 days of incubation at 37°C in an atmosphere of 5% CO $_2$, the cells were radiolabelled with 37 kBq of [^3H]thymidine well $^{-1}$ (Amersham International). After a further 6 to 8 h, each well was harvested onto glass-fiber filter paper, and the incorporation of radioactivity was determined by liquid scintillation counting. Standard errors of the means for triplicate microcultures were usually <10%.

T-cell hybridomas. A T-cell hybridoma (B10.7.37) specific for peptide p61-80 from the *M. tuberculosis* 19-kDa protein was established by a standard fusion

FIG. 1. Schematic diagram of the chimeric protein encoded by plasmid pVW510. Plasmid pVW510 was constructed by forced cloning of the gene encoding the 19-kDa antigen (residues 23 to 159) (with *HpaI* and *BamHI* cloning sites produced by PCR; see Materials and Methods and Results) into the *SmaI* and *BamHI* sites of plasmid pVW500 (19). In this construct the 19-kDa antigen lacking its own signal peptide and lipoylation site was expressed in-frame with the first 36 amino acids of MPB70 and with a threonine residue created at position p37.

protocol (18, 41) with polyethylene glycol 1500 (PEG 1500; Boehringer, Mannheim, Germany) in conjunction with the TcR $\alpha\beta^-$ fusion partner BW5147 (kindly provided by Willi Born, National Jewish Hospital, Denver, Colo.). Once established, hybridomas were assayed for specificity by culturing with antigen and irradiated syngeneic spleen cells for 24 h. The culture supernatants were then removed and tested for their interleukin 2 content by using the HT-2 indicator cell line (41, 42).

RESULTS

Use of the secretory signal peptide of MPB70 to direct the secretion of the 19-kDa antigen of *M. bovis*. The 19-kDa protein was used as a model to test whether the secretory signal peptide of MPB70 could direct the secretion of other mycobacterial antigens across the cytoplasmic membrane of *E. coli*. PCR was used to amplify the gene encoding the 19-kDa antigen from chromosomal DNA extracted from *M. bovis* AN5. The oligonucleotide primers used in this reaction were designed to give a product of 451 bp of mycobacterial DNA which included restriction endonuclease sites at each end to allow cloning into the plasmid vector pVW500 (19) such that the 19-kDa antigen lacking its own signal peptide and lipoylation site would be expressed in-frame with the first 36 amino acids of MPB70 encoded by the plasmid vector (Fig. 1). The restriction sites were also chosen such that they would allow cloning into pBluescript II KS⁺ such that the same protein would be expressed in-frame with the first 40 amino acids of the α -peptide of β -galactosidase encoded by the plasmid vector. A 451-bp product was obtained from the PCR and was ligated into both pVW500 and pBluescript II KS⁺ (which had previously been digested with *SmaI* and *BamHI*) by using the *HpaI* and *BamHI* restriction endonuclease sites encoded by the oligonucleotide primers. The recombinant plasmids were transformed into *E. coli* DH5 α . The identities of the mycobacterial inserts were confirmed by DNA sequence analysis. The recombinant plasmids were designated pVW510 and pVW511, respectively.

Osmotic shock was used to obtain cytoplasmic and periplasmic fractions of *E. coli* DH5 α (pBluescript II KS⁺), *E. coli* DH5 α (pVW510), and *E. coli* DH5 α (pVW511). These fractions and the concentrated culture filtrates were Western blotted with CMA134.1, a monoclonal antibody specific for the 19-kDa antigen (5). The 19-kDa antigen was observed in all three cellular fractions when it was expressed in-frame with the secretory signal peptide of MPB70 (pVW510) (Fig. 2A). Moreover, two forms of the polypeptide were observed in the cytoplasmic and periplasmic fractions, whereas only the lower-molecular-mass form was observed in the culture filtrate. Recombinant 19-kDa antigen was detected only in the cytoplasmic fraction when the secretory signal peptide of MPB70

FIG. 2. (A) Western blots of cellular fractions of recombinant *E. coli* clones incubated with monoclonal antibody CMA134.1. Plasmid pBluescript II KS⁺ is the original plasmid vector. Plasmid pVW510 was constructed by cloning the gene encoding the 19-kDa antigen behind the leader peptide of MPB70 as described in Materials and Methods and Results and in the Fig. 1 legend. Lanes: 1, *E. coli* DH5 α (pVW510) culture filtrate; 2, *E. coli* DH5 α (pVW510) periplasmic fraction; 3, *E. coli* DH5 α (pVW510) cytoplasmic fraction; 4, *E. coli* DH5 α (pBluescript II KS⁺) culture filtrate; 5, *E. coli* DH5 α (pBluescript II KS⁺) periplasmic fraction; 6, *E. coli* DH5 α (pBluescript II KS⁺) cytoplasmic fraction. (B) Western blots of cellular fractions of recombinant *E. coli* clones incubated with monoclonal antibody CMA134.1. Plasmid pBluescript II KS⁺ is the original plasmid vector. Plasmid pVW511 was constructed by cloning the DNA fragment used to construct pVW510 into pBluescript II KS⁺ such that the 19-kDa antigen was expressed in-frame with the first 40 amino acids of the α -peptide as described in Results. Lanes: 1, *E. coli* DH5 α (pBluescript II KS⁺) culture filtrate; 2, *E. coli* DH5 α (pBluescript II KS⁺) cytoplasmic fraction; 3, *E. coli* DH5 α (pBluescript II KS⁺) culture filtrate; 4, *E. coli* DH5 α (pVW511) periplasmic fraction; 5, *E. coli* DH5 α (pVW511) cytoplasmic fraction; 6, *E. coli* DH5 α (pVW511) culture filtrate. (C) Two-dimensional electrophoresis analysis of the periplasmic fraction of *E. coli* (pVW510) expressing the recombinant 19-kDa antigen stained with silver stain. The positions of the 14.3-, 21.5-, 30-, and 46-kDa molecular mass (MW) markers and the acidic and basic poles of the isoelectric focusing gel are shown. Arrows indicate the positions of the isomers of the 19-kDa protein which were confirmed by Western blotting with antibody CMA134.1 (data not shown).

FIG. 3. Two-stage preparative electrophoresis of the recombinant 19-kDa antigen. Each fraction was resolved by SDS-PAGE, and the protein bands were visualized by silver staining. Lane 1, periplasmic fraction of *E. coli*(pVW510); lane 2, fraction obtained following preparative isoelectric focusing; lane 3, purified protein obtained following preparative isoelectric focusing and preparative SDS-PAGE of periplasmic fraction. The arrowhead indicates the position of the recombinant 19-kDa antigen.

was replaced with the first 40 amino-terminal amino acids of the α -peptide of β -galactosidase (pVW511) (Fig. 2B). Silver-stained two-dimensional polyacrylamide gels of the periplasmic fraction of *E. coli* DH5 α (pVW510) revealed that the recombinant 19-kDa antigen was the predominant periplasmic protein and that it existed in four isomeric forms (Fig. 2C). The identities of these isomers were confirmed by Western blotting with CMA134.1 (data not shown).

Purification and characterization of recombinant 19-kDa antigen. The 19-kDa protein was purified from the periplasm of *E. coli* DH5 α (pVW510) by osmotic shock and preparative two-dimensional electrophoresis as described in Materials and Methods. The yield of purified protein was 2.5 mg of soluble protein from 2.5 g (wet weight) of *E. coli* cells (i.e., 1 liter of an overnight culture) which was equivalent to 2.5 mg of purified protein recovered from approximately 40 mg of periplasmic protein. Silver-stained one-dimensional SDS-polyacrylamide gels of recombinant 19-kDa antigen showed that the purification procedure resulted in a single protein band (Fig. 3). The identity of this protein band was confirmed by Western blotting with monoclonal antibody CMA134.1. Two-dimensional silver staining and Western blot analysis of the purified recombinant 19-kDa protein revealed the presence of four isomers of the protein at positions similar to those observed in Fig. 2C for the periplasmic fraction of *E. coli* DH5 α (pVW510) (data not shown). The amino acid sequence of the recombinant 19-kDa protein was determined for the first 17 amino-terminal residues and was found to be identical to the predicted sequence, thus confirming cleavage of the secretory signal peptide of MPB70 from the recombinant protein (Fig. 1).

Antibody recognition of recombinant 19-kDa antigen. Western blotting was used to detect the recognition of the recombinant 19-kDa antigen by a number of monoclonal and polyclonal sera. The antigen was recognized by monoclonal antibody CMA134.1 (Fig. 2), monoclonal antibody TB-23 (Fig. 4, lane 3), monospecific polyclonal antibodies raised against peptides p2 (amino acids 11 to 30) and p15 (amino acids 140 to 159) (Fig. 4, lanes 1 and 2, respectively), rabbit anti-*M. bovis* polyclonal sera and antisera from C57BL/10 mice immunized with killed *M. tuberculosis* H37Ra (data not shown). The antigen was not recognized by monospecific polyclonal antisera raised against peptide p7 (amino acids 61 to 80).

T-cell recognition of recombinant 19 kDa antigen. C57BL/10 mice (groups of three mice each) were immunized with puri-

fied recombinant 19-kDa antigen or PBS emulsified in incomplete Freund's adjuvant, and 7 days later primed lymph node cells were challenged in vitro with the homologous purified 19-kDa antigen (Fig. 5A) or peptide p61-80, the major murine T-cell epitope of the *M. tuberculosis* 19-kDa antigen (15, 16) (Fig. 5B). Lymphocytes from 19-kDa-protein-immunized animals responded strongly when they were challenged in vitro with either the recombinant antigen or p61-80. Although some nonspecific mitogenic activity was observed at higher antigen concentrations (Fig. 5A), significantly lower proliferative responses were observed when lymphocytes from PBS-immunized mice were cultured in vitro with the recombinant 19-kDa antigen. The recombinant 19-kDa antigen was also recognized by a p61-80-specific T-cell hybridoma (Fig. 6). Significantly, the magnitude of the response induced by the purified antigen was considerably better than that seen with the partially purified rGST-19 antigen. These results demonstrate that the purified recombinant 19-kDa antigen induces specific T-cell stimulatory activity both in vivo and in vitro.

DISCUSSION

The intention of the present study was to produce recombinant antigens for the development of sensitive and specific blood-based immunodiagnostic tests of *M. bovis* infections in domestic and wild animals. However, since many *M. bovis* antigens, including the 19-kDa protein, are identical to those of *M. tuberculosis* (2, 5, 46), the production of these recombinant antigens might also be of use in the diagnosis of human tuberculosis. Antigen production in *E. coli* rather than in *M. bovis* reduces the problems associated with the slow generation time of *M. bovis*, the infectivity of the pathogen, and the more complex biochemical composition of mycobacteria. Previously, we have shown that when the *M. bovis* antigen MPB70 was expressed in *E. coli* from the native initiation codon, a soluble form of the protein was secreted into the periplasm of the recombinant *E. coli* strain (19). Production of MPB70 in this way has several advantages for protein purification. Secretion precludes proteolysis of the protein by housekeeping cytoplasmic peptidases and separates it from contaminating cytoplasmic proteins. Thus, we have exploited the secretion of this antigen into the periplasm of *E. coli* as the first step toward protein purification (43).

It has been reported previously that signal peptides can be exchanged among naturally secreted proteins without affecting

FIG. 4. Antibody recognition of the purified recombinant 19-kDa protein. Western blotting of the recombinant 19-kDa protein with antipeptide sera or monoclonal antibody. Lane 1, anti-p2 serum (1/50); lane 2, anti-p15 serum (1/200); lane 3, monoclonal antibody TB-23 (1/1000). MW, molecular mass.

FIG. 5. T-cell response following immunization with the recombinant 19-kDa protein. C57BL/10 mice were immunized subcutaneously in the hind footpads with 25 µg of purified recombinant 19-kDa protein or PBS, and T-cell proliferative responses were determined following challenge in vitro with recombinant 19 kDa protein (A) or peptide p61-80 (B). Data are presented as the mean $\pm$ standard deviation level of incorporation of [³H]thymidine after 3 days of in vitro culture.

secretion (29, 37). However, for some proteins the signal peptide alone is insufficient to direct secretion of the protein into the periplasm of *E. coli*. In some cases, for example, chicken muscle triosephosphate isomerase, this block may be overcome by including 12 or more residues of secretory protein, in this case β -lactamase, between the signal peptide and the isomerase (36). In order to test whether the signal peptide of MPB70 could direct the secretion of other *M. bovis* proteins, the signal peptide and lipoylation site of the 19-kDa antigen of *M. bovis* (which has proved to be refractory to high-level expression of free protein in a range of *E. coli* expression systems [1, 11, 15, 17]) was replaced with the signal peptide of MPB70 along with seven amino acids of the mature protein. Secretion of recombinant 19-kDa antigen into the periplasm and supernatant of recombinant *E. coli* was observed. This was not the case when the signal peptide of MPB70 was replaced with the first 40 amino acids of the α -peptide of β -galactosidase. Secretion of the 19-kDa antigen into the periplasm of *E. coli* facilitated purification of a soluble form of the recombinant protein by preparative two-stage electrophoresis. The yields of a single protein band, as determined by one-dimensional SDS-PAGE, in which the signal peptide of MPB70 had been removed by endopeptidase cleavage in vivo, were consistently 5-fold to 10-fold higher than those for MPB70 (43), i.e., 2.5 mg liter⁻¹. The reason for this difference is unknown. However it might be due to a difference between the two antigens in the requirement for rare tRNAs which, because of the different codon usages of *E. coli* and *M. bovis*, might limit the translation of *M. bovis* proteins in *E. coli*. Alternatively, it might reflect the difference in resistance of the two proteins to proteolytic degradation by the bacterial host.

We have shown previously that expression of MPB70 in *E. coli* gives rise to increased leakage of periplasmic proteins across the outer membrane but that this disruption does not affect the growth of the recombinant bacterium in vitro (19). One explanation for the presence of the recombinant 19-kDa antigen in the culture filtrate is that secreted proteins of mycobacteria may have evolved a hydrophobic tertiary structure to allow diffusion of the protein across the hydrophobic, mycolic acid-rich mycobacterial cell envelope. Consequently, this structure might also allow diffusion of the recombinant protein across the outer membrane of *E. coli*, causing disruption of the membrane and leakage of periplasmic proteins. The requirement for the correct tertiary structure of the protein for diffusion across hydrophobic barriers might explain why only one of the two periplasmic forms of the 19-kDa antigen was observed

in the culture filtrate. In contrast, the native 19-kDa protein of *M. tuberculosis* has been reported to be present in the culture filtrate as a dimer (15). This may reflect posttranslational modifications of the native mycobacterial protein structure such as the presence or absence of the hydrophobic signal peptide and the covalent attachment of fatty acids or carbohydrate moieties (9, 11, 27, 45). Two-dimensional gel electrophoresis of the recombinant 19-kDa antigen revealed four isomers of the protein, which is consistent with the recent report of the presence of four isomeric forms of the native 19-kDa antigen of *M. tuberculosis* (15).

Purified recombinant 19-kDa antigen was recognized by a panel of monoclonal and polyclonal antibodies including murine antisera raised against a species-specific epitope of the 19-kDa antigen of *M. tuberculosis* p15 (residues 140 to 159) (15). However, murine antisera raised against synthetic peptide p7 (residues 61 to 81) did not recognize the recombinant protein. This result is consistent with those of a previous study (15), which demonstrated the cryptic nature of this epitope and which is further evidence that this epitope, which is the dominant murine T-cell epitope of the 19-kDa antigen of *M. tuberculosis* (15–17), probably adopts a different conformation within

FIG. 6. T-cell recognition of recombinant 19-kDa protein. The T-cell hybridoma B10.7.37 specific for p61-80 of the *M. tuberculosis* 19-kDa protein was cultured in vitro (2×10^4 cells per well) in the presence of irradiated syngeneic antigen-presenting cells (3×10^5 cells per well) and antigen as indicated. Culture supernatants were removed after 24 h, and the interleukin 2 content was measured with the HT-2 indicator cell line.

the context of the whole molecule. In contrast, this epitope was presented efficiently for recognition by a p61-80-specific T-cell hybridoma. Moreover, lymphocytes from recombinant 19-kDa-protein-immunized mice responded strongly to challenge in vitro with either the recombinant 19-kDa protein or with peptide p7. In addition, nonspecific mitogenic effects, which are commonly encountered problems associated with recombinant antigens produced in *E. coli*, were relatively low.

In the present study the recombinant 19-kDa antigen was expressed without its lipoylation signal peptide, a region known to contain a number of overlapping epitopes which are frequently recognized by human (32) and bovine (31) T cells. However, this region is highly conserved between mycobacterial species (3, 27) and therefore might contribute to a lack of species specificity if it were used for immunodiagnosis. Moreover, the lack of lipoylation may account for the solubility of the recombinant antigen and its ease of purification. Both lipoylation and glycosylation have been reported for the 19-kDa antigen (11, 27, 45). Since the recombinant protein was expressed in *E. coli* without its lipoylation site, the purified protein was neither lipoylated nor glycosylated, and although it is not known what effects the lack of these modifications might have on the immunological activity of the recombinant polypeptide, lipid (7, 32, 45) and carbohydrate (21) modifications have been shown to influence the immunogenicities of peptides. The ability of *E. coli* to secrete mycobacterial antigens that retain their antigenicities also raises the possibility that attenuated oral vaccines for salmonella might be used to present secreted mycobacterial antigens to the host's immune response from inside the macrophage, thus mimicking the natural route of presentation of these antigens.

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