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Expression of *Mycobacterium tuberculosis* MPT64 in recombinant *Myco. smegmatis*: purification, immunogenicity and application to skin tests for tuberculosis

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SUMMARY

Proteins secreted across the cell wall of mycobacteria are important antigens recognized early in the host response to mycobacterial infection. MPT64 is a 23-kD secreted protein restricted to members of the *Mycobacterium tuberculosis* complex which elicits T cell responses and cutaneous DTH reactions in *Myco. tuberculosis*-infected animals. Patients with tuberculosis and their tuberculin-positive contacts respond to the protein, but recipients of bacille Calmette–Guérin (BCG) vaccine strains lacking the *mpt64* gene do not. In the present study, we describe the development of a unique recombinant mycobacterial vector which secretes the encoded *Myco. tuberculosis* protein MPT64 at high levels into the culture filtrate, from which the protein is isolated by a single-step affinity chromatographic step. The purified protein was recognized by both polyclonal and monoclonal anti-MPT64 antibodies. The T cell reactivity of the protein was confirmed by its ability to stimulate human anti-rMPB64 T cell lines. The *Myco. smegmatis* recombinant MPT64 protein was superior to the *Escherichia coli* rMPB64 protein, which has identical amino acid sequence, in eliciting cutaneous DTH reactions in guinea pigs sensitized with *Myco. tuberculosis*. Animals sensitized with BCG strains lacking the *mpb64* gene failed to respond to MPT64. Similarly, interferon-gamma (IFN-γ) responses in tuberculosis patients and their contacts were higher to the *Myco. smegmatis* form of the protein. The potential of this form of the *Myco. tuberculosis* MPT64 protein as a skin test reagent for tuberculosis is discussed.

Keywords *Mycobacterium tuberculosis* recombinant protein expression immunogenicity

INTRODUCTION

Activation of T cells by mycobacterial proteins is central to the control of tuberculosis (TB) in infected individuals. The genome of *Mycobacterium tuberculosis* encodes more than 1000 proteins, and defining those recognized by human T cells is critical for the development of more effective vaccines and more specific diagnostic reagents. Attention has recently focused on the group of proteins exported across the mycobacterial cell membrane and wall as important targets of the cellular immune responses [1]. These secreted proteins accumulate early in the culture media during active replication of *Myco. tuberculosis* [2], and have been identified on the surface of mycobacteria [3]. Secreted proteins are recognized early in the course of experimental tuberculosis infection [1] and stimulate T cell proliferation and interferon-gamma (IFN-γ) release from peripheral blood mononuclear cells (PBMC) of TB patients [4]. As these proteins are only produced by living mycobacteria, they may be partially responsible for the observed requirements of viable mycobacteria for effective vaccination against *Myco. tuberculosis* infection.

Early culture filtrates of *Myco. tuberculosis* contain a complex mixture of secreted proteins [2]. Some, such as the three closely related 30–32-kD proteins of the antigen 85 family, are widely shared by all mycobacterial species studied. Others such as MPB64 and MBP70 appear restricted to members of the '*Myco. tuberculosis* complex'. MPB64 is a 23-kD protein, present in *Myco. tuberculosis*, virulent *Myco. bovis* and some strains of the vaccine *Myco. bovis* bacille Calmette–Guérin (BCG). Purified native (n) MPB64 stimulates T cell responses in *Myco. tuberculosis*-infected mice [5] and in human TB patients [6]. Furthermore, nMPB64 is capable of eliciting DTH reactions in *Myco. tuberculosis*-sensitized guinea pigs [7]. Recently, we have observed that TB patients and tuberculinpositive recent contacts of TB, but not BCG vaccines, had strong T cell proliferative responses to a recombinant (r) form of MPB64 and MPB70. In the case of MPB64 the reason for this difference is the deletion of the *mpb64* gene from two of the three major vaccine strains of BCG, including BCG-CSL, the form used in Australia. By contrast, both BCG vaccinees and TB patients responded to the MPB59 or 85B protein which is present in a wide range of mycobacteria [6]. On the basis of the apparent

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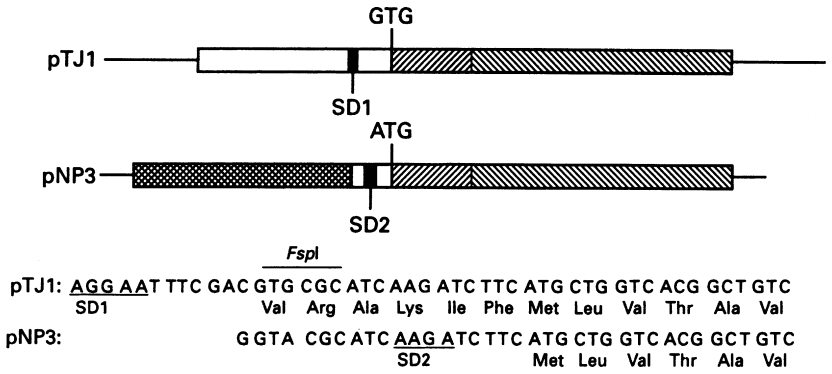

Fig. 1. Shuttle constructs expressing the *mpt64* gene in recombinant *Mycobacterium smegmatis.* Thin bars represent vector sequences. In pTJ1, the whole *mpt64* gene including its putative promoter has been cloned into pRR3. In pNP3, the *mpt64* coding region has been fused to the up-regulated *pBlaF** promoter utilizing a second initiation codon in frame with the original GTG. The resulting signal sequence is truncated of six amino acids. □, *mpt64* promoter; ▨, MPT64 signal sequence; ▧ MPT64 mature protein; ▩, *pBlaF** promoter; ■, Shine–Dalgarno consensus sequence.

specificity of the T cell response to rMPB64 in TB patients and the potency of nMBP64 as a skin test reagent, we and others have proposed that this 23-kD protein would be a suitable candidate as a single protein tuberculin. Early experiments indicated that rMBP64 expressed in *Escherichia coli* was not effective at eliciting DTH in sensitized animals. Therefore, we have investigated the expression of the gene encoding the *Myco. tuberculosis* MPT64 in the fast growing *Myco. smegmatis*.

The development of mycobacterial shuttle plasmids and high efficacy transformation strains of *Myco. smegmatis* [8,9] have permitted the expression of foreign genes in mycobacteria, including those from *Myco. tuberculosis* [10]. The level of expression of exogenous genes from their own promoters has been variable, and inducible promoters from the genes encoding mycobacterial heat shock proteins have been used to increase the level of expression. Recently, a mutant promoter sequence which enhances expression of β-lactamase has been characterized in an amoxycillin-resistant strain of *Myco. fortuitum*. This mutant promoter (*pBlaF**) increased the level of expression of the *E. coli* gene encoding alkaline phosphatase in recombinant *Myco. smegmatis* [11]. We have utilized this *pBlaF** promoter to develop a high expression vector resulting in the secretion of *Myco. tuberculosis* MPT64 from transformed *Myco. smegmatis*. Following a simple purification strategy the *Myco. smegmatis* derived rMPT64 was tested for its immunogenicity in TB patients and in guinea pigs for its suitability as a skin test reagent.

MATERIALS AND METHODS

Bacterial strains and plasmids

Mycobacterium smegmatis strain mc^2155 [9], plasmids pRR3 [12] and pJN30 [11] were supplied by Dr Brigette Giquel (Pasteur Institute, Paris, France). Recombinant *Myco. smegmatis* mc^2155 and *Myco. tuberculosis* were grown in Middlebrook 7H9 medium (Difco Labs, Detroit, MI) supplemented with ADC enrichment medium (Difco) and kanamycin (25 μg/ml).

Antigen and antibodies

Recombinant MPB64 was expressed as a fusion protein with glutathione-S-transferase in *E. coli* containing the pGEX-2T vector, and purified on glutathione agarose as described previously [6]. MPB64 purified from culture filtrate of *Myco. tuberculosis* was a kind gift of Dr S. Nagai (Tokyo, Japan).

Polyclonal MPB64 antisera was raised in rabbits by three intramuscular immunizations with 1 mg of rMPB64 in Freund's incomplete adjuvant (FIA) at weekly intervals. Murine anti-MPB64 MoAb, L24B4, was kindly supplied by Dr A. Andersen (Statens Serum Institute, Copenhagen, Denmark).

Cloning of MPT64 gene into recombinant Myco. smegmatis

Genomic DNA from *Myco. tuberculosis* was prepared by phenol-chloroform extraction [13]. The gene for MPT64 was amplified from *Myco. tuberculosis* genomic DNA by the polymerase chain reaction (PCR) using the primers MPB64-N, 5′ TAG AGT ACT GGA TCC TCG ATC TGC TAG3′; and MBP64-C, 5′ TAG AGT ACT GAA TTC TAG GCC AGC ATC3′, based on the sequence of MPB64 [14] with the addition of *Sca*I restriction sites. Thirty amplification cycles (1 min at 94°C, 1 min at 50°C, 1 min at 72°C) were performed in a total volume of 100 μl. The 913-bp fragment obtained, consisting of the 672-bp coding region and 141 bp up-stream, included the putative -10 and -35 consensus promoter sequences reported for *mpb64* (Fig. 1). After digestion with *Sca*I, the PCR product was inserted into the unique *Sca*I site of shuttle vector pRR3 to give rise to pTJ1. To express the *mpt64* gene under the control of the up-regulated mycobacterial promoter *pBlaF**, the 913-bp PCR fragment was digested with *Fsp*I and *Sca*I and subcloned into pJN30 immediately down-stream from the *pBlaF** promoter sequence. This gave rise to pNP3 (Fig. 1). Sequence analysis was performed by the dideoxy chain termination reaction. These shuttle plasmids were expanded in *E. coli*, purified and then electroporated into *Myco. smegmatis* mc^2155 at 2·5 kV, 200 Ω and 25 mF in 0·2 cm gap electroporation cuvettes (BioRad, Richmond, CA). Positive clones were screened for expression of the *mpt64* gene after 2–3 days culture in 7H9 medium plus kanamycin (25 μg/ml).

MPT64 expression from Myco. smegmatis *TJ1 and NP3*

rMPT64 protein expression was compared in recombinant *Myco. smegmatis* containing the *mpt64* gene bearing plasmids TJ1 and NP3 and *Myco. smegmatis* containing vector JN30 alone.

Individual colonies from 7H11 agar plus kanamycin plates were inoculated into 5 ml of 7H9 broth plus kanamycin and grown for 3 days with shaking at 37°C. Cells were pelleted at 6000 ***g*** for 10 min. The supernatant was collected and protein precipitated with five volumes of ice-cold acetone. The cells were resuspended in PBS with 1% Triton X-100, 50 mM benzamidine and 100 μM phenylmethylsulphonyl flouride (PMSF) and sonicated on ice at 35 W for 3×30 s. The sonicate was pelleted at 10 000 *g* and supernatant collected.

The levels of protein expression were compared following SDS-PAGE in 15% reducing gels and electroblotting onto nitrocellulose at 250 mA for 1 h. Blots were blocked with 1% milk powder in PBS for 1 h and then probed with a rabbit polyclonal anti-MPB64 antisera (1:1000) or with MPB64 MoAb L24B4 (undiluted culture supernatant). Following incubation with goat anti-rabbit or anti-mouse immunoglobulin conjugated to alkaline phosphatase, colour was developed with nitro-blue terazolium, 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate.

Purification of MPT64 by affinity chromatography

Log-phase (40 h) *Myco. smegmatis* NP3 culture (5 ml) was inoculated into 400 ml of 7H9 broth containing 5% ADC (5% bovine serum albumin (BSA), 2% dextrose, 0·03% catalase) and incubated for 3 days at 37°C with shaking. Cells were pelleted at 6000 ***g*** for 20 min and the supernatant was removed, sterile filtered and the protease inhibitor PMSF (1 mM) and sodium azide (0·01%) were added. Supernatants were concentrated 100-fold on a YM-10 membrane using an Amicon 8400 stirred ultrafiltration cell.

Purified rabbit anti-MPB64 IgG was isolated by ammonium sulphate precipitation and Protein A Sepharose chromatography and coupled to cyanogen-bromide-activated Sepharose 4B following the manufacturer's instructions (Pharmacia Uppsala, Sweden). The concentrated NP3 supernatant was applied to the affinity column equilibrated in PBS at a rate of 1 ml/min and allowed to recirculate for 4 h. Non-specifically bound material was removed with PBS containing 0·5 M sodium chloride, and the bound material eluted with 0·1 M diethylamine pH 11·0. Following dialysis, the eluate was freeze-dried. Affinity-purified rMPT64 was further purified for some experiments by gel filtration on a Superose 12 column.

Contamination of the *Myco. smegmatis* product with mycobacterial lipoarabinomannan (LAM) was checked by immunoblotting 5 μg of the affinity-purified rMPT64 and 5 μg of LAM-B (a kind gift of Professor P. Brennan, Colorado State University, CO) with the MoAb L9 [15]. The presence of the major secretory antigen MPB59 (antigen 85) was also examined by immunoblots with a polyclonal anti-antigen 85 rabbit antisera [16]. Endotoxin in all protein preparations was measured by the Limulus Amoebocyte lysate assay (Sigma, St Louis, MO).

Reactivity of Myco. smegmatis *rMPT64 with human T cell line*

The affinity-purified rMPT64 was tested with three T cell lines derived from peripheral mononuclear cells of TB patients to the *E. coli* rMPB64 (Roche, in preparation). T cells (2×10^4) and irradiated PBMC (10^5) were added in 200 μl in the wells of a round-bottomed 96-well plate. *Escherichia coli*-derived rMPB64 and *Myco. smegmatis* rMPT64 were added in triplicate wells over a range of final concentrations from 10 to 0·01 μg/ml. The cells were incubated for 72 h at 37°C in 5% CO_2 and pulsed for 6 h with 0·5 μCi ^{3}H-thymidine per well and then harvested. The incorporated thymidine was counted by liquid scintillation spectroscopy.

IFN-γ production in TB patients and contacts

Thirty-one TB patients or healthy tuberculin-positive contacts and six BCG vaccinees were tested in a whole blood IFN-γ assay (*Quanti*feron-*TB*; CSL, Melbourne, Australia). Aliquots (1 ml) of heparinized blood were incubated overnight at 37°C in 5% CO_2 in the presence of *Myco. tuberculosis* tuberculin, 10 μg *E. coli* rMPB64 or 10 μg *Myco. smegmatis* rMPT64. After 24 h, supernatants were removed and IFN-γ was measured by a MoAb capture ELISA according to the manufacturer's instructions.

Induction of DTH in Myco. tuberculosis- *and BCG-sensitized guinea pigs*

In initial experiments, outbred female guinea pigs 10–12 weeks old were sensitized by the intradermal injection of 1 mg (wet weight) of glutaraldehyde-killed BCG Tokyo [7] and 1 month later challenged intradermally with 1 μg tuberculin purified protein derivative (PPD), 10 μg of *E. coli* rMPB64 or 10 μg of *Myco. smegmatis* rMPT64. The area of induration was measured 24 h later. A second group of animals were sensitized by intradermal injection of 1 mg (wet weight) gamma-irradiated *Myco. tuberculosis*, BCG-CSL or 1 ml of PBS. Three months later, the animals were challenged intradermally with PPD or *Myco. smegmatis* rMPT64.

RESULTS

Expression of rMPT64 from Myco. smegmatis

DNA sequencing confirmed that the 913-bp PCR fragment contained the gene for the *Myco. tuberculosis* protein, termed MPT64 (data not shown). The sequence is identical to that of the *Myco. bovis* BCG *mpb64* gene apart from a single base change which does not affect the amino acid sequence [17]. Expressions of the *mpt64* gene in *Myco. smegmatis* from the clone TJ1 (Fig. 1) resulted in modest levels of protein in the culture filtrate and the cell sonicate (Fig. 2). This indicated that the 913-bp PCR fragment contains the promoter sequences necessary for the expression of the genes in *Myco. smegmatis*. Further, the secretion of the recombinant protein into the culture filtrate indicated that the *Myco. tuberculosis* secretory signal was recognized in *Myco. smegmatis*. To increase the levels of protein expression, two plasmids including the *blaF** promoter were constructed. When the promoter was inserted up-stream of the 913-bp fragment in the NP1 construct no expression of protein was evident. Digestion of the 913-bp fragment with the restriction endonucleases *Fsp*I at a site 18 bp from the start of the *mpb64* sequence and *Sca*I at a site introduced by PCR resulted in a 692-bp fragment. This fragment was cloned into JN30 immediately down-stream of the *pBlaF** promoter to yield pNP3. The original GTG start codon was lost in this construct, but a second translation start at the ATG codon 18 bp down-stream was recognized, since the preceding sequence (AAGA) closely resembles a Shine Dalgarno sequence (Fig. 1). Translation initiation at this methionine effectively deleted the first six amino acids of the signal sequence when expressed in *Myco. smegmatis*. The level of rMPT64 protein expression was increased several-fold in both the sonicate and culture filtrate (Fig. 2). Purification of the protein by affinity chromatography from culture filtrates of NP3 yielded 3·8 mg/*l* of rMPT64, with a small contaminant of BSA from the culture enrichment media. The yield of protein from the culture filtrate was approximately six times that

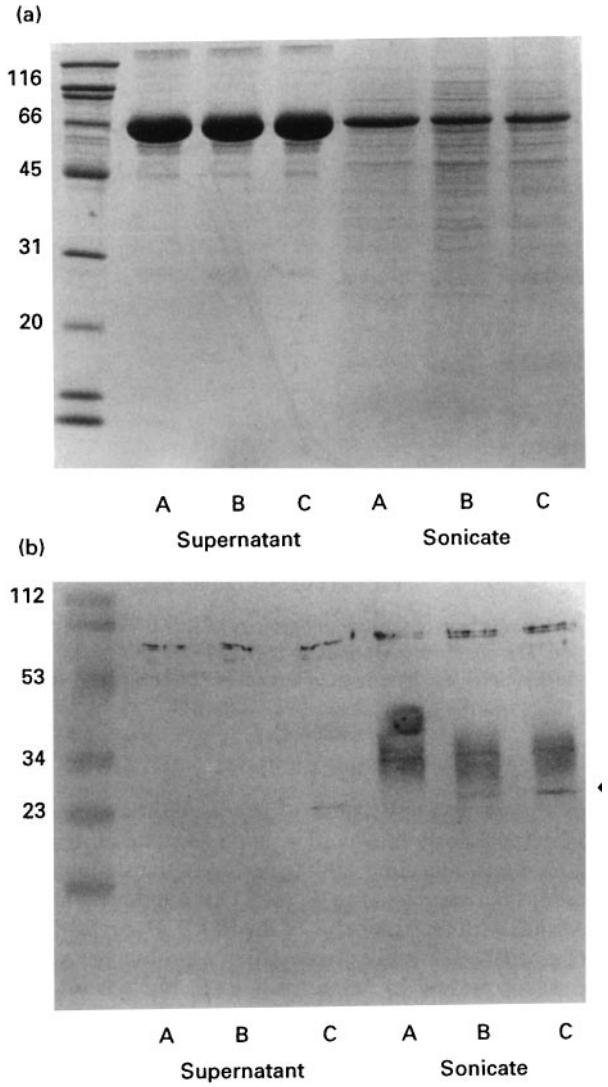

Fig. 2. MPT64 protein expression in supernatants and sonicates of recombinant *Mycobacterium smegmatis* transfected with the following plasmids: A, control JN30; B, TJ1 using *mpt64* promoter; C, NP3 using *pBlaF** promoter, were assessed by SDS–PAGE and coomassie blue stain (a) and immunoblotting (b) with polyclonal rabbit anti-MPB64 antisera.

from the cell sonicate. This affinity-purified product was free of lipopolysaccharide (LPS) (<0·125 Endotoxin Units per ml) as measured by the Limulus test, and the mycobacterial cell wall component LAM as assessed by immunoblotting (data not shown). There was no evidence of copurification of secretory mycobacterial proteins of the antigen 85 family as assessed by immunoblotting (data not shown). Therefore affinity-purified preparations of rMPT64 were used for immunological studies. Further purification with gel filtration removed the minor BSA component, resulting in a single 23-kD band which was reactive with the MPB64 MoAb L24B4 (Fig. 3).

Reactivity of rMPT64 with human T cells

Short-term culture filtrates of *Myco. tuberculosis* containing MPT64 and the purified rMPB64 protein are major targets of the human T cell response to *Myco. tuberculosis* [4,6]. To investigate the immunogenicity of the *Myco. smegmatis*-expressed protein, proliferative responses of human T cell lines raised to rMPB64 from TB patients and their contacts were tested with both forms of the protein. As shown in Fig. 4, the specific T cell lines proliferated strongly to the *Myco. smegmatis* rMPT64.

An alternative method of assessing immune responsiveness to mycobacterial proteins in humans has been the detection of IFN-γ in whole blood assays [18]. We observed marked differences in the induction of IFN-γ secretion between the *E. coli* rMPB64 and the *Myco. smegmatis* rMPT64 proteins in TB patients, their contacts and BCG vaccinees (Table 1). The amount of IFN-γ released by individual patients in response to *Myco. smegmatis* rMPT64, but not *E. coli* rMPB64, was significantly correlated with the response to PPD (Spearman rank correlation coefficient $r_s = 0{\cdot}6$; $P = 0{\cdot}006$). Further, the highest levels of IFN-γ to both PPD and MPT64 were observed in recent contacts of TB patients compared with BCG vaccinees. These responses parallel the pattern of proliferative responses to rMPB64 observed in TB patients and contacts [6].

Reactivity of rMPT64 in DTH reactions

Previously we have noted the failure of the 30-kD (MPB59) recombinant protein expressed in *E. coli*. from the pGEX-2T vector to elicit a DTH reaction in BCG-sensitized guinea pigs. These animals were reactive to the native form of the protein isolated from culture filtrate (data not shown). This was also observed with the *E. coli* rMPB64 which failed to elicit a cutaneous reaction in guinea pigs sensitized with BCG Tokyo, a strain naturally expressing the MPB64 protein. Sensitized animals gave a strong DTH reaction to 1 μg tuberculin PPD (mean induration 15·5 mm (s.d. = 1·0)), but failed to respond to 10 μg of the *E. coli* rMPB64. By contrast, the *Myco. smegmatis* rMPT64 (10 μg) elicited a DTH reaction with a mean induration of 10 mm (s.d. = 2·8).

BCG-CSL vaccine, the BCG strain used for vaccination in Australia, has previously been shown to lack the *mpb64* gene. To investigate the ability of rMPT64 to discriminate between BCG and *Myco. tuberculosis* sensitization in skin tests, we tested the *Myco. smegmatis* rMPT64 in guinea pigs sensitized with irradiated *Myco. tuberculosis* or BCG-CSL (Table 2). Skin test reactivity to PPD was seen in all animals from both sensitized groups, but reactions to rMPT64 were found only in the *Myco. tuberculosis*-sensitized animals. There was no reaction to rMPT64 in control animals which were PPD-negative. The diameter of induration caused by 1 μg of rMPT64 was equivalent to that induced by 1 μg of PPD.

DISCUSSION

Transcription and translation of *Myco. tuberculosis* genes in mycobacterial hosts should allow more accurate processing of gene products, including folding and post-translational modification, than expression in *E. coli*. This hypothesis has been established with the expression of the 19-kD *Myco. tuberculosis* protein, where native-like glycosylation was observed when the gene was expressed in *Myco. smegmatis*, but not in *E. coli*. Furthermore, the recombinant *Myco. smegmatis* protein was more immunogenic when tested in murine 19-kD T cell lines than the *E. coli* counterpart. Expression in this system, under the control of the native 19-kD promoter, yielded approximately nine times more 19-kD protein than from an equivalent culture of

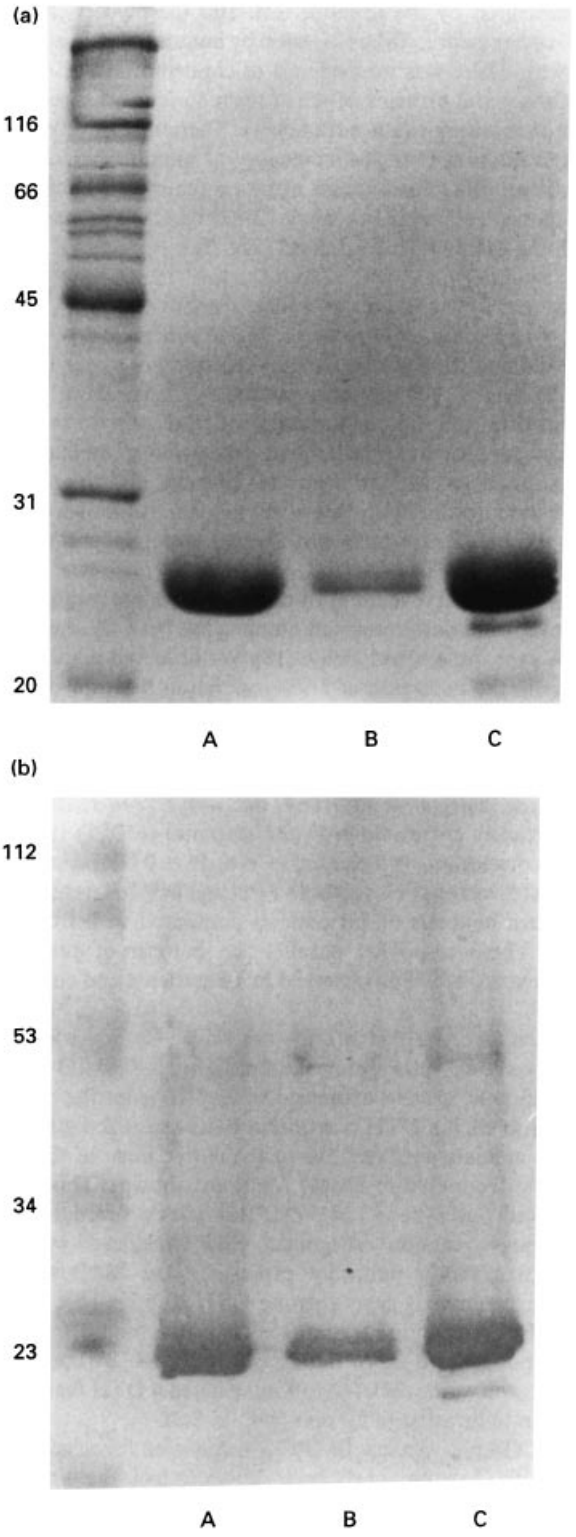

Fig. 3. The following preparations: A, *Escherichia coli* rMBP64; B, *Mycobacterium smegmatis* rMPT64 purified by affinity chromatography and gel filtration; C, native MPT64, were assessed by SDS–PAGE and coomasie blue staining (a) and immunoblotting with MoAb L24B4 (b).

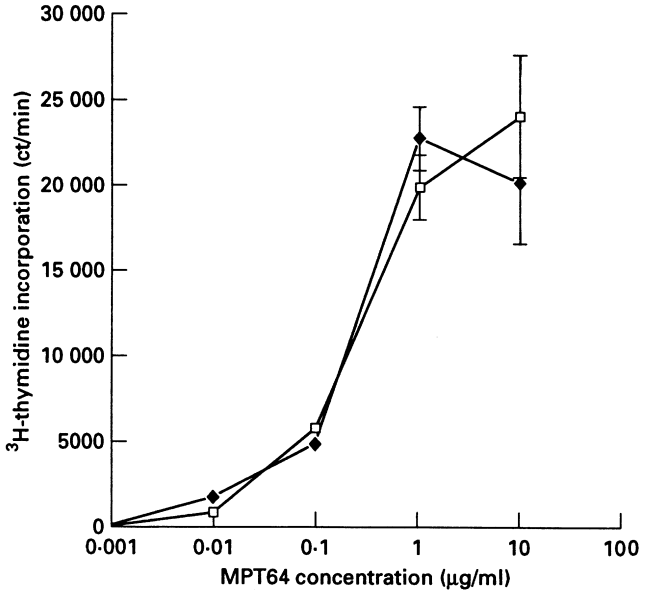

Fig. 4. Comparison of the immunogenicity of *Escherichia coli* rMPB64 (□) and *Mycobacterium smegmatis* rMPT64 (◆) as assessed by the specific thymidine incorporation of human MPB64-reactive T cell lines.

Myco.tuberculosis [10]. In the present study, recombinant *Myco. smegmatis* secreted the tuberculosis protein MPT64 at high levels, allowing the isolation of the protein by a single-step affinity chromatography step from culture filtrate. The purified protein was as immunogenic as the *E. coli* recombinant protein in antibody binding and *in vitro* proliferative assays, and superior in the induction of skin DTH reactions and the stimulation of IFN-γ from human T cells.

Expression of *Myco. tuberculosis* proteins in rapidly growing mycobacteria can be further improved by the addition of stronger mycobacterial promoters in the constructs. Although *mpt64* was expressed from its own promoter in *Myco. smegmatis*, the level of expression was at least doubled when *mpt64* was placed under control of the *pBlaF** promoter isolated from an amoxycillin-resistant strain of *Myco. fortuitum*. This has previously been shown to increase the level of expression of β-lactamase in *Myco. fortuitum* and the expression of *E. coli* alkaline phosphatase in recombinant *Myco. smegmatis* [11]. Interestingly, the modified secretory signal sequence, shortened by six amino acids in construct NP3, was still effective at exporting rMPT64 from *Myco. smegmatis*. This resulted in more than six-fold higher levels of rMPT64 in the culture filtrate than in sonicated organisms (Fig. 2), and permitted a simple purification procedure. Under the *pBlaF** promoter the levels of rMPT64 isolated by affinity chromatography (3·8 mg/*l*) were equivalent to those obtained from expression of *mpb64* in the *E. coli* pGEX-2T vector (3·6 mg/*l*), and significantly higher than those recovered from culture filtrate of *Myco. tuberculosis* (0·3 mg/*l*; S. Nagai, personal communication [19]). Purification of nMPT64 from culture filtrate, in which it constitutes only 0·1% of the total protein, involves the use of at least four serial chromatographic procedures [19]. By contrast, purification from *Myco. smegmatis* NP3 from culture filtrate was technically simple and provided a higher yield of rMPT64 free of other mycobacterial components. Other strong promoters are being characterized in *Myco. smegmatis*, and these may allow the development

Table 1. Interferon-gamma production in tuberculosis patients and contacts induced by tuberculin purified protein derivative (PPD) and rMPT64 purified from *Mycobacterium smegmatis* but not rMPB64 from *Escherichia coli*

Antigen	Median IFN-γ, U/ml (range)		
Subjects (*n*)	PPD	*E. coli* rMPB64	*Myco. smegmatis* rMPT64
BCG vaccinees (6)	2·0 (1–5)	1·0 (0–4)	9·5 (4–22)
Contacts (14)	53·5 (3–304)**	2·5 (0–46)	51 (4–187)*
Active TB (10)	20·5 (5–97)**	2·0 (1–9)	10·5 (0–88)
Inactive TB (7)	13 (0–87)	0	15 (0–29)

Significant difference in IFN-γ levels compared with BCG vaccinees as determined by the Mann–Whitney *U*-test (*$P < 0{\cdot}02$; **$P < 0{\cdot}005$).

of even more effective expression systems for the production of exogenous proteins.

The MPT64 protein is restricted to the *Myco. tuberculosis* complex, which includes *Myco. bovis*. The gene, however, has been deleted from some strains of the vaccine form of *Myco. bovis* (BCG) during multiple subcultures from the original isolate [20]. The absence of *mpb64* from the commonly used strains BCG-Pasteur and BCG-Glaxo and their derivatives, including BCG-CSL, results in significant differences in the proliferative responses to the protein between BCG vaccinees and subjects infected with *Myco. tuberculosis* [6]. Such differences may form the basis of skin tests to distinguish between TB infection and BCG vaccination. This is supported by the observation in the present study (Table 1) that IFN-γ release in response to the *Myco. smegmatis* rMPT64 is significantly higher in contacts of TB patients compared with BCG vaccinees. Further, DTH reactivity to rMPT64 develops in *Myco. tuberculosis*-, but not BCG-sensitized, guinea pigs (Table 2). This is consistent with the recent report that nMPT64 also elicited DTH in *Myco. tuberculosis*-sensitized guinea pigs, but not in those exposed to BCG-Copenhagen, a strain which also lacks the *mpb64* gene [21].

The reasons for the difference in the ability of the two preparations of recombinant MPT64 to induce DTH reactions remain unresolved. There was no significant contamination of either preparation with LPS, a potent inducer of IL-10 which can down-regulate cutaneous skin test reactions [22]. Carbohydrate components of mycobacterial cell walls such as LAM are potent inducers of tumour necrosis factor (TNF) release, but there was no LAM in the *Myco. smegmatis* rMPT64 and no contamination with other mycobacterial secreted proteins, such as the 30-kD 85B antigen. Because of differences in glycosylation of the *Myco. tuberculosis* 19-kD antigen in *E. coli* and *Myco. smegmatis* [10], we investigated the glycosylation state of rMPT64 by immunoblotting with concanavalin-alkaline phosphatase (data not shown). There was no evidence of glycosylation in either form of the recombinant protein from *E. coli*. or *Myco. smegmatis* or in native MPB64 from BCG. The tertiary conformation of the two recombinant forms was similar to that of the native protein, as MoAb L24B4, which reacts with a conformational epitope [17], showed similar binding characteristics to the proteins. Although rMPB59 (antigen 85B) expressed from pGEX-2T vector in *E. coli* also failed to elicit DTH (data not shown), this may not be a general property of recombinant mycobacterial proteins expressed in *E. coli*. Haga and colleagues [23] recently demonstrated that rMPB64 was extremely potent in eliciting DTH using cleaved rMPB64 expressed in *E. coli* as a fusion protein with maltose binding protein.

Other secreted proteins have been found to be differentially expressed in *Myco. tuberculosis* and BCG, including the *Myco. tuberculosis* 38-kD [24], MPB70 [25] and the 10-kD ESAT-6 proteins [26]. A combination of these *Myco. tuberculosis*-restricted proteins may be of greater utility as a skin test reagent than when used individually. Moreover, the form in which these proteins are expressed and purified will have a significant effect on their potency. The demonstration that *Myco. smegmatis* produces large quantities of secreted recombinant protein with strong immunogenicity suggests that it will be an appropriate vector to express other candidate antigens for use as important diagnostic tools.

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Table 2. Recombinant MPT64 from *Mycobacterium smegmatis* elicits DTH reactions in outbred guinea pigs sensitized with *Myco. tuberculosis* but not in BCG-CSL-sensitized animals

Recall antigen	PPD (1 μg)		rMPT64 (1 μg)	
	No. positive	Mean induration (mm)	No. positive	Mean induration (mm)
Myco. tuberculosis-sensitized	3/3	13·6	2/3	13·5
BCG-sensitized	4/4	13·5	0/4	0
Control	0/4	0	0/4	0

PPD, Purified protein derivative.

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