INTRODUCTION

Sequencing of the genome of Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), has revealed that approximately 10% of the genome encodes two families of glycine-rich proteins termed PE and PPE on the basis of their characteristic Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs near the N-terminal domain (Cole et al., 1998; Cole & Barrell, 1998). The PE protein family includes 69 proteins with a conserved N-terminal domain. Their role in tuberculosis is unknown, but it has been speculated that they may have an important immunological significance. In this investigation, the immunogenicity of the ppe44 (Rv2770c) gene product in BALB/c mice infected subcutaneously or intravenously with Mycobacterium bovis bacille Calmette-Guérin (BCG) was evaluated. Mice infected subcutaneously developed high titres of anti-PPE44 IgG1 antibodies, while PPE44-specific IgG2a antibodies were absent at all times tested. PPE44-primed cells from draining lymph nodes and spleen produced low levels of IFN-γ, and a moderate degree of delayed-type hypersensitivity was observed following PPE44 intracutaneous challenge. In mice infected intravenously, the anti-PPE44 IgG1 antibody response was markedly higher compared with the subcutaneous infection; anti-PPE44 IgG2a antibodies at titres approximately 0.5–2.0 log10 lower than IgG1 were detected. Interferon (IFN)-γ production in PPE44-stimulated spleen-cell cultures was transient. These results indicate that PPE44 represents a novel mycobacterial antigen expressed during subcutaneous and intravenous infection by M. bovis BCG in BALB/c mice. Both infection models seem to polarize the immune response to PPE44 towards a Th2 phenotype, as testified by the IgG1 isotype being predominant over IgG2a and by the low IFN-γ and delayed-type hypersensitivity responses.

In previous studies aimed at searching for M. tuberculosis genes involved in virulence based on the assumption that the virulent strain M. tuberculosis H37Rv and the attenuated mutant H37Ra must necessarily differ in the expression of virulence genes, we found that the gene Rv2770c, now termed ppe44, is downregulated in H37Ra (Rindi et al., 1999). This observation, together with the finding that nucleotide sequences of ppe44 were found only in species belonging to the M. tuberculosis complex (Rindi et al., 2001), prompted us to consider the ppe44 gene product as a potential virulence factor. In the present paper, as a first step towards evaluating the role of the ppe44 gene product in tubercle infection, we investigated the immunogenicity of PPE44 in BALB/c mice infected with Mycobacterium bovis BCG by subcutaneous and intravenous routes.

Abbreviations: BCG, bacille Calmette–Guérin; DTH, delayed-type hypersensitivity; IFN, interferon; PPD, tuberculin protein-purified derivative; TB, tuberculosis.
METHODS

Bacterial strains and growth conditions. M. tuberculosis H37Rv and M. bovis BCG (Pasteur strain) were from a collection maintained in our department. Strains were grown in liquid Middlebrook 7H9 medium (Difco Laboratories) supplemented with 0.25% Tween 80 (Difco Laboratories) and albumin–dextrose complex (ADC) [consisting of 0.5% albumin, fraction V (Sigma), 0.085% NaCl and 0.2% glucose]. Middlebrook 7H10 plates supplemented with 10% oleic acid–ADC enrichment (Becton Dickinson) were used for determining the number of c.f.u.

*Escherichia coli* of c.f.u. enrichment (Becton Dickinson) were used for determining the number of c.f.u. When required, ampicillin was included at a concentration of 100 \( \mu \text{g ml}^{-1} \).

Cloning, production and purification of PPE44. The ppe44 gene was amplified from the genomic DNA of M. tuberculosis H37Rv using upstream (5'-GATTTTGGAGCTCTACCTCGC-3') and downstream (5'-TCAGAGAGCGCTGCTTGG-3') primers. The PCR-generated fragment was cloned in *E. coli* XL-1 Blue into the pQE-30 UA expression vector in frame with an N-terminal His tag (Qiagen). The integrity of the coding region was verified by sequencing the inserted DNA fragment according to the manufacturer's recommendations (Qiagen). The recombinant plasmid was then transformed into *E. coli* M15(pREP4) host cells for expression. A 5 ml culture of recombinant *E. coli* was induced to express recombinant PPE44 (rPPE44) by the addition of 1 mM IPTG at the mid-exponential phase of growth.

Cells were harvested at various times post-induction, suspended in 1 \( \times \) SDS sample buffer and denatured by heating at 100 °C for 5 min. The target protein was identified by SDS-PAGE. Purification of rPPE44 was performed by metal chelate column chromatography with Ni-NTA acid resin according to the manufacturer’s instructions (Qiagen). Purified rPPE44 was shown by SDS-PAGE to be free of any significant amount of *E. coli* proteins and migrated at its predicted size of 39.5 kDa (Fig. 1). rPPE44 reacted with an anti-His antibody probe in an immunoblot assay (data not shown). The endotoxin level was determined to be less than 10 endotoxin units mg\(^{-1}\) using the Limulus amoebocyte lysate assay (Sigma).

**Mice and M. bovis BCG infection.** BALB/c female mice were purchased from Harlan Italy, maintained under pathogen-free conditions and used at 7 weeks of age. Mice were inoculated either subcutaneously at the tail base or intravenously with 2.5 \( \times \) 10\(^8\) or 5 \( \times \) 10\(^8\) c.f.u. BCG, respectively. Mice were sacrificed at various times after infection for evaluation of immune responses.

Interferon (IFN)-\(\gamma\) assay. Spleens and inguinal lymph nodes were aseptically removed and single-cell suspensions were prepared in culture medium consisting of RPMI 1640 supplemented with 1-glutamine, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum (Euroclone). Erythrocytes in the spleen-cell suspensions were lysed with NH\(_4\)Cl/Tris solution (Sigma). Mononuclear cells were cultured in a volume of 200 \( \mu \text{l} \) in 96-well plates (Corning Costar) at a concentration of 3 \( \times \) 10\(^5\) cells per well in the presence of medium alone, or in medium containing rPPE44 (1 \( \mu \text{g ml}^{-1} \)), tuberculin protein-purified derivative (PPD: 1 \( \mu \text{g ml}^{-1} \), Staten Serum Institute) as a positive control antigen or concanavalin A (1.5 \( \mu \text{g ml}^{-1} \), Sigma). Cells were incubated for 5 days at 37 °C in 5% CO\(_2\) and supernatants were harvested and assayed for IFN-\(\gamma\) by a standard ELISA using commercially available monoclonal antibodies according to the manufacturer's instructions (Euroclone). Recombinant IFN-\(\gamma\) was used as a standard.

Delayed-type hypersensitivity (DTH) to rPPE44. At 2 and 4 weeks after subcutaneous infection of BALB/c mice with BCG, the DTH response was measured by determining footpad swelling after intra-cutaneous injection of 5 \( \mu \text{g} \) rPPE44 in 50 \( \mu \text{l} \) PBS into one hind footpad. As a positive control, 5 \( \mu \text{g} \) PPD was similarly injected into the contralateral footpad. Footpad swelling was measured at 24 and 48 h post-injection with an engineer's micrometer with a minimum increment of 0.01 mm.

Antibody assay. For the evaluation of antibodies against rPPE44 and PPD, serum samples of infected mice were tested by ELISA. Briefly, ELISA plates (Probind) were coated overnight at 4 °C with 1 \( \mu \text{g per well of rPPE44 or PPD in carbonate buffer (pH 9.6). The plates were subsequently post-coated for 1 h with PBS, pH 7.4, containing 1% BSA to block available sites for non-specific binding. Twofold dilutions of sera in PBS/0.05% Tween 20 were added to duplicate wells and incubated for 1 h. IgG isotypes were revealed by goat anti-mouse IgG1 (Sigma) or IgG2a (Bethyl Laboratories), conjugated to biotin according to standard procedures, and by HRP-conjugated goat anti-biotin antibody (Sigma). The enzyme reactions were carried out with tetramethylbenzidine (Sigma) and stopped with 0.05 M H\(_2\)SO\(_4\); the absorbance was measured at 450 nm. All steps were performed at room temperature. Post-coating was done with 150 \( \mu \text{l per well; antigens, samples, conjugate, substrate and H\(_2\)SO\(_4\) were added in volumes of 100 \( \mu \text{l per well. The anti-rPPE44 serum titre was considered as the highest dilution giving optical readings greater than a cut-off value calculated as the mean absorbance of six sera from uninfected mice \( \pm 3 \text{ SD} \) at the initial serum dilution of 1:100.**

RESULTS AND DISCUSSION

The immunogenicity of PPE44 was investigated by evaluating humoral and cellular immune responses to rPPE44 in BALB/c mice infected subcutaneously or intravenously with *M. bovis* BCG.

For the subcutaneous infection, BALB/c mice were inoculated at the base of the tail with 2.5 \( \times \) 10\(^8\) c.f.u. BCG. At various time points after infection, groups of four or five
mice were killed, sera were collected to evaluate anti-PPE44 IgG1 and IgG2a antibody responses, and spleens and draining inguinal lymph nodes were removed to assay IFN-γ production in vitro. As shown in Fig. 2, infected mice developed anti-rPPE44 IgG1 antibodies starting from the second week of infection; IgG1 titres peaked at 4 weeks post-infection and subsided thereafter. No anti-rPPE44 IgG2a antibody was found at any tested time. A similar antibody response was observed against PPD. Spleen cells of infected mice cultured in the presence of rPPE44 produced IFN-γ from the second week of infection onwards, but at levels lower than those produced by PPD-stimulated cells. IFN-γ was detected in the supernatants of lymph node cells only at the very beginning of the infection (1–2 weeks) (Fig. 3), indicating that PPE44-specific, IFN-γ-producing cells are induced locally and then spread to the spleen.

The ability of PPE44 to prime a DTH response, a CD4+ T-cell mediated response that can be regarded as a reflection of the strength of the Th1 response, was investigated in groups of mice infected subcutaneously with BCG and injected intracutaneously into one hind footpad with rPPE44 at 14 and 28 days post-infection. As a positive control, PPD was similarly injected into the contra-lateral footpad; uninfected mice injected with the test antigens were used to assay the non-immune tissue reactivity. As shown in Table 1, at 14 days post-infection the DTH response to rPPE44, evaluated as footpad swelling, was basically absent at 24 and 48 h after challenge; at 28 days post-infection, however, a weak reactivity was observed at both tested times. In contrast, PPD injection resulted in high levels of DTH at both 14 and 28 days post-infection. Uninfected control mice showed negligible footpad swelling to both antigens.
For the intravenous infection, BALB/c mice were inoculated with $5 \times 10^6$ c.f.u. BCG and the immunological responses were evaluated at various time points as stated above. As shown in Fig. 4, high levels of anti-rPPE44 IgG1 were detected from week 4 of infection onwards with peak titres approximately 1 log$_{10}$ higher than those observed in the subcutaneous infection. PPE44-specific IgG2a were also detected in the sera of infected animals starting from week 4 of infection at titres approximately 0.5–2.0 log$_{10}$ lower than IgG1. Anti-PPD IgG1 and IgG2a antibody responses showed similar kinetics, although some delay was observed in the outcome of the anti-PPD IgG2a response. IFN-$\gamma$ production by spleen cells cultured in the presence of rPPE44 (Fig. 5) peaked at week 4, subsiding to low or undetectable values by week 8 post-infection. PPD-induced IFN-$\gamma$ production showed a similar pattern, but with a significantly higher peak value at week 4 of infection.

Based on the IgG1/IgG2a ratio, which is one parameter indicating whether a Th1 or a Th2 immune response dominates the immune response, our data suggest that in BALB/c mice both subcutaneous and intravenous infection polarize the immune response to PPE44 antigen towards a Th2 phenotype; this was particularly evident in the subcutaneous infection, where no PPE44-specific IgG2a anti-body, indicative of a Th1-type response, was detected. The low production of IFN-$\gamma$ in vitro by spleen cells from infected mice stimulated with rPPE44 and the relatively weak DTH response to rPPE44 corroborated the Th2 bias. Importantly, however, IgG1/IgG2a ratios similarly biased towards a predominant Th2-type response were also detected for PPD both in the subcutaneous and in the intravenous infection, which indicated that the predominance of the Th2 response in our models was of a more general nature and not necessarily linked to PPE44.

Of the few other PPE proteins that have been studied in detail, some have been shown to induce strong humoral responses (Choudhary et al., 2003; Chakhaiyar et al., 2004), while a number of them (Mtb39A/Rv1196, Mtb41/Rv0916c and PPE68/Rv3873) turned out to be potent T-cell antigens recognized by $M. tuberculosis$-infected individuals, but not by PPD-negative donors (Dillon et al., 1999; Oktels et al., 2003; Skeiky et al., 2000). In particular, in PPE68/Rv3873, a cell envelope protein that contributes to the immunogenicity of the RD1 region of $M. tuberculosis$, a T-cell epitope has been mapped (Demangel et al., 2004). In all cases, however, the immune response to these PPE proteins was accompanied by the production of high levels of IFN-$\gamma$, suggesting that their recognition was mediated preferentially by Th1 cells. Furthermore, some were able to confer substantial lung protection against challenge with aerosolized $M. tuberculosis$ comparable to that induced by BCG vaccination (Dillon et al., 1999; Skeiky et al., 2000).

As Th1 helper T cells producing IFN-$\gamma$ are thought to be essential for resistance to TB both in mice and humans (Cooper et al., 1993; Flynn et al., 1993; Jouanguy et al., 1996; Newport et al., 1996), the low level of production of IFN-$\gamma$ in cultures of rPPE44-primed lymphoid cells from infected BALB/c mice might indicate that the possibility of a protective immune response against PPE44 is poor. However, it can be argued that IFN-$\gamma$ production in vitro does not necessarily correlate directly with the induction of protective immunity (Li et al., 1999; Morris et al., 2000). Moreover, as the immune response to different mycobacterial antigens is
influenced by genes of the major histocompatibility complex (Huygen et al., 1993, 1994), the low IFN-γ production in response to PPE44 observed in our experiments might be due to the mouse strain used.

Indeed, it has been reported that, following intravenous infection with M. bovis BCG, lymphocytes from C57BL/6 and BALB.B10 mice (H-2b haplotype) released significantly higher levels of IFN-γ in response to various mycobacterial antigens than those from BALB/c mice (H-2d haplotype) (Huygen et al., 1992). In agreement with these findings, when we tested the immune response to PPE44 and PPD in C57BL/6 mice infected intravenously with BCG, we found higher levels of IFN-γ compared with those obtained in BALB/c mice (Table 2). Furthermore, it should be pointed out that BALB.B10 mice (H-2b haplotype) released significantly in infection with the mouse strain used.

**Table 2. IFN-γ production by spleen-cell cultures from BALB/c and C57BL/6 mice intravenously infected with M. bovis BCG**

Data are expressed as mean ± SD of triplicate cultures from three or four mice. IFN-γ production in unstimulated spleen-cell cultures was 31 ± 9 and 30 ± 6 pg ml⁻¹ for BALB/c mice and 121 ± 39 and 139 ± 7 pg ml⁻¹ for C57BL/6 mice at 6 and 10 weeks post-infection, respectively.

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<th>Weeks post-infection</th>
<th>IFN-γ production (pg ml⁻¹)</th>
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<td>PPE44</td>
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<td>BALB/c</td>
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<td>6</td>
<td>193 ± 62</td>
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Work is in progress in our laboratory to assay different delivery systems of the PPE44 antigen in mice aimed at enhancing Th1-type immune responsiveness to clarify whether this novel mycobacterial antigen can be considered as a desirable candidate TB vaccine.

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**REFERENCES**


