A PE Protein Expressed by *Mycobacterium avium* Is an Effective T-Cell Immunogen

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Infection of mice with *Mycobacterium avium* or immunization with a novel PE gene expressed by *M. avium* (MaPE) showed that a dominant T-cell immune response was elicited. Immunization with an MaPE DNA vaccine protected mice against an aerosol challenge with *Mycobacterium tuberculosis*, suggesting that mycobacteria express PE antigens with cross-protective T-cell epitopes.

Little is known about the structure, function, or immunological response to the PE proteins encoded by the subfamily of PE genes found throughout the genome of *Mycobacterium tuberculosis* and other mycobacteria (3, 6, 11). These PE genes encode proteins that range in size from ~30 to ~110 amino acids, and most contain a characteristic Pro-Glu (PE) amino acid motif near the N terminus. Similar sequences are found as the N-terminal domain of the larger subfamily of proteins that contain polymorphic glycine repeat sequences (PE_PGRS) (2, 6). Studies that have used reverse transcriptase PCR (RT-PCR) and microarray analyses (10, 12, 16, 17) indicate that certain PE genes are expressed by *M. tuberculosis*. There is also some evidence that the PE 35 gene found in RD1, a multigene region that is absent in *Mycobacterium bovis* BCG strains, can elicit an immune response (4, 5), but no function has been assigned to this gene. Since the *Mycobacterium avium* genome contains only a few PE genes and no PE_PGRS genes, we are investigating the PE genes of this mycobacterium. In this report, we describe the immunogenic properties of one PE protein expressed by *M. avium* (MaPE).

*M. avium* strain 104, *M. tuberculosis* Erdman, and *M. bovis* BCG Pasteur were obtained from Frank Collins (CBER/FDA). Initially, the MaPE gene was identified in the unannotated *M. avium* genome sequence available from TIGR (http://www.tigr.org) as contig 3273, nucleotides 748277 to 748577. The gene was amplified by PCR from *M. avium* chromosomal DNA using primers introducing a 5′ HindIII site and a 3′ BamHI site for cloning into the DNA vaccine vector pJW4303 or a 5′ NdeI site and a 3′ XhoI site for cloning into the expression vector pET24b, which incorporates a His tag at the C terminus. All constructs were sequenced to verify the expression vector pET24b, which incorporates a His tag at the C terminus. All constructs were sequenced to verify the nature of the final product. For purification of recombinant His-tagged MaPE, the plasmid carrying the MaPE insert in pET24b was freshly transformed into *Escherichia coli* BL21(DE3)(pLys), and the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Protein was purified using Ni chromatography as described previously (7). MaPE expression was assayed by RT-PCR as described previously (9), using the specific primers 5′-ATGTCGTTCGTG ACCACACAGCCCGGAG (forward) and 5′-TCAGAGGGCC GCTGCGGCCTTG (reverse). C57BL/6 mice (*n* = 5) were infected intraperitoneally with 5 × 10⁶ mycobacteria, and after 30 or 100 days, splenocytes were used to measure cytokines, following ex vivo restimulation in the presence of primary murine bone marrow macrophages (BMMO), with 5 μg/ml of purified recombinant MaPE. Cytokines were measured as described previously by capture enzyme-linked immunosorbent assay (ELISA; BD Biosciences, San Diego, CA) (15). CD4⁺ and CD8⁺ populations were prepared using magnetic beads and enrichment columns (magnetic cell sorting system; Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. DNA immunization with 200 μg DNA per injection per mouse and protection assays with the mouse aerosol challenge model were performed by measuring the colonization of *M. tuberculosis* in mouse tissue as previously described (15).

A screening of the genomes of *M. avium* subsp. *paratuberculosis* (http://www.cbc.umn.edu/ResearchProjects/AGAC/Mptb) and *M. avium* strain 104 (unannotated; http://www.tigr.org) indicates that these mycobacteria contain six PE genes. One PE gene that was chosen for study, MaPE (Fig. 1), is 89.9% homologous with the amino acid sequences of PE 18 and PE 19, which are found in the ESAT-6 gene cluster region 5 of *Mycobacterium tuberculosis* H37Rv (6, 13). The sequence of MaPE is also 61% homologous to the PE domain of PE_PGRS 33 found in *M. tuberculosis*, which has been the focus of our previous studies (1, 8, 9). Using RT-PCR and MaPE-specific primers, mRNA expression by *M. avium* was demonstrated in vitro and in infected macrophages up to 6 days postinfection (Fig. 1). The identity of the MaPE product was confirmed by sequencing, and the results show that this PE antigen is expressed in antigen-presenting cells infected with *M. avium*.

To investigate the host immune response against the MaPE antigen, C57BL/6 mice were infected with an intraperitoneal injection of 5 × 10⁶ *M. avium* 104 organisms, and splenocytes as well as sera were collected at various time points. As shown...
In Fig. 2, in vitro restimulation with purified MaPE of splenocytes isolated from *M. avium*-infected mice resulted in a significant secretion of gamma interferon (IFN-γ) at 100 days postinfection (Fig. 2A) (~2 ng per ml). No specific IFN-γ was released in the culture supernatants when splenocytes were incubated with recombinant PE_PGRS 33 (8) as a control antigen (data not shown). Subsequently, starting with splenocytes collected from mice at 100 days postinfection, CD4+ and CD8+ T cells were separated by magnetic immunobeads and stimulated with purified MaPE antigen. As shown in Fig. 2C, CD4+ T cells released significant amounts of IFN-γ (25 ng per ml). CD8+ T cells also secreted specific IFN-γ in the presence of MaPE antigen but much less than the CD4+ T cells (Fig. 2D). No reactivity was observed when pooled sera from mice infected with *M. avium* 104 were used in Western blot analyses against purified recombinant MaPE protein, compared to an anti-His antibody positive control which recognizes the recombinant protein (data not shown). However, we cannot rule out the possibility that a lack of a humoral immune response to MaPE might be due to differences between the recombinant protein used here and native MaPE, which could contain modifications that serve as antibody epitopes. Together, these results indicate that infection of C57BL/6 mice with *M. avium* 104 elicits a T-cell-mediated immune response to the MaPE antigen.

To investigate the potential of MaPE as an immunogen, a nucleic acid-based vaccine was constructed by inserting the MaPE gene into the vector pJW4303 (7). As was observed for *M. avium* infection, in C57BL/6 mice there was no production of antibodies to MaPE following three immunizations of 200 μg each of MaPE DNA (data not shown). Similarly, no serological activity with the PE antigen was found when the sera were tested by ELISA (data not shown). Splenocytes were collected from mice immunized with MaPE DNA or with the vector only 3 weeks following the final immunization, and IFN-γ was measured by cytokine ELISA following stimulation with MaPE. As shown in Fig. 2B, significant amounts of specific IFN-γ (~4 ng per ml) were released by splenocytes from MaPE DNA-immunized mice compared with vector-immunized mice.

Since both *M. tuberculosis* and *M. bovis* BCG contain numerous PE genes that are similar to MaPE, infection or vaccination with these organisms could elicit immune responses that are cross-reactive with the *M. avium* MaPE protein. To investigate this possibility, C57BL/6 mice were aerosol challenged with *M. tuberculosis* Erdman or infected subcutaneously with *M. bovis* BCG Pasteure, and splenocytes were collected from the infected animals at 6 and 13 weeks and incubated with MaPE antigen. In the ex vivo assay, a significant IFN-γ response was elicited by recombinant MaPE in both *M. tuberculosis* (2.7 ng IFN-γ/ml)- and *M. bovis* BCG (2.1 ng IFN-γ/ml)-infected animals (Fig. 3). These results suggest that both *M. tuberculosis* and *M. bovis* BCG express PE antigens containing T-cell epitopes similar to those found in MaPE and that MaPE might elicit T-cell immunity that protects against challenge with virulent mycobacteria. To investigate this, C57BL/6 mice were immunized with MaPE DNA as described above and then aerosol challenged with *M. tuberculosis* Erdman strain 4 weeks after the last immunization. Immunization with MaPE DNA gave significant protection against a low-dose aerosol challenge with *M. tuberculosis* (Table 1). MaPE vaccination resulted in a 0.53 log reduction (*P* < 0.01) in the number of CFU in the lung 30 days postchallenge and reduced dissemination to the spleen, with a 0.54 log reduction (*P* < 0.05) in numbers of bacterial colonies compared to those in nonimmunized mice.
In this study, we have focused on one PE protein, MaPE, which is 89.9% identical to both PE 18 and PE 19 found in M. tuberculosis H37Rv (6). Genomic searches suggest that M. avium may contain as few as 6 PE genes, compared to ~40 PE genes present in M. tuberculosis (6, 11), and no PE_PGRS genes. This makes M. avium potentially more practical for investigating the properties of the PE family of genes. In this report, we have shown that (i) C57BL/6 mice infected with M. avium for up to 3 months contain both CD4+ and CD8+ T cells that respond to stimulation with purified recombinant MaPE by releasing antigen-specific IFN-γ; (ii) infected mice produce no antibody reactive with recombinant MaPE, as determined by immunoblotting and serum ELISA; and (iii) similar IFN-γ T-cell-mediated immune responses with no antibody responses were found when C57BL/6 mice were immunized with a nuclease acid-based MaPE vaccine. The T-cell response to MaPE is not limited to M. avium but is observed following M. bovis BCG vaccination of mice or infection of C57BL/6 mice with M. tuberculosis. Since M. bovis and M. tuberculosis have a large number of similar PE genes (6, 11, 14), this is likely due to antigenically cross-reactive T-cell epitopes found within PE proteins. Expression of PE antigens homologous with MaPE by M. tuberculosis would explain the reasonable levels of protection observed in the M. tuberculosis aerosol challenge model following immunization of the mice with MaPE DNA. It will be of interest to evaluate protection against M. avium infection following immunization with the MaPE vaccine and to determine whether MaPE contributes to cross-reactive immunity provided by exposure to environmental mycobacteria, which has implications for the use of both the BCG vaccine and diagnostics such as purified protein derivative. It will also be of interest (i) to investigate the ability of other PE antigens to elicit T-cell-mediated immunity and to protect against tuberculosis and (ii) to determine if they should be included in multiantigenic vaccines for tuberculosis.

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TABLE 1. Reduction in the number of Mycobacterium tuberculosis CFU in mouse tissues following immunization with an MaPE DNA vaccine*  

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>No. of CFU in the:</th>
<th>Lung</th>
<th>Spleen</th>
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<tbody>
<tr>
<td>Naïve</td>
<td>6.54 ± 0.15</td>
<td>5.24 ± 0.18</td>
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</tr>
<tr>
<td>Vector control</td>
<td>6.40 ± 0.10</td>
<td>5.31 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>MaPE</td>
<td>6.01 ± 0.08 (0.53)</td>
<td>4.70 ± 0.27 (0.54)</td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td>5.25 ± 0.25 (1.3)</td>
<td>4.20 ± 0.35 (1.0)</td>
<td></td>
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</tbody>
</table>

* The data represent the pooled responses from five mice and show the standard deviation from the mean. Data were analyzed by one-way analysis of variance, and the significant differences between the means were measured using Tukey’s test. Numbers in parentheses indicate log reductions in the MaPE or BCG vaccine group compared with numbers in nonimmunized mice. The protection experiment was performed three times and gave similar results.


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