DETECTION OF CD4/CD8 RATIO IN MICE IMMUNIZED WITH A BICISTRONIC PLASMID CONSTRUCT ENCODING A PPE GENE OF MYCOBACTERIUM AVIUM PARATUBERCULOSIS AND A CYTOKINE GENE OF MURINE GAMMA INTERFERON *

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ABSTRACT
In the present study a gene encoding PPE protein of M. a. paratuberculosis (FJ032182) was cloned with murine IFN-γ in a mammalian bicistronic vector pIRES 6.1 to elucidate the role of gamma interferon on the PPE gene for determination of CD4/CD8 ratio in post immunized mice. Flow cytometric analysis with mononuclear mice splenocytes on 42nd day post immunization revealed significant reduction of the CD4/CD8 ratio in the mice group immunized with pIRES PPE/IFN (1.65±0.0001) compared to the pIRES group (2.92±0.0003). These result suggested that co-expression of murine IFNγ in conjunction with PPE protein significantly enhanced the CMI response.

Key words: CD4/CD8, Gamma interferon, PPE, Mycobacterium avium paratuberculosis.

INTRODUCTION
M. a. paratuberculosis is an intracellular pathogen which multiplies mainly inside mononuclear phagocytes in the primary stage of infection and it is evident that cell mediated immune response control the resulting pathology. It is responsible for paratuberculosis (Johne’s) disease, a chronic granulomatous enteritis of domestic and wild ruminants (Harris and Barletta, 2001; Olsen et al., 2002). Numerous secretory proteins of various mycobacterial species have shown to be potent immunogens and can be used as subunit vaccines (Mullerad et al., 2002 a, b and 2003; Koets et al., 2006), either alone or with adjuvants. Expressions of T cell antigen in prokaryotic vector have failed to induce CTL and cytokine response. However expression of T cell antigen in a mammalian vector for eliciting CD4+ T cell response and CD 8+ cytotoxic T cell response to generate immunity have been reported in a number of animal models ( Martin et al., 2000; Chambers et al., 2002 Pardini et al., 2006 and Sechi et al.,2006). Cytokines also (mainly IFN-γ, TNF-γ, IL-10 etc.) play a major role in the protective immune response against mycobacterial diseases (Coussens et al., 2004). Co-expression of T cell antigen with co-stimulatory molecules in a bicistronic eukaryotic system made the DNA vaccine more effective (Barouch et al., 2002). Moreover, expression of two T cell antigens in eukaryotic bicistronic system may also be useful for enhancing protective immunity. It has been shown that PPE (Proline-Proline- Glutamic acid rich) family proteins may play important roles as T-cell antigens in M. tuberculosis and/or other mycobacterial species (Okkels et al., 2003; Skeiky et al., 2000). In the present work it has been elucidated the effect of gamma interferon on a T cell stimulatory PPE antigen (FJ032182) by means of comparative analysis of CD4/CD8 ratio in an immunized murine model.

MATERIALS AND METHODS
Bacterial strain of M. a. paratuberculosis 316F were obtained from Central Diengenees Kunding Tiek Institute, Lelystad, The Netherlands Biological Products Division of IVRI, Izatnagar, and later maintained at Gene Expression Lab, Division of Animal Biotechnology. IVRI, Izatnagar. A set of primers was designed for the specific amplification of the PPE gene of M.a.paratuberculosis based on

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the sequence information of *M. a.paratuberculosis* str. k10, complete genome Gen Bank Accession No. AE016958 (C terminal antigenic region of the locus_tag MAP_1507). Similarly, one set of primer was designed for amplification of murine interferon gamma gene ORF based on sequence information (Gene bank Accession No. NM_008337). The primers were synthesized by Integrated DNA Technologies, USA. The nucleotide sequences of these primers shown in Table 1. Bicistronic vector pRES 6.1 was supplied from Clontech, USA. Host strain DH5α of *E. coli* were supplied by MBI Fermentas, Germany. The genomic DNA from *M. a. paratuberculosis* was isolated by the method of Portillo *et al.* (1991) with a few modifications. Specific amplification of the 1080 bp PPE gene from the genomic DNA of *M. a. paratuberculosis* was carried out using the specific primers pIRES MAP PPE F and pIRES MAP PPE R. Also PCR was carried out to amplify a 467 bp murine interferon gamma gene using specific primers IFN F and IFN R.

The gel purified PCR products containing the appropriate restriction sites *NheI* & *EcoRI* for PPE gene fragment and *XbaI* & *Not I* for gamma gene was digested with these respective enzymes. Now frame A and frame B of pRES vector was released by digesting with the enzymes *NheI* & *EcoRI* and *XbaI* & *Not I* respectively. The reaction was carried in a 50 µl reaction volume containing 15 µl (1 µg) of digested PCR products, 5 µl of 1X Tango buffer and 2 µl of each of the restriction enzymes. The reaction mixture was incubated overnight in a water bath at 37°C and the digested product was checked on 1% agarose gel. Two sets of 10 µl ligation mixture, each containing 4 µl of the digested PCR products, 1 µl of digested vector, 1 µl of ligase buffer, 1 µl of PEG-4000 and 1 µl of T4 DNA ligase were made separately, where in one of the set contain vector and the PPE gene and another set contain vector, PPE gene and gamma interferon gene. The reaction was carried out overnight at 22°C in a water bath. Each of the ligated mixture was transformed separately into competent DH5α cells, grown and selected on LB agar plates containing Ampicillin (100 µg/ml). The positive clones were designated as pIR PPE and pIR PPE/IFN. Large scale purification of the plasmid constructs pIR PPE and pIR PPE/IFN was done using QIAGEN megakit.

Swiss albino mice supplied by laboratory Animal Section, IVRI Izatnagar were maintained on ration comprising wheat dalia 62%, maize 30%, wheat bran 7%, salt 1% and mineral mixture 25 ppm with 5 ml milk per mice. The animals were divided into four groups namely A, B, C and D. They were immunized with the purified recombinant plasmid as shown in the Table 2.

On the 42nd day after immunization, about 5ml of sterile RPMI 1640 medium was injected into the peritoneal cavity of each mouse, gently massaged and the mice were left in the cage for 5 min. Then the mice were sacrificed by CO₂ asphyxiation. Abdominal cavity was cut open. Spleens were harvested from sacrificed mice and made into a single cell suspension. The cells suspended in RPMI-1640 were layered over Ficoll-Paque PLUS and mononuclear splenocytes were isolated by density gradient centrifugation at 1350 X g for 30 min. Splenocytes thus obtained were used for Flow cytometric analysis. 100 µl of cell suspension containing 2×10⁵ splenocytes were incubated with 20 µl of antibody cocktail (containing monoclonal antibodies against mouse CD3e, CD4 and CD8a) for 30 min in the dark. Appropriate Isotype controls were also maintained. Subsequently, the cells were washed twice with PBS and aliquots of 10,000 cells analyzed per sample by FACS calibur (Becton Dickinson).

**RESULTS AND DISCUSSION**

The bicistronic constructs which were generated after cloning the *NheI* & *EcoRI* digested PPE gene fragment into frame A and *XbaI* & *Not I* digested gamma gene in the frame B of the plasmid vector pRES 6.1 designated as pIRPPE/IFN.

<table>
<thead>
<tr>
<th>Primer Code</th>
<th>Sense/ Antisense</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIRES MP PPE F</td>
<td>Sense</td>
<td>5’-GCC GCT AGC ATG TGG GTC CAG GCC GC-3’ - 29mer</td>
</tr>
<tr>
<td>pIRES MP PPE R</td>
<td>Antisense</td>
<td>5’-GCC GAA TTC TTA CTC GGT TCC AGC GTC GC-3’ – 29mer</td>
</tr>
<tr>
<td>IFN F</td>
<td>Sense</td>
<td>5’-GCC GCT AGA ATT GAC GCT ACA CAC TGC-3’ 27mer</td>
</tr>
<tr>
<td>IFN R</td>
<td>Antisense</td>
<td>5’-CCG CGG CCG CTC AGC AGC GCC CAC TGG TT-3’ 28mer</td>
</tr>
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</table>
Similarly a monocistronic construct pIRPPE was also generated after cloning of digested PPE PCR product in the frame A of the same vector.

Four groups of mice were immunized with 100µg of purified plasmid constructs, plasmid vector (Mock) and TE controls in two doses on 0 and 35th day to study the immune responses.

Splenocytes collected from each group of mice were subjected to flow cytometric analysis after labeling with CD4 and CD8 specific monoclonal antibodies conjugated with appropriate fluorescent tags. There was a significant decrease in the CD4/CD8 ratio in the mice group immunized with plasmid construct pIR PPE/IFN (1.6522678) compare to the pIR PPE group (2.9247191) (Table 3 and Fig.1).

The use of cytokines as adjuvant is known to enhance immune responses when they were administered during the development of immune response against a particular antigen (Anderson, 2001; Kaufmann, 2001). IFNγ is the key cytokine observed in immunological responses to mycobacterial infections (Stable et al.1996). It is the defining cytokine of Th1 subset, and activates macrophages for microbicidal activity. It induces IL12, which causes Th cells to differentiate in to Th1 subset (Goldsby et al., 2003).

In the present work it has been studied the adjuvant properties of IFNγ for enhancing the T cell immune response against a PPE antigen of M. a. paratuberculosis in immunized mice using a plasmid construct pIR PPE/IFN. Accordingly plasmid constructs pIR PPE, pIR PPE/IFN and plasmid pIRES as control were used for immunization in murine model and the immune responses elicited were studied by Flow cytometric analysis to detect CD4/CD8 ratio. Mononuclear splenocytes isolated from plasmid constructs immunized mice groups were subjected to flow cytometric analysis to find the CD4 and CD8 positive cells. The CD4/CD8 ratio was significantly reduced in the group pIR PPE/IFN followed by pIR PPE as expected. The reduction in the ratio may be correlated to the increase in the CD8 count thereby confirming the effect of CMI response. The monocistronic construct pIR PPE also elicited milder CMI response in comparison to pIR PPE/IFN. This revealed that the presence of IFNγ synergised the T cell response of PPE protein and provided an adjuvant effect.

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REFERENCES


