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Cell-mediated immune response to epitopic MAP (multiple antigen peptide) construct of LcrV antigen of *Yersinia pestis* in murine model

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ABSTRACT

Yersinia pestis is the causative agent of plague. Cellular immunity seems to play an important role in defense against this disease. The subunit vaccine based on V (Lcr V) antigen has been proved to be immunogenic in animals and in humans. The multiple antigen peptide (MAP), incorporating all the relevant B and T cell epitopes is highly immunogenic in mice through intranasal route of immunization in PLGA particles containing CpG-ODN as an immunoadjuvant inducing humoral and mucosal immune response. In the present study, cell-mediated immune response using same MAP was studied in murine model. Primary and memory T cell responses were studied in outbred and inbred mice immunized intranasally with MAP in the presence of two immunoadjuvants (Murabutide and CpG-ODN). All the three compartments (Spleen, Lamina propria and Peyer's patches) of the lymphoid system showed increased lymphoproliferative response. Highest lymphoproliferative response was observed especially with CpG-ODN. Cytokine profile in the culture supernatant showed highest Th_1 and Th_{17} levels. FACS analysis showed expansion of both $CD4^+$ and $CD8^+$ T cells producing gamma-interferon, perforin and granzyme-B with major contribution from $CD4^+$ T cells.

1. Introduction

Yersinia pestis, a causative agent of plague, recently being classified as a re-emerging disease [1–4]. Cell-mediated immunity (CMI) to *Y. pestis* is important for vaccine-mediated protection against plague. Adoptively transferring *Y. pestis* stimulated T cells provided protection against *Y. pestis* in a fully antibody independent model, suggesting that vaccine-stimulated T cells are directly responsible for the observed protection [5]. Vaccine primed cytokine production by T cells help in protection, but they also contribute protection via direct lysis of the infected cells [20]. In addition, $CD4^+$ T cells can exert cytolytic activity on MHC class II bearing targets [22]. Involvement of $CD8^+$ T cell-mediated immune responses in LcrV DNA vaccine have been evaluated in protection against lethal *Y. pestis* challenge [6]. Currently, the fraction 1 (F1) and V proteins have received the most attention, as vaccination with them protected small animals as well as sub human primates against pneumonic plague [7–10]. Our laboratory is working on peptide based vaccine on V antigen for quite some time, which offers an alternative approach for plague vaccine [11–13]. This Study has been extended in designing MAP (Multiple antigen peptide), incorporating all the protective B and T cell epitopes of V antigen with inbuilt immunoadjuvants, thereby showing all individual epitopes contributed overall mucosal and humoral immune response in different strains of mice [14].

Above finding led us to study the involvement of cell–mediated immunity and cytokine milieu in murine model after intranasal immunization with the same MAP with two adjutants using PLGA (Poly-lactide-co-glycolide) as delivery vehicles. Cellular immunity contributed by MAP as well as individual peptides was studied in detail. We found significantly higher T cell proliferative response and cytokine secretion (IL-2, IL-12, IFN-γ, TNF-α, IL-1β & IL-17A) except IL-4 in culture supernatant. We also found significant number of $CD4^+$ and $CD8^+$ T cells secreting IFN-γ, perforin and granzyme-B. These findings strongly advocate suitability of such subunit vaccine for plague disease.

2. Materials and methods

2.1. Mice

Outbred (Swiss Albino) and inbred [$H\text{-}2^d$ (BALB/c) & $H\text{-}2^b$ (C57BL/6)] mice of age group 6–8 weeks were procured from central animal facility AIIMS, NEW Delhi, India. All experimental groups consisted of six animals. All the animals were provided food and water *ad libitum*. All experiments were conducted in accordance with the guidelines of CPCSEA (for the care and use of laboratory animals), Ministry of Social Justice, and Government of India

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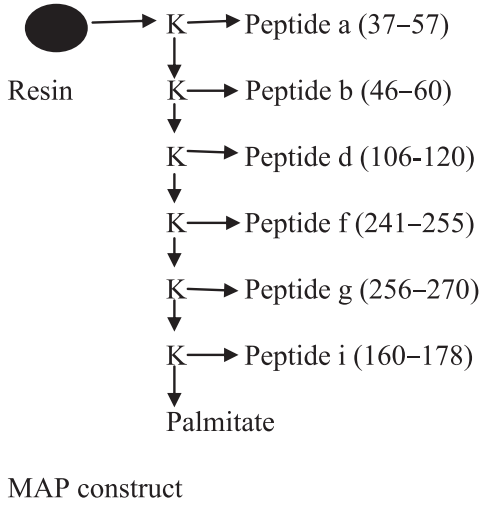
and adopted by the Ethics Committee on animal experimentation by AIIMS, New Delhi.

2.2. Antigen

Multiple antigen peptide (MAP), containing the relevant B and T cell epitopes of V antigen was synthesized using F-moc chemistry as described [14]. Resin and lysine (K) were used as linker for adding B and T cell epitopes of V antigen to the MAP construct. Finally MAP was entrapped in PLGA particles.



MAP construct

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2.3. Adjuvants

The adjutants used in the study were CpG-ODN 1826, murabutide and alum.

2.4. Immunization

Mice were immunized with 20 μg of MAP (entrapped in microsphere) in 20 μl of saline intranasally. Mice were anesthetized by isoflurane and MAP was given to both the nostril with a micropipette on day 0 followed by 10 μg of MAP boosted on 10th day. A second booster dose was given on 35th day for studying memory response. CpG-ODN and murabutide (5 μg/animal) were administered by physically mixing with microsphere, where as Alum was used under identical experimental condition for comparison purpose.

2.5. Formulations

Formulations	MAP (μg)	Adjuvant (μg)	Volume in saline (μl)
MAP+Alum (MAlum)	20	20	20
MAP+CpG (MCpG)	20	5	20
MAP+Murabutide (MMBT)	20	5	20
MAP+CpG+Murabutide (MCpGMBT)	20	(2.5 + 2.5)	20

2.6. T cell proliferation assay

Immunized mice were sacrificed on 21st day for effector and 60th day for memory response. Splenocytes (SP), Lamina propria (LP) and Peyer's patches (PP) (devoid of B cells by panning with rabbit anti-mouse immunoglobulin) cells were prepared as per reported protocol [12]. Briefly, 1×10^6 cells/well were plated with RPMI–1640 media supplemented with 10% FCS at 37 °C, in a humidified atmosphere with 5% CO_2 for 72 h. Cells were *in vitro* stimulated with MAP (5, 10, 15 μg/well), V antigen (10 μg/well) and individual peptides (10, 25, 50 μg/well). Concanavalin A was taken as positive control. Cells were left unstimulated as negative control. After 72 h cells were pulsed with 0.5 μCi of tritiated thymidine and stimulation index was calculated by measuring thymidine incorporation as described [12].

2.7. Cytokine estimation in culture supernatants

Culture supernatant was centrifuged at 5000 rpm for 5 min and cytokines were estimated using sandwich ELISA as par manufacturer's instructions (eBioscience, USA).

2.8. Flow cytometry and intracellular cytokine staining

SP, LP and PP cells were isolated from the mice primed with MAP along with CpG and *in vitro* stimulated with MAP or individual peptides on day 21. Cells were stimulated with different formulations and Brefeldin A was added in order to accumulate cytokines. After 48 h, cells were subjected to surface staining with monoclonal antibodies anti mouse CD8-APC/Cy7 (BioLegend), CD4-PE-Cy7 (eBiosciences). After permeabilization, cells were treated with anti mouse perforin–FITC, granzymeB-PE and IFN-γ PerCP-Cy5.5 (eBiosciences) and finally cells were captured in BD FACS Canto flow cytometer. 10,000 live events were used and analyzed using WINMID software as described [13]. Suitable isotype controls were used. The results were expressed as percentage of cells showing the marker out of a gated population.

2.9. Statistical analysis

The data on T cell proliferation assay and cytokine levels were analyzed using nonparametric kruskal-walli's one way analysis of variance by ranks. Mean value and standard deviation were calculated using Student's two-tailed *t*-test. FACS statistical analysis was performed using SPSS version 12.0.1 for windows. Analysis of data was performed using one–way ANOVA. Results are considered statistically significant if $p \leqslant 0.05$.

3. Results

3.1. V-MAP showed higher in vitro T cell proliferative response

Mice were primed *in vivo* with MAP of V antigen and SP, LP & PP cells were *in vitro* stimulated with MAP, native V antigen and individual peptides respectively. Higher cellular response was observed in all the three compartments in both inbred and outbred strains. The lymphoproliferative response in lymphoid organs of mice, which were primed with MAP immunogen and *in vitro* stimulated with MAP, was higher as compared to native V antigen primed and stimulated with V antigen (Fig. 1A). However when MAP primed lymphocytes were *in vitro* stimulated with native V antigen, the lymphoproliferation was similar to MAP + Alum (MAlum) group (Fig. 1A). Secondly, the lymphoproliferative response was observed in all the three compartments when MAP primed cells were stimulated with individual peptides. Peptide d and f had shown higher cellular response than other peptides (Table 1A).

The memory response on day 60, showed slightly higher SI value (insignificant) compared to effector response observed on day 21 in outbred as well as inbred (H-2^b & H-2^d) strain in all the three