Developing subunit immunogens using B and T cell epitopes and their constructs derived from the F1 antigen of *Yersinia pestis* using novel delivery vehicles

Leenu Sabhnani a, Monika Manocha a, Kurella Sridevi a, Donthamsetty Shashikiran b, Ravi Rayanad c, Donthamsetty Nageswara Rao a,

**Abstract**

*Yersinia pestis* is the etiological agent of pneumonic and bubonic plague. As the currently licensed vaccines for plague have their own limitations, there is a need for a rational and more effective form of a subunit vaccine to combat both forms of the disease. Newer methods of antigen delivery coupled with adjuvant offer an alternative approach toward a plague vaccine. In order to develop a new generation vaccine against plague, we chose an immunodominant, outer membrane capsular protein, F1 of *Y. pestis*. The immunogenicity of the peptide sequences, predicted to possess B (three sequences, B1, B2 and B3) and T (two sequences, T1 and T2) cell determinants, was studied in a murine model with different genetic backgrounds, using alhydrogel and liposomes as delivery vehicles. All the peptide sequences are immunogenic in all mouse strains and showed primary and secondary immune response. B2 peptide was found to be most immunogenic, followed by B1 and B3 peptides. Chimeras made between B and T structures proved highly immunogenic and the antibody levels are comparable with native F1 antigen, thereby proving that T1 and T2 are helper sequences. Interestingly, the liposome mode of immunization was found to be more immunogenic and generated higher affinity antibodies than the alum-based preparation. Immunization using a mixture of all the peptides further proved B2 to be immunodominant. The IgG isotype profile showed predominance of IgG1, IgG2b followed by IgG2a for all the formulations irrespective of mode of antigen delivery. Lymphocyte proliferation of spleen cells primed in vivo with peptides, B-T conjugates and F1 antigen followed by in vitro stimulation with these antigens in soluble (medium) and particulate (liposome) form, showed dose-dependent stimulation of T cells, while B-T constructs showed a higher stimulation index, comparable to F1 antigen. The liposome mode of antigen presentation showed higher lymphoproliferation of spleen cells. Of all the peptides tested, T1 and T2 sequences showed the highest stimulation indices. The pattern of cytokine levels was in the following order: interferon-γ > interleukin-2 > interleukin-4. In vivo protective studies of the B-T conjugates revealed that BIT1 and a mixture of conjugates showed a survival rate of 10 days. Thus, the study highlights the importance of B and T cell epitopes as peptide-based immunogens, being a serious alternative for plague vaccine.

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**Keywords:** F1 antigen; B cell peptide; T cell peptide; Liposome; Alum; In vivo protection

1. Introduction

*Yersinia pestis* is the etiological agent of both forms of plague, which is still active in various regions of the world. Currently, the licensed procedure for immunizing individuals against plague involves parenteral administration of killed whole-cell suspension of *Y. pestis* [3]. This approach has shown protection against bubonic plague, but failed to prevent infection in the lungs (pneumonic plague). Moreover, the vaccine requires repeated boosters to maintain peak antibody levels [4]. This necessitates the search for acellular vaccines against plague that should consist of immunogenic subunits derived from the parent organism. Two of the most prominent antigens are fraction 1 (F1)
2. Materials and methods

2.1. Peptide sequences, conjugates and peptide cocktail

1. B1: NH2-FTTKVIGKDSRDFDISPKV-COOH (aa 105–123)
2. B2: NH2-FFVRSIGSKGKLAAGKYTDATV-COOH (aa 142–165)
3. B3: NH2-TSQDGNHHQFT-COOH (aa 96–106)
4. B4: NH2-DDFFVRSIGSKGKLS-COOH (aa 141–154)
5. T1: NH2-VNGENLGDDVLAT-COOH (aa 123–137)
6. T2: NH2-TGSQDFFVRSIG-COOH (aa 137–148)

2.2. Mice

Inbred mice of age group 6–8 weeks [C57BL/6 (H-2b), BALB/c (H-2d), CBA/J (H-2k) and FVB/J (H-2n)] were procured from the breeding facilities of the National Institute of Immunology (NII), New Delhi. Outbred mice were procured from the breeding facilities of the Central Animal House, AIIMS, New Delhi. All experimental groups consisted of five or six animals. All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals as promulgated by CPCSEA, Ministry of Social Justice, Government of India and adopted by the Ethics Committee on animal experimentation by AIIMS, New Delhi.

2.3. Liposomal formulation

Liposomes were prepared according to our protocol reported previously [9]. In brief, multilamellar vesicles (MLVs) were prepared by dissolving phosphatidylcholine, cholesterol and phosphatidylglycerol in a molar ratio of 7:4:1 in chloroform and methanol and subsequently evaporated to dryness. These MLVs thus obtained were suspended in 1 ml of double-distilled water (DDW) and probe-sonicated to form small unilamellar vesicles (SUVs). The SUVs formed were kept at room temperature for 1 h for stabilization and later the solution was centrifuged at 5000 rpm for 15 min to remove the aggregated lipids.
peptide or peptide conjugates (5.0 mg ml\(^{-1}\)) were added to the SUVs, freeze-dried and subsequently lyophilized. The dry powder obtained was rehydrated with 1.0 ml DDW. After dilution with 5.0 ml phosphate-buffered saline (PBS, 0.01 M, pH 7.4) the suspension was ultracentrifuged at 100,000 \(\times g\) for 1 h at 4\(^\circ\)C. The liposomal pellet was washed twice with PBS and finally suspended in 0.2–0.5 ml PBS and stored at \(-70^\circ\)C. The percentage entrapment of peptide in liposomes was estimated in the supernatants by the bicinchoninic acid method [10] and was found to be in the range of 40–45%. The size of the liposomes was determined by scanning electron microscope and they were in the range of 40–60 nm.

### 2.4. Immunization protocol

Mice were immunized with various combinations of peptide and peptide conjugates:

1. peptide alone in alhydrogel: 50 \(\mu\)g
2. peptide alone in liposome: 25 \(\mu\)g
3. B-T conjugates in alhydrogel: 50 \(\mu\)g
4. cocktail of peptides: 50 \(\mu\)g (10 \(\mu\)g each)
5. F1 antigen in liposome: 10 \(\mu\)g

The optimum antigen for alhydrogel-adsorbed, liposome-entrapped preparation was standardized by dose–kinetic study. For booster immunization, the amount of antigen was reduced to half the primary dose. The liposome preparation was also adsorbed on alhydrogel before immunization. In the case of alhydrogel and liposome preparation, mice were immunized in the footpad on days 0, 21 and 35. Mice were bled on days 28, 40 and 60. The serum was stored at \(-20^\circ\)C until use.

### 2.5. Antibody measurement

In enzyme-linked immunosorbent assay (ELISA), 100 \(\mu\)l of peptide–bovine serum albumin (BSA) conjugate (100 ng per well) was used to coat the 96-well microtiter plates (Immunolon2, Dynatech, VA, USA). After washing and blocking with skimmed milk powder, a single dilution of the test serum (1:100) or a serial two-fold dilution (1:400–102,400) of pooled antisera was used for measuring peptide-specific total IgG levels and peak antibody titers, respectively. Titers were assessed as the highest serum dilution giving an absorbance (0.15) higher than that of pre-immune sera. The color was developed using OPD as chromogen and \(H_2O_2\) as the substrate and absorbance was read at 490 nm.

### 2.6. Estimation of IgG isotypes

For estimating IgG isotypes, mice sera (dilution 1:100) from different immunization groups were incubated for 2 h at 37\(^\circ\)C, with different peptide-BSA conjugates coated on ELISA plates. The IgG isotype binding was detected using secondary goat anti-mouse IgG specific for each subclass (dilution 1:1000, Sigma isotyping kit) for 2 h at 37\(^\circ\)C, followed by tertiary antibody (rabbit anti-goat IgG–horseradish peroxidase (HRP) conjugate, 1:1000 dilution). The absorbance was read as described above.

### 2.7. Binding affinity of the anti-peptide antibodies (K\(_D\))

The binding affinities of the anti-peptide antibodies in various immunizations were determined by measuring the dissociation constant (K\(_D\)) [11]. In brief, mice serum (1:200 dilution) was incubated with different concentrations of the peptide (0.1–10 nM) for 15 h at 20\(^\circ\)C, so as to attain antigen–antibody equilibrium. The antigen–antibody complexes were transferred onto the wells of the microtiter plates previously coated with the respective peptide capture antigen (100 ng per well). After washing three times with PBS containing 0.05% Tween 20, the plates were incubated with goat anti-mouse IgG–HRP (1:1000 dilution) for 90 min at 37\(^\circ\)C. The color was developed using OPD and hydrogen peroxide as substrate. The reaction was stopped with 8 N \(H_2SO_4\) and absorbance was read at 492 nm. Dissociation constants were then calculated using regression analysis and a simplification of the mathematical equation of Scattered and Klotz [11]:

\[
\frac{A_o - A}{A_o} = \frac{1 + K_D}{a_o}
\]

where \(A_o\) is the absorbance without free antigen, \(A\) is the absorbance with free antigen and \(a_o\) the concentration of free antigen. This equation permits the determination of \(K_D\) even when the concentration of specific antibody is not known.

### 2.8. Antigen-induced splenocyte proliferation

For studying T cell recognition properties, outbred mice were immunized subcutaneously with F1 antigen (10 \(\mu\)g) and individual peptides/peptide conjugates (50 \(\mu\)g) adsorbed on alhydrogel. Animals were boosted with half the dose of the antigen on day 8. Mice were killed on day 15. Single cell suspensions of splenocytes (devoid of B cells by panning with anti-mouse immunoglobulins) were prepared and cultured in 96-well plates at 2 \(\times\) 10\(^5\) cells per well in RPMI 1640 medium supplemented with gentamicin and streptomycin (50 \(\mu\)g ml\(^{-1}\)) and 10% heat-inactivated fetal calf serum.

The cells in each well were then stimulated in vitro with peptide/peptide conjugate/F1 antigen dissolved in medium (25, 50 and 100 \(\mu\)g ml\(^{-1}\)) or entrapped in liposomes (10, 25 and 50 \(\mu\)g ml\(^{-1}\)) in a final volume of 200 \(\mu\)l. Phytohemagglutinin (2 \(\mu\)g ml\(^{-1}\)) was used as a positive control. Medium and peptide-free liposome served as a negative control. After 72 h of culture, 50 \(\mu\)l of the culture supernatant was collected and stored at \(-70^\circ\)C for cytokine estimation. The cultures were pulsed with \(^3\)H-thymidine (specific activity 6.4 Ci mmol\(^{-1}\), BARC, Trombay, Mumbai, India)
Fig. 1. Peptide-specific end-point ELISA titers (reciprocal values, in thousands) of different strains of inbred mice, immunized using alhydrogel (described in Section 2).
at 0.5 μCi per well. The cells were incubated for 18 h and then harvested onto glass fiber disks, and thymidine incorporation was measured by a liquid scintillation counter. Proliferative responses were expressed as stimulation index (SI). All cultures were taken in triplicate.

2.9. Cytokine measurement

The culture supernatants were centrifuged at 5000 rpm for 15 min, filtered through 0.22-μm pores and assayed for cytokine levels using sandwich ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.10. Statistical analysis

The data on peptide-specific IgG levels, IgG subclasses, K0 measurement, T cell proliferation and cytokine levels were compared by non-parametric, Kruskal–Wallis one-way analysis of variance by ranks. The levels of significance were compared between peptide(s) in alhydrogel versus peptide(s) in liposomes and between the strains.

2.11. In vivo protective studies

BALB/c mice (six in each group) were immunized with respective B-T conjugate adsorbed on alhydrogel (30 μg per animal) or conjugate mixture (10 μg of each conjugate) through the intramuscular route in the hind leg on days 1, 7 and 14. On day 21, animals were boosted with half the dose of the conjugate at the same inoculation site. The control group consisted of six mice immunized with adjuvant. F1 antigen (10 μg) was used under identical experimental conditions as a positive control. On day 28, each group was challenged intraperitoneally with live Y. pestis bacteria (1 × 10^7 bacteria in 0.1 ml saline per animal, strain 195-p). The survival time was monitored for all the groups for over 10 days. Survivors were culled after this time period.

3. Results

3.1. Humoral response to individual and conjugated peptides

The antibody response in different mouse strains was observed in terms of peak antibody titer and total subclass-specific IgG levels. In general, mice bearing haplotype H-2q and H-2b showed higher antibody levels as compared to the other two mice strains H-2k and H-2d, irrespective of the nature of the peptide used. Low peak antibody titers were observed for all the peptides in all the strains on day 28. Mice bearing the haplotypes H-2q and H-2b produced peak titers of 3200–6400, while the other two strains produced peak titers of 1600–3200 on day 28. The peak antibody titers rose to 6400–25 600 on day 42 for most of the peptides. When peptide-specific peak antibody titers were measured on day 60, there was a decline with peak titers ranging from 1600 to 12 800, depending upon the nature of the peptide and strain used. However, for peptides B1, B2, B3 and T1 the peak titers were maintained at the same levels in the mice bearing the haplotypes H-2q and H-2b. In general, B2 and T1 peptides showed higher immunogenicity than the other three peptides and the magnitude of the order of peak antibody titers was observed as B2 > T1 > B1 ≈ T2 > B3, with the order of responding mouse strains being H-2q > H-2k > H-2b > H-2d (Fig. 1a–e).

The delivery of the same peptide antigens in liposomes elicited higher peak antibody titers up to 25 600, which was statistically higher (P < 0.05) as compared to the alhydrogel-based preparations. Though there was a small fall in peak antibody titer on day 60, this fall was insignificant on day 42. Interestingly, liposomal delivery produced consistent peak antibody titers with all the peptides in all the mouse strains, which was not the case with the alhydrogel-based preparations (Fig. 2a–e).

When the humoral response was studied for peptide conjugates delivered in alhydrogel, it showed very high peptide-specific peak antibody titers in all the strains and in all the bleeds (Fig. 3a–f). The peak antibody titers for either constituent peptide or the conjugate ranged from 3200 to 12 800 on day 28. By day 42, the antibody titers rose to 51 200 whereas a few peptide conjugates showed peak titers of 102 400. By day 60, the peak antibody titers were maintained at the same level or a marginal fall was observed with the constituent peptides. The B2T1 conjugate showed the highest antibody titers, followed by B1T1 > B2T2 > B1T2 > B3T1 > B3T2.

Outbred strains consistently produced high peak antibody titers in all the bleeds with all the conjugates. The peak titers for F1 antigen in all the strains were 64 000 on day 28 and showed a booster response to 102 400 on day 42. However, by day 60, the titers were the same as at the primary bleed or higher (data not shown). It is important to mention that peak antibody titers exhibited by B-T conjugates are comparable with anti-F1 antibodies.

3.2. IgG subclass

Delivery of all the peptides in alhydrogel generated both IgG1 and IgG2a/2b isotypes in all the strains. Initially low levels of these isotypes were observed on day 28. By day 42, there was a concomitant rise in either IgG1 or IgG2a/2b isotypes or both the isotypes, which was statistically significant (P < 0.01). Incidentally, these isotype levels were maintained at the same level or a marginal fall in their levels was observed on day 60, which was statistically insignificant (data not shown).

The levels of IgG3 isotypes were negligible in all the bleeds with all the peptides. In general, IgG1 and IgG2b are the major isotypes produced followed by IgG2a, irre-
Fig. 2. Peptide-specific end-point ELISA titers (reciprocal values, in thousands) of different strains of inbred mice, immunized using liposomes (described in Section 2).
Fig. 3. Peptide- and conjugate-specific end-point ELISA titers (reciprocal values, in thousands) of outbred and different strains of inbred mice, immunized using alhydrogel (described in Section 2).
Fig. 4. Peptide-specific IgG isotype levels of different strains of inbred mice, immunized using liposomes. Data are presented as mean ± S.D.
Fig. 5. Peptide conjugate-specific IgG isotype levels of outbred and different strains of inbred mice, immunized using liposomes. Data are presented as mean ± S.D.
3.3. Humoral response to a cocktail of peptides

The humoral response to a mixture of all the peptides was studied in outbred and three inbred strains of mice. High B2 peptide-specific IgG levels were detected when compared to other peptides. Thus, the order of response was B2 > B1 > B3 > T1 > T2 with B2 peptide showing a stronger anamnestic response as compared to other peptides in all the strains (data not shown).

3.4. Dissociation constant (K_D) of the peptide antisera

The affinity of the antibodies raised against different peptide formulations (alhydrogel and liposomes) was studied by measuring K_D values in low and high responder strains. The serum chosen for each peptide was that on day 42 (Table 1). Alhydrogel-based peptide sera showed K_D values in the range of 17.5–60.1 nM, while liposome-based antisera showed K_D values of 9.07–18.2 nM, which was statistically significant (P < 0.01) when compared to alhydrogel-based preparation. There was a two- to fourfold lower K_D value with liposome-based preparations when compared to alhydrogel, indicating that the affinities of the antibodies by this delivery route were higher than those of the alhydrogel-based preparations.

3.5. Antigen-induced T cell proliferation

To characterize the prevalence of peptide-specific T lymphocytes, mice were first primed either with native antigen (F1) or with peptide conjugates of B-T and spleen cells re-stimulated in vitro, with native antigen/peptide corresponding to the nature of the peptide or formulation used. In general, liposomal delivery of antigen produced higher isotype levels (P < 0.05) and these levels were maintained even on day 60 as compared to alhydrogel-based preparations (Fig. 4a–e).

When the IgG isotype profile was observed for peptide conjugates delivered in alhydrogel in outbred as well as inbred strains, the predominant isotypes were either IgG1 or IgG2a or IgG2b or both (Fig. 5a–f). The primary immune response showed low levels of these isotypes while in subsequent bleeds, these isotypes were significantly higher (P < 0.01) and the levels were maintained until day 60. All the strains and all the peptide conjugates showed very high IgG1, or IgG2a or IgG2b isotype levels in all the bleeds. Though peptide B3 showed lower isotype levels than other peptides, its conjugate showed similar levels of these isotypes with minimal strain-to-strain variation. The IgG3 levels detected in all bleeds were negligible with all the peptides. The major isotype produced by F1 antisera is predominantly IgG1 and IgG2b in all the four strains. In subsequent bleeds, there was a rise in IgG1 isotype, while levels of IgG2b were maintained at the same level. The levels of IgG2a are almost consistent in all the bleeds (data not shown).

### Table 1

| Peptide | Vehicle | Haplotypes | K_D (nM) |
|---------|---------|------------|----------|
| B1      | Alhydrogel | H-2 (low responder) | 49.5 ± 0.02 |
|         |          | H-2 (high responder) | 23.2 ± 0.07 |
| Liposomes | H-2 (low responder) | 15.2 ± 0.03 |
|         |          | H-2 (high responder) | 10.5 ± 0.07 |
| B2      | Alhydrogel | H-2 (low responder) | 41.7 ± 0.05 |
|         |          | H-2 (high responder) | 17.5 ± 0.04 |
| Liposomes | H-2 (low responder) | 15.2 ± 0.03 |
|         |          | H-2 (high responder) | 9.98 ± 0.02 |
| B3      | Alhydrogel | H-2 (low responder) | 60.1 ± 0.05 |
|         |          | H-2 (high responder) | 34.2 ± 0.07 |
| Liposomes | H-2 (low responder) | 28.2 ± 0.07 |
|         |          | H-2 (high responder) | 21.4 ± 0.02 |
| T1      | Alhydrogel | H-2 (low responder) | 43.2 ± 0.04 |
|         |          | H-2 (high responder) | 19.5 ± 0.07 |
| Liposomes | H-2 (low responder) | 14.1 ± 0.07 |
|         |          | H-2 (high responder) | 9.07 ± 0.06 |
| T2      | Alhydrogel | H-2 (low responder) | 48.9 ± 0.05 |
|         |          | H-2 (high responder) | 19.2 ± 0.08 |
| Liposomes | H-2 (low responder) | 9.58 ± 0.05 |
|         |          | H-2 (high responder) | 10.0 ± 0.02 |

### Table 2

| Peptide | F1 antigen | Conjugate | S P | S P | S P | S P |
|---------|------------|-----------|-----|-----|-----|-----|
| B1      |            |           | 10.1 (50) | 13.6 (25) | 5.9 (50) | 9.3 (25) | 13.8 (50) | 16.0 (25) | 12.1 (25) | 15.4 (25) | 12.7 (50) | 16.1 (25) | 13.3 (50) | 15.1 (25) |
| B2      |            |           | 7.5 (50) | 11.9 (25) | 6.0 (50) | 9.6 (25) | 10.2 (50) | 24.1 (25) | 16.9 (50) | 19.8 (25) | 12.1 (50) | 17.2 (25) | 17.8 (50) | 19.2 (25) |
| B3      |            |           | 4.8 (50) | 7.1 (25) | 6.3 (25) | 7.3 (25) | 14.8 (50) | 17.9 (25) | 13.2 (25) | 15.4 (25) | 12.7 (50) | 16.1 (25) | 13.3 (50) | 15.1 (25) |
| T1      |            |           | 15.4 (50) | 23.2 (25) | 15.1 (25) | 21.1 (25) | 14.2 (50) | 14.8 (25) | 13.2 (25) | 14.9 (25) | 9.4 (50) | 12.4 (25) | 10.5 (25) | 10.5 (25) |
| T2      |            |           | 12.2 (50) | 20.1 (25) | 7.9 (25) | 14.2 (25) | 13.8 (25) | 14.9 (25) | 9.4 (50) | 12.4 (25) | 10.5 (25) | 10.5 (25) | 12.2 (50) | 17.2 (25) |

The numbers in parentheses indicate the peptide/conjugate/F1 antigen concentration (µg ml⁻¹) showing maximum SI.

Formulations: S, soluble; P, particulate.

i-j: P < 0.001; a-b, c-d, k-l: P < 0.01; e-f, g-h, m-n, o-p, q-r, s-t: P < 0.05.
conjugate/constituent peptides either in soluble form (medium) or in particulate form (liposomes) (Table 2). All three peptides B1, B2 and B3 showed SIs of 4.8–10.1 at 50 μg ml⁻¹ when presented in soluble form in vitro, while the same peptides showed a higher SI (7.15–13.6) at a lower pulsing antigen concentration of 25 μg ml⁻¹ in particulate form. T1 and T2 peptides showed maximal proliferative indices, both in soluble form and in particulate form. T1 and T2 peptides required half the dose of antigen pulsed in vitro of B1, B2 and B3 peptides for producing T cell activation. The increasing order of SI observed for different peptides was T1 > T2 > B3 > B1 > B2. When peptide-primed splenocytes were stimulated in vitro with F1 antigen, moderate stimulation was observed, which was marginally less as compared to above with both the formulations (Table 2). When T1-primed splenocytes were stimulated in vitro with different peptides, B1, B2 and B3 (50 μg ml⁻¹) showed SIs of 3.0–7.8 and 5.9–10.6 in soluble and particulate form, respectively. However, under identical conditions T1 and T2 peptides (25 μg ml⁻¹) showed SIs of 8.6–9.2 and 13.0–14.5 in medium and liposomes, respectively (data not shown). Interestingly, F1-primed splenocytes showed very high SIs (22.2 and 29.6) with homologous antigen (25 μg ml⁻¹), in soluble and particulate form, respectively (data not shown).

When conjugate-primed splenocytes were stimulated in vitro with the respective conjugate or constituent peptide or F1 antigen, high stimulation was observed (SI 10.1–16.9) in soluble form (Table 2). However, there was a marginal increase in SI (10.5–24.1) when these antigens were presented in particulate form. Notably, the constituent T cell peptides always showed a higher SI, irrespective of how they were presented, compared to constituent B cell peptides.

### 3.6. Cytokine levels

The culture supernatants obtained from spleen cells primed and stimulated with F1 antigen showed high levels of cytokines, i.e. IL-2 in soluble (318 pg ml⁻¹) and particulate form (355 pg ml⁻¹). In culture supernatants obtained from spleen cells primed with F1 antigen and pulsed in vitro with different peptides, IL-2 levels were maximal (225–300 pg ml⁻¹) for T1 and T2 peptides, while the other three peptides B1, B2 and B3 showed low IL-2 levels (115–185 pg ml⁻¹). Similarly, peptide B1-, B2- and B3-primed

### Table 3

| Primed with | Pulsed with | IFN-γ (pg ml⁻¹) | IL-2 (pg ml⁻¹) | IL-4 (pg ml⁻¹) |
|-------------|-------------|-----------------|----------------|----------------|
| B1 peptide  | B1 (M)      | 200             | 160            | 45             |
|             | B1 (L)      | 232             | 115            | 68             |
|             | F1 (M)      | 200             | 122            | 60             |
|             | F1 (L)      | 224             | 118            | 80             |
| B2 peptide  | B2 (M)      | 332             | 210            | 100            |
|             | B2 (L)      | 300             | 238            | 120            |
|             | F1 (M)      | 240             | 160            | 62             |
|             | F1 (L)      | 260             | 145            | 68             |
| B3 peptide  | B3 (M)      | 280             | 110            | 35             |
|             | B3 (L)      | 288             | 140            | 40             |
|             | F1 (M)      | 230             | 110            | 60             |
|             | F1 (L)      | 254             | 95             | 62             |
| T1 peptide  | T1 (M)      | 440             | 250            | 98             |
|             | T1 (L)      | 700             | 255            | 125            |
|             | F1 (M)      | 566             | 215            | 40             |
|             | F1 (L)      | 600             | 238            | 60             |
| T2 peptide  | T2 (M)      | 510             | 220            | 35             |
|             | T2 (L)      | 800             | 280            | 60             |
|             | F1 (M)      | 446             | 255            | 90             |
|             | F1 (L)      | 676             | 200            | 110            |
| F1 antigen  | F1 (M)      | 860             | 318            | 68             |
|             | F1 (L)      | 940             | 355            | 102            |
|             | B1 (M)      | 266             | 160            | 59             |
|             | B1 (L)      | 200             | 181            | 96             |
|             | B2 (M)      | 234             | 150            | 30             |
|             | B2 (L)      | 206             | 185            | 62             |
|             | B3 (M)      | 232             | 140            | 30             |
|             | B3 (L)      | 200             | 115            | 34             |
|             | T1 (M)      | 600             | 260            | 48             |
|             | T1 (L)      | 690             | 280            | 56             |
|             | T2 (M)      | 400             | 225            | 62             |
|             | T2 (L)      | 480             | 300            | 64             |
and stimulated cultures showed moderate IL-2 levels (110–238 pg ml\(^{-1}\)), while T1 and T2 peptide-primed cultures showed high IL-2 levels (250–255 and 220–280 pg ml\(^{-1}\), respectively). There was no significant difference between the two formulations, i.e. soluble versus liposome mode of antigen presentation (Table 3).

Conjugate-primed and -pulsed cultures showed undoubtedly higher IL-2 levels in all the formulations, which were statistically significant (\(P < 0.05\)) as compared to B1, B2 and B3 peptide-pulsed cultures. T1- and T2-pulsed cultures always showed higher IL-2 levels, with all the combinations. T1 peptide showed an edge over T2 peptide, in generating higher IL-2 levels. Interestingly, F1-primed and -pulsed cells showed higher IL-2 levels and these levels are comparable to T1- and T2-pulsed cultures (Table 4).

F1-primed and -pulsed spleen cells produced high levels of interferon-\(\gamma\) (IFN-\(\gamma\)) (860–940 pg ml\(^{-1}\)), irrespective of

| Primed with conjugates | Pulsed with | IFN-\(\gamma\) (pg ml\(^{-1}\)) | IL-2 (pg ml\(^{-1}\)) | IL-4 (pg ml\(^{-1}\)) |
|------------------------|------------|-------------------------------|-----------------------|----------------------|
| B1T1                   | B1T1 (M)   | 640                           | 170                   | 40                   |
|                        | B1T1 (L)   | 960                           | 280                   | 48                   |
|                        | F1 (M)     | 480                           | 120                   | 40                   |
|                        | F1 (L)     | 510                           | 140                   | 38                   |
|                        | B1 (M)     | 216                           | 128                   | 35                   |
|                        | B1 (L)     | 224                           | 140                   | 60                   |
|                        | T1 (M)     | 248                           | 240                   | 62                   |
|                        | T1 (L)     | 272                           | 200                   | 64                   |
| B1T2                   | B1T2 (M)   | 780                           | 305                   | 112                  |
|                        | B1T2 (L)   | 1100                          | 375                   | 108                  |
|                        | F1 (M)     | 470                           | 245                   | 70                   |
|                        | F1 (L)     | 560                           | 248                   | 76                   |
|                        | B1 (M)     | 248                           | 145                   | 62                   |
|                        | B1 (L)     | 224                           | 140                   | 64                   |
|                        | T2 (M)     | 460                           | 132                   | 56                   |
|                        | T2 (L)     | 414                           | 145                   | 54                   |
| B2T1                   | B2T1 (M)   | 600                           | 148                   | 68                   |
|                        | B2T1 (L)   | 890                           | 285                   | 66                   |
|                        | F1 (M)     | 500                           | 145                   | 102                  |
|                        | F1 (L)     | 580                           | 198                   | 108                  |
|                        | B2 (M)     | 202                           | 220                   | 55                   |
|                        | B2 (L)     | 250                           | 225                   | 40                   |
|                        | T1 (M)     | 264                           | 180                   | 45                   |
|                        | T1 (L)     | 280                           | 240                   | 95                   |
| B2T2                   | B2T2 (M)   | 800                           | 295                   | 62                   |
|                        | B2T2 (L)   | 1200                          | 355                   | 70                   |
|                        | F1 (M)     | 700                           | 220                   | 60                   |
|                        | F1 (L)     | 680                           | 215                   | 56                   |
|                        | B2 (M)     | 360                           | 210                   | 36                   |
|                        | B2 (L)     | 380                           | 205                   | 35                   |
|                        | T2 (M)     | 332                           | 155                   | 45                   |
|                        | T2 (L)     | 340                           | 280                   | 65                   |
| B3T1                   | B3T1 (M)   | 322                           | 255                   | 65                   |
|                        | B3T1 (L)   | 360                           | 280                   | 80                   |
|                        | F1 (M)     | 304                           | 160                   | 46                   |
|                        | F1 (L)     | 366                           | 240                   | 72                   |
|                        | B3 (M)     | 220                           | 240                   | 50                   |
|                        | B3 (L)     | 248                           | 205                   | 70                   |
|                        | T1 (M)     | 200                           | 138                   | 38                   |
|                        | T1 (L)     | 260                           | 230                   | 46                   |
| B3T2                   | B3T2 (M)   | 355                           | 195                   | 60                   |
|                        | B3T2 (L)   | 340                           | 205                   | 80                   |
|                        | F1 (M)     | 268                           | 180                   | 64                   |
|                        | F1 (L)     | 305                           | 200                   | 70                   |
|                        | B3 (M)     | 200                           | 138                   | 56                   |
|                        | B3 (L)     | 204                           | 155                   | 62                   |
|                        | T2 (M)     | 220                           | 138                   | 60                   |
|                        | T2 (L)     | 246                           | 130                   | 80                   |
the way the antigen was presented to the cells, while peptide B1-, B2- and B3-pulsed spleen cells produced low levels of IFN-\(\gamma\) (200–266 pg ml\(^{-1}\)), whereas T1- and T2-pulsed spleen cells produced maximal IFN-\(\gamma\) levels (400–690 pg ml\(^{-1}\)). The levels of IFN-\(\gamma\) were statistically higher (\(P<0.01\)) with T1 and T2 peptides as compared to B1, B2 and B3 peptides, with T1 peptide showing an edge over T2 peptide. In the case of individual peptide-primed and -pulsed spleen cells, T1 and T2 peptides showed significantly (\(P<0.01\)) higher IFN-\(\gamma\) levels in the particulate form of presentation (700–800 pg ml\(^{-1}\)) than in soluble form (440–510 pg ml\(^{-1}\)), while the other three peptides (B1, B2 and B3) produced low levels of IFN-\(\gamma\) (200–332 pg ml\(^{-1}\)) (Table 3).

With conjugate-primed and -pulsed spleen cells, comparatively higher IFN-\(\gamma\) levels (600–1200 pg ml\(^{-1}\)) were produced, while the particulate form of presentation still produced significantly higher (\(P<0.01\)) levels of IFN-\(\gamma\) as compared to the soluble form. Somehow, conjugates of B3 produced low levels of IFN-\(\gamma\) (322–360 pg ml\(^{-1}\)). Surprisingly, conjugate-primed and -pulsed spleen cells with individual constituent peptides (B1, B2, B3, T1 and T2) showed higher levels of IFN-\(\gamma\) for T1 and T2 peptides than B1, B2 and B3 (Table 4).

Low levels of IL-4 were observed for all the formulations. Spleen cells primed and pulsed with F1 antigen showed low levels of IL-4 (68–102 pg ml\(^{-1}\)), while peptide-pulsed cultures produced 30–96 pg ml\(^{-1}\) in both soluble and particulate form (Table 3). Peptide-primed and -stimulated spleen cells produced very low levels of IL-4 (35–125 pg ml\(^{-1}\)). Spleen cells primed with peptides and pulsed with F1 antigen also produced low levels of IL-4 (60–110 pg ml\(^{-1}\)) in any of the formulations used. Also in the case of conjugates, the spleen cells produced very low levels of IL-4 (40–112 pg ml\(^{-1}\)), either with conjugates or with constituent peptides (Table 4).

### 3.7. In vivo protective studies

Mice challenged with *Y. pestis* bacteria in alhydrogel survived until day 4. However, the groups immunized with BIT1 and the mixture of all conjugates showed high protection, comparable with the F1 antigen (Table 5), and the survival rate was nearly 80% on day 10.

### 4. Discussion

Human plague infection is associated with exposure to *Y. pestis*-infected rodents/lice or by inhalation of infectious aerosols. Even though live and killed vaccines are available to prevent plague, the reactogenicity, long immunization schedule, frequent boosters to maintain peak antibody levels and lack of demonstrable efficiency against pneumonic plague have limited their applicability for humans. Intensive efforts have been focused on the development of a subunit vaccine against plague, comprising two major virulence factors of *Y. pestis*, F1 and V antigen [12]. Several studies have shown that the protective immunity against plague is mediated in part by antibodies directed against the F1 antigen, which is a major envelope protein [13]. Thus, F1 antigen formed the basis of the new generation plague vaccine. Though a great deal of work has been done on native F1 antigen, peptide-based vaccines using the immunogenic potential of antigenic sites have not been reported. The present study is a step forward in this direction for generating humoral and cellular responses to B and T cell antigenic sites on F1 antigen in a murine system.

It is a generalized observation that peptide-based vaccines are weak immunogens and they undergo rapid degradation in the bloodstream. Therefore, these vaccines have to be supplemented with potent adjuvants through novel delivery vehicles. Thus, the liposomal mode of antigen delivery was the basis of the present study. Initially, the immunogenicity of F1 antigen was studied in four inbred strains of mice. All strains produced very high antibody titers after immunization with a low dose of antigen in liposomes, indicating that F1 is a highly immunogenic molecule. This observation encouraged us to study the

| Group          | Control | F1 | BIT1 | B1T2 | B2T1 | B2T2 | B3T1 | B3T2 | B4T1 | B4T2 | Conjugate mixture |
|----------------|---------|----|------|------|------|------|------|------|------|------|------------------|
| Day 1          | L D     | L D | L D  | L D  | L D  | L D  | L D  | L D  | L D  | L D  | L D              |
| Day 2          | 6 0 6 0 | 6 0 | 6 0  | 6 0  | 6 0  | 6 0  | 6 0  | 6 0  | 6 0  | 6 0  | 6 0              |
| Day 3          | 1 2 5 1 | 5 1 | 3 1  | 3 2  | 1 3  | 1 2  | 0 1  | 0 1  | 2 0  | 1 1  | 5 1              |
| Day 4          | 0 1 4 1 | 5 0 | 2 1  | 1 2  | 0 1  | 0 1  | 0 0  | 0 2  | 0 0  | 0 0  | 0 0              |
| Day 5          | 0 4 0 0 | 0 2 | 0 0  | 1    |    |    |    |    |    |    |                  |
| Day 6          | 0 4 0 | 4 1 |    |    |    |    |    |    |    |    |                  |
| Day 7-10       | 0 4 0 0 |    |    |    |    |    |    |    |    |    |                  |

Groups of six mice each were immunized with different B-T conjugates/mixture of B-T conjugates/F1 antigen. They were boosted and challenged with living *Y. pestis* on day 28. The survival time was monitored for all the groups for 10 days. L, live; D, dead.
immunogenicity of some of the predicted peptide sequences in mice with different genetic backgrounds, through immunization in liposomes and alhydrogel. Inbred strains are expected to give consistent results, because they lack the genetic variation that characterizes a normal outbred population. This implies that different haplotypes may present the same antigen differently and may subsequently produce a different immune response. When the immunogenicity of all the peptides was measured in terms of peak antibody titers, all were immunogenic, both in alhydrogel and in liposomes, and peptide B2 proved to be most immunogenic compared to the other peptides. This is in accordance with our earlier observation that B2 peptide is the immunodominant sequence of F1 antigen [6]. However, liposomal delivery of antigen proved more immunogenic than alum-based immunization in generating high affinity antibody levels and the levels persisted over a longer duration with half the immunizing dose. It appears that liposomal delivery of the antigen may be responsible for internalization, and endosomal transport of the peptides for stimulating high antibody levels and activating high affinity B cell clones. In order to maintain consistent peak antibody levels and memory response, the B cell and T cell determinants should be derived from the same protein antigen. Hence, we made a construct between putative B and T sequences and their immunogenicity was studied in both inbred and outbred strains of mice. The immunogenicity study indicated that all the B-T conjugates produced very high antibody levels by influencing B cell activation. The secondary response with the conjugates was better than with the individual peptides, even with alum immunization. This increased antibody level is possible, provided that the length of the B-T constructs containing the T helper sequence creates a conformation that is more efficiently processed and presented than that of the individual peptide. The antibody levels were quite comparable to those with the native F1 antigen. Interestingly, even the constituent peptides showed high antibody levels. This study clearly indicates that the chosen T1 and T2 sequences possess T-helper activity. To our surprise, the conjugates made between T1 peptide and B1, B2 and B3 always showed a higher immune response than the ones made with T2 peptide.

It is generally accepted that the level of antibodies, their subclass and avidity are important factors in preventing disease. F1 antigen entrapped in liposomes generated primarily IgG1 and IgG2b isotypes, followed by IgG2a in all the strains. Similarly, the delivery of individual peptides either in alhydrogel or in liposomes produced IgG1 and IgG2a or IgG2b isotypes in all the strains. The study clearly indicates the activation of both CD4+ TH1 and TH2 phenotypes that may be essential for the clearance of pathogens from the circulation. Again, both IgG2a and IgG2b isotype antibodies are known to be cytophilic in nature. The subclass distribution with conjugates again showed a mixed TH1/TH2 type response, in which both IgG1 and IgG2a or IgG2b isotypes were elevated. Though IgG1 isotype possesses lower protective capacity than IgG2a or IgG2b, in Yersinia infections increased levels of IgG1 isotype have been correlated with protection [14]. A similar isotype distribution as observed in the present study with peptides, peptide conjugates and native F1 antigen probably shows identical antigen-presenting cell (APC) processing pathway and cytokine secretion.

During the study on the immunogenicity of a physical mixture of all the peptides, again the B2 peptide proved more immunogenic than the other peptides. The study further proves that the B2 sequence appears to be highly accessible to the B lymphocytes and may be a surface motif on the F1 antigen. Overall, the study indicates that the predicted sequences are immunogenic with no genetic restriction of the immune response, and outbred strains show an equal or higher immune response as compared to inbred strains.

The affinity of the antibodies was studied for all the peptides in both delivery systems, using sera obtained from high and low responder strains. The K_D value is two- to four-fold lower with liposomal delivery as compared to alhydrogel delivery, indicating that the affinity of the antibodies in the former case is superior. This could explain the slow sensitization of high affinity B cell clones using the particulate mode (liposomes) of delivery of peptide antigens.

Cell-mediated immunity (CMI) is important for a plague vaccine, as it eliminates bacilli from intracellular sites (lymph nodes etc.). The ability of the peptides or peptide conjugates of F1 antigen to induce a CMI response was determined by assaying the splenocyte proliferative response and cytokine secretion in outbred mice. When F1 antigen/individual peptide-primed spleen cells were stimulated in vitro with native antigen or respective peptides, high stimulation was observed for T1 and T2 peptides, which showed lymphoproliferation similar to F1 antigen, thereby confirming that these predicted sequences are potential T cell epitopes. This is in accordance with the predictive algorithms of the F1 antigen, where these two regions possess folded amphipathic β structure. Similarly, when peptide-primed spleen cells were stimulated in vitro with F1 antigen or the respective peptide, high T cell proliferation was observed with T1 and T2 peptides, thus reconfirming these as T cell epitopes. Liposomal presentation of antigen in vitro with the primed spleen cells always produced high lymphoproliferation and required half the antigen dose compared with the soluble mode of presentation of the antigen. This observation is further supported by different conjugates showing, in the presence of T1 and T2 peptides, a lymphoproliferative response similar to that of F1 antigen. Also, the conjugate-primed spleen cells stimulated in vitro with conjugates showed higher SIs than the constituent peptides. This probably explains the generation and expansion of
high affinity T cell clones. Thus, the overall observation on humoral and cellular responses conclusively proves B1, B2 and B3 peptides to be B cell epitopes and T1 and T2 to be T cell epitopes.

T lymphocytes and their products are essential both in regulating specific antibody formation and in inducing antibody-independent immunity in various infections. On activation of T cells by the APCs and on interaction with the antigen, T cell subsets (i.e. TH1 and TH2) are activated which are reported to play a crucial role in infectious diseases. CD4+ T cells that produce predominantly TH1 cytokines are inflammatory T cells and potentiate CMI. The TH1 cytokines activate macrophages and natural killer cells through IL-2 help to expand and activate CD8+ T cells. When F1-primed/peptide-primed spleen cells were stimulated in vitro with the respective peptide or F1 antigen, high levels of IFN-γ were observed. T1 and T2 peptides produced significantly higher IFN-γ levels compared to B1, B2 and B3 peptides. The particular mode of peptide antigen presentation consistently produced high IFN-γ and IL-2 levels when compared to the soluble form of antigen presentation. The same holds for the peptide conjugates. This study clearly indicates that the predicted T cell epitopes are indeed T helper epitopes and are able to interact with a wide range of MHC molecules. Low levels of IL-4 were observed with all of the formulations regardless of the mode of antigen presentation to the primed spleen cells. Taking the cytokine data together, a more pronounced CD4+ TH1-driven response was observed, which was reflected in the generation of IgG2a/IgG2b antibodies. During the in vivo protection studies, mice immunized with F1, B1T1 and a mixture of conjugates showed higher protection and the animals survived for about 10 days. Surprisingly, the conjugates of either B2T1 or B2T2 did not show good protection in vivo. This may be attributed probably to (a) B1T1 showing a higher lympho proliferative response than B2T1 or B2T2, though the difference in humoral response was comparatively not visible, or (b) the fact that in the B1T1 conjugate the two sequences are proximal to each other and they might be positively selected, while in the B2T2 sequence the two sequences are overlapping with a sequence FFVRSIG and thus may be negatively selected, or, finally, (c) the fact that T1 peptide consistently showed higher proliferative and antibody responses than T2 peptide.

In conclusion, the study highlights the importance of peptide-based immunogens of F1 antigen as an alternative to conventional vaccines against plague.

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