**Pseudomonas aeruginosa** flagellin as an adjuvant: superiority of a conjugated form of flagellin versus a mixture with a human immunodeficiency virus type 1 vaccine candidate in the induction of immune responses

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In the present study, the adjuvant activity of flagellin was compared, in the conjugated and mixed forms, against a peptide vaccine from human immunodeficiency virus type 1 (HIV-1) p24–Nef vaccine candidate. Mice were immunized with the HIV-1 p24–Nef peptide with flagellin in both conjugated and mixed forms. Lymphocyte proliferation, CTL activity, and IL-4 and IFN-\(\gamma\) cytokines were evaluated by bromodeoxyuridine, carboxyfluorescein succinimidyl ester and ELISA methods, respectively. At the same time, the frequency of IFN-\(\gamma\)-producing T-lymphocytes, as well as total and isotype-specific antibodies, were assessed by ELISPOT and indirect ELISA, respectively. Our experimental results showed that the immunized mice with the HIV-1 p24–Nef conjugated or mixed forms of flagellin led to increases in the proliferative responses and Th1 cytokine pattern. The conjugated form of vaccine led to increased CTL activity and a Th1 cytokine pattern of immune responses, as well as an IgM isotype of humoral responses in comparison with the mixed form. The flagellin-conjugated vaccine seems to be more potent in increasing vaccine immunogenicity.

**INTRODUCTION**

In recent decades, human immunodeficiency virus (HIV) infection has been considered to be one of the most important risk factors threatening global health. In this light, an effective candidate vaccine against HIV-1 is highly demanded. It should be noted that a suitable adjuvant is a critical component of a vaccine to induce protective immune responses (Parker, 2002). A main limitation of
current vaccines against HIV-1 is the inability to induce protective immune responses against virus infection (Mahdavi et al., 2009; Parker, 2002). The use of immunological adjuvants is considered to be one possible strategy to increase vaccine immunogenicity and its protective effects. Adjuvants, originally defined by Ramon (1924), are able to dramatically increase the immunogenicity and potency of candidate vaccines (Perez et al., 2013). Adjuvants include a wide spectrum of substances that increase immune responses against vaccines (Azmi et al., 2013; Fakharzadeh et al., 2013). Therefore, the development of new adjuvants or adjuvant–immunotherapeutic approaches may lead to the generation of more immunogenic vaccines. One of the most important mechanisms of action of adjuvants is maturation and activation of antigen-presenting cells such as dendritic cells (DCs) through specific receptors like Toll-like receptors (TLRs) (Schmid et al., 2011; Hou et al., 2008). Not only can DCs and other antigen-presenting cells be activated by a variety of TLR signalling pathways, but DCs, when activated by TLRs, also promote the production of inflammatory cytokines, upregulate co-stimulatory molecules (CD80, CD86 and CD40) and induce the expression of MHC class II molecules (Hou et al., 2008). These changes help DCs process and present antigens more efficiently, stimulating immune responses effectively (Toyota-Hanatani et al., 2008). In addition, recent studies have indicated that flagellin (FlIC) is recognized as a pathogen-associated molecular pattern (PAMP) and, as a carrier protein or adjuvant, it is able to increase immune responses to the main antigen, which may be a very small protein or peptide (Qian et al., 2015; Bates et al., 2011). Because of its high immunogenicity and protective effects, bacterial flagellin can be used as a novel candidate for vaccine design (Faezi et al., 2014). Several studies have shown the adjuvant activity of the FlIC molecule in combination with various vaccine models (Asadi Karam et al., 2013; Girard et al., 2014; Lee et al., 2006; Mizel and Bates, 2010; Yin et al., 2013). However, key questions remain such as how the FlIC molecule should be used in order to maximize its adjuvant activity. Should we physically conjugate the FlIC molecule to the vaccine or would mixing it with the vaccine be sufficient to enable its adjuvant activity? The development of new approaches in the application of adjuvants (e.g. using the adjuvant in the mixed form or linking it to a vaccine) may lead to a more immunogenic vaccine. Therefore, in the present study, we aimed to evaluate the adjuvant properties (mixed or conjugated form) of FlIC with an HIV-1 peptide vaccine model by measuring the cellular and humoral immunological parameters.

**METHODS**

**Mice.** Inbred female BALB/c mice (6–8 weeks old) were purchased from the Pasteur Institute (Karaj, Iran). The mice were housed for 1 week before the experiments, given free access to food and water, and maintained in a light/dark cycle with lighting (12/12 h) at 20–22 °C. All mouse experiments were carried out in accordance with the Animal Care and Use Protocol of the Pasteur Institute of Iran.

**HIV-1 p24–Nef fusion peptide.** The HIV-1 p24–Nef fusion peptide from the p24 (aa 159–173) and Nef (aa 102–117) proteins was synthesized according to the solid-phase method by GL Biochem Company (Shanghai, China). The peptide was purified using high-performance liquid chromatography to obtain a peptide with a purity exceeding 95% for experiments.

**Production and purification of recombinant flagellin.** In a previous study, the type A flagellin (Fla-A) gene was cloned and expressed as a His-tagged protein in a bacterial expression system (Faezi et al., 2014). Briefly, the *Pseudomonas aeruginosa* type A flagellin gene (*flIC*) was first cloned into the expression vector pET-28a and then expressed in *Escherichia coli* BL21(DE3) and finally purified through Ni2+ affinity chromatography.

**Conjugation procedure.** For covalent conjugation of the recombinant FlIC to HIV-1 p24–Nef peptide based on the previous studies, a standard protocol was followed (Gauthier & Klok, 2008; Lateef et al., 2007). Briefly, in a separate container, 1 mmole FlIC was reacted with an excess amount of acetic anhydride as a carboxyl protecting group agent for at least 1 h. The protected peptide was separated using a dialysis bag to remove any impurities. In another container, 1 mmole HIV1 p24–Nef was activated by excess amounts of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and 10 mg N-hydroxysulfosuccinamide in PBS and the reaction was stirred for at least 10 min. The protected FlIC was then gently added dropwise into the activated HIV-1 p24–Nef container and the reaction was mixed for 48 h. The fidelity of conjugation and the finishing time for the reaction completion were estimated by TLC. The conjugate was purified using dialysis, as well as by Sephadex G-75 column chromatography (data not shown).

**Vaccine preparation.** The conjugated and mixed forms of HIV-1 p24–Nef/FlIC and p24–Nef peptide alone were prepared in Montanide ISA70. Briefly, all of the candidate vaccines were mixed with Montanide ISA70 at a ratio of 3 : 7 and vortexed vigorously for 1 h. After vaccine preparation, 100 μl mixture containing 20 μg vaccine candidate was used for immunization.

**Experimental groups and immunization schedule.** The mice were divided into five groups containing 12 mice in each. Mice in the first three groups were immunized intradermally on day 0 with 100 μl HIV-1 p24–Nef/FlIC conjugate formulated in Montanide ISA70, a mixture of p24–Nef and FlIC formulated in Montanide ISA70, or p24–Nef peptide formulated in Montanide ISA70, respectively. The two control groups were injected with Montanide ISA70 or PBS on day 0 by the same route of immunization. All experimental groups were boosted on days 21 and 42 after immunization under the same conditions.

**Lymphocyte proliferation assay.** Three weeks after the third immunization, the spleens of experimental mice (*n*=6) were removed under sterile conditions and suspended in sterile cold PBS containing 2% FBS. Red blood cells were lysed with lysis buffer and the cell suspension was adjusted to 2 × 10^6^ cells ml^-1^ in RPMI 1640 (Gibco) supplemented with 10% FBS, 4 mM l-glutamine, 1 mM sodium pyrovate, 50 μM 2-mercaptoethanol, 100 μg streptomycin ml^-1^ and 100 IU penicillin ml^-1^. Cell suspension (100 μl) was dispensed into 96-well, flat-bottomed culture plates (Nunc) and stimulated with 10 μg fusion peptide ml^-1^. Phytohaemagglutinin A (5 μg ml^-1^; Gibco), unstimulated wells and culture medium were used as positive controls, negative controls and blanks, respectively. All experiments were done in triplicate. After 72 h of cell culture, 20 μl bromeo-deoxyuridine (BrDU; Roche) was added to each well and the plates were further incubated at 37 °C for 18 h. After incubation, the plates were centrifuged at 300 g for 10 min, the supernatant was carefully aspirated, the plates were dried and 200 μl fixation/denaturation buffer
was added to each well for 30 min. The plates were aspirated and 100 μl anti-BrdU–HRP conjugate as a secondary antibody was added and incubated for 2 h. The plates were then washed five times with PBS, and tetramethylbenzidine substrate was added to the wells and incubated for 5 min in the dark at room temperature. The reaction was stopped by adding 100 μl 1 M H₂SO₄. The absorbance of each well at 450 nm (A₄₅₀) was determined. The absorbance of blank wells was subtracted from all other wells, and the stimulation index (SI) was calculated according to the following formula:

\[ SI = \frac{A_{\text{stim}}}{A_{\text{unstim}}} \]

In vivo CTL assay. To detect the cytotoxicity of the candidate vaccine, a carboxylfluorescein succinimidyl ester (CFSE; Invitrogen) method was used (Nakagawa et al., 2011). Splenocytes from naïve mice were stained with 5 μM (high intensity) or 0.5 μM (low intensity) CFSE. The high-intensity CFSE-labelled cells were loaded with 20 μg relevant peptide ml⁻¹, and the low-intensity CFSE-stained cells (0.5 μM) were used as a peptide-unpulsed control. The cells were then transferred intravenously (3 × 10⁵ cells of each) into the experimental groups of mice. Twenty hours later, lymphocytes were isolated from the spleen and the cell suspension was washed and resuspended in cold PBS. The red blood cells were lysed and flow cytometry analysis was carried out to demonstrate the difference in separation patterns based on the intensity of CFSE staining. The percentage of killing of the high- and low-intensity CFSE-stained cells was calculated and the specific killing of HIV-1 p24–Nef-pulsed (CFSEhigh) target cells was calculated as follows:

\[ \text{Specific killing} (\%) = 1 - \left(\frac{[\text{CFSE}^{\text{high}}/\text{CFSE}^{\text{low}}]}{[\text{CFSE}^{\text{high}}/\text{CFSE}^{\text{low}}]} \right) \times 100 \]

ELISA of cytokines. Three weeks after the second booster, a total of 4 × 10⁶ spleen cells were placed in each well of a 24-well plate in complete RPMI 1640, stimulated in vitro with 10 μg p24–Nef fusion peptide ml⁻¹ and incubated at 37 °C in 5% CO₂; in the other wells, unstimulated samples were prepared. For individual mice, three wells were considered as stimulated cells and three wells as unstimulated cells. Three days after antigen recall, the supernatants were removed and the concentration of IFN-γ and IL-4 was estimated using an ELISA kit (Quantikine; R&D Systems) according to the manufacturer’s instructions. The concentration of each sample (pg ml⁻¹) was calculated according to a standard curve and the absolute cytokine production of each mouse was calculated after subtraction of the value of the unstimulated well from the stimulated well. To calculate the IFN-γ: IL-4 ratio, the quantity of IFN-γ in each mouse was divided by that of IL-4 and the ratio of IFN-γ: IL-4 was reported as a cytokine pattern.

IFN-γ ELISPOT assay. The frequency of IFN-γ-producing splenocytes was analysed using an ELISPOT assay (Mabtech). Briefly, a total of 4 × 10⁵ spleen cells were placed in each well of a 96-well microplate and in vitro stimulation was carried out with 10 μg p24–Nef peptide ml⁻¹. Phytohaemagglutinin was added to some wells as a positive control for stimulated cells, and wells containing unstimulated cells and RPMI 1640 were used as negative controls. The plates were incubated at 37 °C in 5% CO₂ for 24 h. After in vitro restimulation, the plates were washed five times with washing buffer. Next, 100 μl anti-mouse IFN-γ in PBS containing 0.5% FBS was added to the wells and the plates were incubated for 2 h at room temperature. The plates were washed five times with washing buffer and incubated for 1 h at room temperature with 100 μl 1:1000-diluted streptavidin–alkaline phosphatase conjugate. After a final wash, the spots were developed by adding 100 μl BCIP/nitro-blue tetrazolium substrate to the wells and incubating for 30 min at room temperature in the dark. The plates were rinsed three times with distilled water and dried at 4 °C. The spots were counted using a stereomicroscope (Nikon). The number of specific IFN-γ-producing lymphocytes was calculated by subtracting the number of spots in unstimulated wells from those in the stimulated wells.

ELISA of antibodies and their isotypes. Specific antibodies were determined using an optimized indirect ELISA method. Briefly, 100 μl p24–Nef fusion peptide (10 μg ml⁻¹ in PBS) was added to 96-well ELISA V-bottom plates (Nunc) and incubated for 24 h at 37 °C. The wells washed with PBS containing 0.05% Tween 20 (washing buffer) and blocked for 1 h at 37 °C with 5% skimmed milk in PBS (blocking buffer). The plates were washed with washing buffer and 100 μl 1:100-diluted serum was added to each well and incubated at 37 °C for 2 h. The wells were washed five times with washing buffer and incubated for 2 h with 100 μl 1:7000 dilution of anti-mouse conjugated to HRP (Sigma). The wells were washed five times and incubated for 30 min with 100 μl tetramethylbenzidine substrate in the dark. The reaction was stopped with 1 M H₂SO₄ and the absorbance was measured at A₄₅₀ using an ELISA plate reader. Detection of specific IgG1, IgG2a, IgG2b, IgG3 and IgM subclasses was carried out using goat anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM secondary antibodies (Sigma) according to the manufacturer’s instructions.

Statistical analysis. The number of mice in each experiment was four to seven for all groups. All experiments were carried out in triplicate and the data are expressed as means ± SD for each experiment. All statistical analyses were carried out using a Mann–Whitney U test. In all cases, P < 0.05 was considered to be statistically significant.

RESULTS

Lymphocyte proliferation

Evaluation of lymphocyte proliferation in the experimental groups was carried out with a BrdUb/ELISA-based method. Antigen recall was done for 3 days and then lymphoproliferative activity was reported as the SI of individual mice. All vaccine-immunized groups showed significant differences when compared with the control groups (P ≤ 0.009) (Fig. 1). Immunization of mice with p24–Nef/FliC in both mixed and conjugated forms significantly increased lymphocyte proliferation in comparison with the p24–Nef-immunized group (P = 0.016 and P = 0.009, respectively). Immunization of mice with the p24–Nef/FliC conjugate showed significantly increased lymphocyte proliferation in comparison with the mixed form (P = 0.021). There was no significant difference between the control groups (P > 0.05).

Cytotoxicity assay

The cytotoxicity activity of the experimental groups was analysed by using an in vivo CTL assay method. Fig. 2 shows that all vaccine-immunized groups (p24–Nef/FliC conjugate and mixed forms and p24–Nef immunized groups) led to induce CTL activity as compared with the adjuvant (Montanide-70) and PBS control groups (P ≤ 0.014). Immunization of mice with p24–Nef/FliC in both conjugated and mixed forms showed increased CTL activity in comparison with the p24–Nef-immunized group (P = 0.004 and P = 0.174, respectively). Immunization of mice with conjugated p24–Nef/FliC resulted in increased CTL activity at a border line level (P = 0.068) in comparison with the mixed form. There was no significant difference between the control groups (P > 0.05).
Determination of the IFN-γ : IL-4 ratio in the experimental groups showed that all vaccine-immunized groups (conjugated and mixed form of p24–Nef/FliC and p24–Nef) increased their IFN-γ : IL-4 ratio in comparison with the Montanide-70 and PBS control groups (P<0.016). Immunization of mice with p24–Nef/FliC in both conjugated and mixed forms was found to significantly increase the IFN-γ : IL-4 ratio in comparison with the p24–Nef-immunized group (P<0.009). Immunization of mice with the p24–Nef/FliC conjugate form in comparison with the mixed one significantly increased the IFN-γ : IL-4 ratio (P<0.028).

There was no significant difference between the control groups (Fig. 3).

**IFN-γ ELISPOT assay**

The frequency of IFN-γ -secreting lymphocytes was evaluated using an ELISPOT assay (Fig. 4). The results showed that all vaccine-immunized groups (p24–Nef/FliC conjugate and mixed forms and p24–Nef) were able to significantly increase the number of IFN-γ -secreting lymphocytes in comparison with the control groups (P<0.006). Immunization of mice with the p24–Nef/FliC conjugated form significantly increased the number of IFN-γ -secreting lymphocytes in comparison with the p24–Nef-immunized group (P<0.01). However, immunization of mice with p24–Nef/FliC in the mixed form did not increase the number of IFN-γ -secreting lymphocytes in comparison with the p24–Nef-immunized group (P=0.927). Immunization of mice with the p24–Nef/FliC conjugate in comparison with the mixed form increased the number of IFN-γ -secreting lymphocytes (P=0.076). There was no significant difference between the control groups (P>0.05).

**Total antibodies**

The results of total antibody showed that all vaccine-immunized groups (p24–Nef/FliC conjugate and mixed forms and the p24–Nef immunized group) significantly increased total antibodies in comparison with the control groups (P<0.011) (Fig. 5). Immunization of mice with the p24–Nef/FliC conjugate and mixed forms did not increase the total antibodies in comparison with the p24–Nef-immunized group (P=0.378 and P=0.338, respectively). Immunization of mice with the p24–Nef/FliC conjugate in comparison with the mixed form did not increase the total antibodies (P=1). There was no significant difference between the control groups (P>0.05).
body was evaluated using an indirect ELISA method. Experimental mice were immunized with vaccines and total anti-gated vaccine resulted in significantly increased IgM titres in comparison with the candidate vaccine (Kaiser & Akira, 2002). Flagellin proteins are able to stimulate TLR5 signalling and to reinforce the immune response against vaccines (Mizel & Bates, 2010). Flagellin causes the maturation of DCs by increasing the production of TNF-α, as well as the reinforcement of innate and acquired immune responses by inducing the production of IFNs (Arimilli et al., 2008). In this study, the adjuvant activity of the flagellin molecule was evaluated in a peptide vaccine model in conjugated and mixed forms. In the conjugated form, the flagellin molecule was joined to the HIV-1 p24–Nef candidate vaccine using a chemical method to produce a conjugated form of the vaccine. In the mixed form, flagellin was mixed with HIV-1 p24–Nef protein. The adjuvant activity of flagellin was then compared for these two forms.

The results of lymphocyte proliferation assays showed that immunization with the HIV-1 p24–Nef candidate vaccine significantly increased lymphocyte proliferation compared with the control groups, and utilization of FljC either in the mixture or conjugated to the vaccine caused a significant increase in lymphocyte proliferation compared with the vaccine-only group. In addition, the application of FljC in a conjugated form caused a significant increase in lymphocyte proliferation compared with the mixed form. Lymphocyte proliferation is a criterion of the cellular immune response (Mahdavi et al., 2011) and our findings suggest that FljC could stimulate a cellular immune response. Our previous study on the FimH antigen of uro-pathogenic E. coli showed that FljC as an adjuvant increased the lymphocyte proliferation response against the FimH antigen (Asadi Karam et al., 2013).

The cytotoxic activity of CD8+ T-lymphocytes was evaluated as another criterion for cellular immune responses. The results showed that the vaccine candidate when either mixed with or conjugated to FljC was more effective in induction of CTL activity in comparison with the vaccine-only group. However, injection of the conjugated form of the vaccine induced a higher level of cytotoxic activity in comparison with the mixture.

In a study on a cancer vaccine model, it was shown that immunization with an E6/E7 candidate vaccine in combination with flagellin as a TLR5 agonist induced CTL activity and suppressed tumour growth, and increased the survival rate of cancer-bearing mice. The authors suggested that stimulation of TLR5 has an important role in the induction of CTL activity against tumours (Nguyen et al., 2013a). It was also shown in their next study that flagellin improved CTL activity against E6/E7 vaccine in a therapeutic cancer vaccine model (Nguyen et al., 2013b). Another study showed that flagellin is able to improve CTL activity and that it induced an ovalbumin-specific CTL response (Braga et al., 2008). These findings confirm our results in the induction of a CTL response in utilization of FljC as an adjuvant.

In order to analyse the cytokine pattern, IFN-γ and IL-4 cytokines were evaluated. The results indicated that the
conjugated and mixed forms of the vaccine with FliC caused a significant increase in the ratio of IFN-γ : IL-4 compared with the vaccine-only group. However, the conjugated form of the vaccine also caused a significant increase in the ratio of IFN-γ : IL-4 versus the mixed form. Thus, the conjugated form of the vaccine with FliC was more potent than the mixture in the induction of a Th1 pattern in the immune response.

Many studies have demonstrated that flagellin can polarize immune response towards a Th1 pattern. The study by Bargieri et al. (2010) on a 19 kDa *Plasmodium falciparum* merozoite surface protein-1 (MSP119) showed that immunization with the flagellin-conjugated form induced a higher level of IFN-γ cytokine compared with the vaccine-only group (Bargieri et al., 2010). In another study, immunization with an FimH vaccine in combination with flagellin

**Fig. 6.** Specific IgG1, IgG2a, IgG2b, IgG3 and IgM isotypes in the experimental groups. Results are shown as means ± SD for IgG1 (a), IgG2a (b), IgG2b (c), IgG3 (d) and IgM (e) for the experimental groups.
increased the IFN-γ level in comparison with the vaccine alone (Asadi Karam et al., 2013). However, in the study on MSP109, it was shown that immunization with the flagellin-conjugated form of MSP109 was more effective than with a mixture in the induction of a Th1 pattern (Bargieri et al., 2010). These results confirm our findings in the induction of a Th1 pattern using P. aeruginosa flagellin as an adjuvant. Additionally, the frequency of IFN-γ-producing lymphocytes was analysed and, due to in vitro short-term exposure to the antigen, the response was related to the function of the memory effector cells (Calarota et al., 2008; Li et al., 2008; Mahdavi et al., 2011). The results of the IFN-γ ELISPOT as a memory response showed that both the mixture and the conjugated forms of the vaccine successfully increased the number of IFN-γ-producing lymphocytes compared with the vaccine-only group, and the conjugated form acted more effectively than the mixture in the induction of memory IFN-γ-producing T-lymphocytes.

The study by Caron et al. (2005) on TLR-5 agonists showed that effector memory CD4+ T-cells are very sensitive to TLR5 stimulation, such that after stimulation with flagellin they suddenly proliferate and produce IFN-γ (Caron et al., 2005). DCs are important in the induction of memory T-cells, and strong induction of DCs results in more efficient memory T-cell formation, and thus it is likely that TLR-5 engagement via FliC on DCs results in better memory formation (Thibault et al., 2009; Vicente-Suarez et al., 2009).

The results of the antibody assay indicated that the vaccine candidate in all three forms – mixed, conjugated and alone – significantly induced specific antibodies compared with the control groups, and the FliC adjuvant – either conjugated or as a mixture – had only a mild effect on antibody responses in comparison with the vaccine-only group and increased only specific IgM in comparison with the vaccine-only group. In the study by Honko et al. (2006), it was demonstrated that flagellin is a highly effective adjuvant and promotes a strong humoral immune response against Yersinia pestis antigens in both murine and non-human primate models, which finally lead to a protective response following challenge. They also found that flagellin may be an efficacious adjuvant for use in humans. However, many studies have shown that FliC as an adjuvant can increase specific antibodies in combination with various vaccines (Eom et al., 2013; Karam et al., 2013; Lee et al., 2006), although we did not achieve this effect. In fact, our results with respect to antibody responses showed a weak effect of FliC on the total antibodies and only an increase in the IgM isotype was observed. This may be due to the nature of our vaccine candidate. In fact, our vaccine candidate was basically CTL based, and there was no ideal B-cell epitope in its backbone (Mahdavi et al., 2010). However, other parameters, including the method of injection, vaccine dose and other factors, may be implicated in the achievement of such results.

Over all, the present study provides some immunological evidence that the behaviour of the FliC molecule as an adjuvant varies according to the mode of utilization in the vaccine formulation. However, the results of this study showed that conjugation of flagellin to a vaccine resulted in better adjuvant activity than when the flagellin was simply mixed with the vaccine. More studies are needed in order to clarify other aspects of these immune responses.

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