



Cationic nanoglycolipidic particles as vector and adjuvant for the study of the immunogenicity of SIV Nef protein

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ABSTRACT

The objective of this study was to test the immunogenicity of SIV Nef protein formulated in cationic nanoglycolipidic particles of 100 nm of diameter. In parallel, the adjuvant effect of these nanoglycolipidic particles was compared in similar experiments using GST-Nef in association with the commonly strongest used complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant in association with MDP or MDP alone. Our results showed that these particles do not alter the integrity of our immunogen GST-Nef, which remains stable for more than three months at 4 °C. We demonstrated that in the presence of nanoglycolipidic particles antibodies against Nef were produced since the first injection and remained stable after the third injection with high titers for long lasting periods as observed with CFA and IFA/MDP adjuvant. The analysis of immunoglobulin isotype profiles of antibodies generated by the different protocols of immunization showed the preponderance of IgG1 isotypes suggesting the predominance of Th2-type immune response.

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1. Introduction

All trials to develop an efficient vaccine against HIV have failed. The assay using soluble envelope glycoprotein gp120 (AIDS VAX gp120) allowed production of antibodies which are unable to protect human from HIV-1 infection (Pitisuttithum et al., 2006). Similarly the vaccination with recombinant adenoviruses, serotype 5, expressing Gag, Pol and Nef proteins, proved unable to control efficiently HIV-1 infection despite the production of CD8 cytotoxic immune responses (Buchbinder et al., 2008; McElrath et al., 2008). However, it is interesting to note that in a recent assay a small, but significative protection was observed for the first time in the vaccinated group when compared with the control. In this assay performed in Thailand volunteers were primo-immunized with canary pox viral vector encoding for gp120, Gag and Pol and then boosted with soluble gp120 (Rerks-Ngarm et al., 2009). Taking into account these data, the success of future vaccines will depend on the development of new immunogens, vectors and adjuvant. To this end, a deeper understanding of the immunogenicity of every viral protein, associated with various vectors and adjuvant, becomes essential.

To this end, we analyzed in this study the immunogenicity of SIV Nef protein. The *nef* gene is conserved among primate lentiviruses.

It is among the first viral gene expressed after HIV infection. This suggests a critical role for *nef* gene product in the viral cycle and therefore in the pathogenesis of HIV and simian immunodeficiency virus (SIV) infections. The *nef* gene product of HIV-1 and SIV is a cytoplasmic regulatory protein with a molecular weight of 25–27 kDa (Franchini et al., 1986; Kaminchik et al., 1994). In addition, Nef enhances viral replication in primary peripheral blood mononuclear cells *in vitro* (Baba et al., 1999; Foster and Garcia, 2008; Jere et al., 2010; Miller et al., 1994), a property shown to be a reflection of enhanced infectivity of the HIV-1 *in vivo* (Chowers et al., 1994). Nef also induces a dramatic down regulation of CD4 and MHC-I molecules from the cell (Foster and Garcia, 2008; Garcia and Miller, 1991; Guy et al., 1987; Noviello et al., 2008). This effect on CD4 expression has been proposed as a mechanism of prevention for cell super-infection and also as a viral escape mechanism (Benson et al., 1993). Various reports provide evidence that Nef alters signal transduction pathways and induces a lack of cell responsiveness when surface receptors are stimulated (Baur et al., 1994; Collette et al., 1996; De and Marsh, 1994; Fackler et al., 2007; Rudolph et al., 2009; Stolp et al., 2009). It has also been reported that Nef protein is expressed on the surface of infected human T cells and can interact with uninfected T cells via its carboxy terminal region (Otake et al., 1994; Raymond et al., 2011). However, Nef can also suppress T cells growth and thus be involved in the selective depletion of CD4+ cells in HIV infection (Daniel et al., 1992).

Moreover, Nef protein has been shown to be required for SIV pathogenicity *in vivo* (Baba et al., 1999; Kestler et al., 1991). Indeed, adult rhesus macaques, infected with SIV bearing a large deletion in *nef* gene, exhibit low viral loads and do not develop AIDS disease

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(Chakrabarti et al., 1995; Kestler et al., 1991). In addition, it has been shown in the SIV model that vaccine induced Nef specific cytotoxic T lymphocytes (CTL) can control early replication after challenge of macaques by pathogenic SIV (Sinclair et al., 1997). This Nef mediated protection is related to its capacity to down regulate MHC-I expression on HIV-1 infected cells. It is also interesting to note that anti-Nef specific CTL were detected in uninfected children born from HIV infected mothers (Collins et al., 1998). Moreover, studies of long-term survivors of HIV-1 infection also indicate that Nef can be an important determinant for clinical outcome (Greene et al., 2010). In several long-term nonprogressors, the efficient control of HIV-1 infection seems to be associated with deletion in *nef* gene (Deacon et al., 1995) or with an unusual high frequency of defective alleles and functionally defective Nef protein (Mariani et al., 1996).

Thus, Nef protein appears to be an effective candidate for an HIV vaccine since it is produced early during the viral life cycle. Targeting the immune responses against Nef could aid in preventing HIV-1 infection and in controlling the viral load by blocking the first steps of viral cycle. However, it is important to generate, in addition to cellular immune response, a specific humoral immune response against Nef that can lead to the induction of anti-Nef antibodies. Those antibodies could then block membrane and extracellular activities of Nef.

The major problems in using subunit vaccines, such as a Nef vaccine, are that purified proteins are generally poorly immunogenic and alum is the single adjuvant currently approved for humans. Thus, other types of adjuvant, such as liposomes, virosomes, ISCOM, TLR ligands, cytokines and nanoparticles are under investigation. In the present work we tested the capacity of cationic nanoglycolipidic particles (NP) to enhance the antibody response against SIV GST-Nef protein.

These latter NP vectors constitute a new family of biodegradable nanoparticles. They are capable of associating with different types of immunogens. Indeed, the composition of the NP can be modified to optimize vector-immunogen association (De Miguel et al., 2000; Major et al., 1997; von Hoegen, 2001). Accordingly, several studies have used the NP as vectors associated with different viral immunogens including those of rabies (Castignolles et al., 1996), hepatitis B (Debin et al., 2002), Influenza (von Hoegen, 2001) and CMV viruses (Priour et al., 1996). In addition to their function as vectors, these NP possess an intrinsic adjuvant effect and are compatible with both systemic and mucosal administrations (Debin et al., 2002).

In the present work we tested the capacity of NP cationic nanoglycolipidic particles to enhance the antibody response against SIV GST-Nef protein. This response will be compared with those obtained in the presence of the commonly strongest adjuvant (complete Freund's adjuvant, incomplete Freund adjuvant in association with MDP or MDP alone).

2. Material and methods

2.1. Construction, production, expression and purification of glutathione-S-transferase-Nef fusion protein (GST-Nef_{SIV mac 251})

The plasmid expressing *gst-nef_{SIV mac 251}* was constructed and produced as previously described for GST-Nef_{HIV-1LAI} (Cazeaux et al., 2002; Moureau et al., 2002) and used for transformation in competent DH5 α cells. Briefly, transformed *E. coli* DH5 α cells were grown in LB complete medium and centrifuged after 5 h induction in isopropyl β -D-1-galactopyranoside (IPTG). After GST-Nef protein solubilization from the bacterial lysate, a one step purification of GST-Nef protein was conducted by affinity chromatography on glutathione agarose (Sigma Chemical Co. – USA). Purification of glutathione S-transferase (GST) protein was expressed in DH5 α cell

transformed with pGEX-2T vector and solubilization of the protein was performed as described for GST-Nef.

2.2. Nanoglycolipidic particles (NP) and formulation of GST-Nef

The nanoglycolipidic particles were prepared from the maltodextrin as described previously (Major et al., 1997). These cationic particles were composed of polysaccharide (77%) and a mixture of DPPC and cholesterol (70:30, w/w). Formulation of GST-Nef was performed by simple mixing the protein and the nanoglycolipidic particles in a ratio of 1/40 (w/w).

2.3. Immunogens

GST-Nef was formulated with cationic nanoglycolipid particles of about 100 nm in diameter or were mixed with: (i) complete Freund's adjuvant (CFA), (ii) muramyl dipeptide (MDP), (iii) association of incomplete Freund's adjuvant (IFA) and MDP.

2.4. Immunization of mice

Female BALB/c mice (H-2d) were obtained from IFFA Credo (France) and kept in standard housing with rodent chow and water available *ad libitum*, on a 12 h light/dark cycle. Experiments were performed in compliance with French and European regulations on animal experimentation and were approved by the local animal experiments ethical committee. BALB/c mice were merged into four groups of five. Six to eight weeks old mice were used for subcutaneous immunizations. Five mice were immunized with 10 μ g of recombinant GST-Nef containing nanoglycolipidic particles (200 μ L) or with GST-Nef in association with Complete Freund's Adjuvant or Muramyl Dipeptide or with Complete Freund's adjuvant associated with Muramyl Dipeptide. Injections were repeated subcutaneously on each 3 weeks interval with the same antigen doses.

2.5. Samples

Animals were bled for serum at day 0, 14, 35, 58 and 98. More precisely sera were obtained from blood samples collected by retro-orbital punctures 2 weeks after each injection.

2.6. Enzyme linked immune sorbent assay: ELISA

Serological responses to measure anti-Nef antibodies titers were performed by an end-point enzyme linked immunosorbent assay (ELISA). Maxisorb microtiter wells (Nunc, Denmark) were coated either with 50 μ L of recombinant GST-Nef or with GST at 10 μ g/mL in phosphate buffered saline (PBS) pH7.4 for 2 h at 37 °C. Wells were blocked with 200 μ L of PBS supplemented with 5% milk for 1 h at 37 °C and then washed with PBS containing 0.2% Tween20. Wells were then incubated with 50 μ L of various sera dilutions. Sera were diluted in PBS with 0.5% milk and 0.05% Tween20 and were incubated for 2 h at 37 °C. After extensive washing, absorbed antibodies were detected with 50 μ L of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako S.A., France). After one hour of incubation followed by washing, 100 μ L of O-phenylenediamine dihydrochloride (OPD) (Sigma, France) dissolved in phosphate citrate buffer, pH5, activated with H₂O₂ were added as a substrate. Colour development was stopped after 20 minutes by adding 30 μ L of 4 N H₂SO₄ and the 490/600 nm absorbance ratio was measured. Antibody titers were expressed as the reciprocal of the final dilution which gave an absorbance ratio of 0.2.

For the determination of the levels of GST-Nef specific IgG1 and IgG2a responses, peroxidase conjugated goat anti-mouse IgG1

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