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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.

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 (Prieur 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$_{HIV\text{-}1LAI}$ (Cazeaux et al., 2002; Moureau et al., 2002) and used for transformation in competent DH5α cells. Briefly, transformed *E. coli* DH5α cells were grown in LB complete medium and centrifuged after 5 h induction in isopropyl β-D1- 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 DH5α 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 libithum*, 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 μg of recombinant GST-Nef containing nanoglycolipidic particles (200 μL) 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 μL of recombinant GST-Nef or with GST at 10 μg/mL in phosphate buffered saline (PBS) pH7.4 for 2 h at 37 °C. Wells were blocked with 200 μL 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 μL 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 of 50 μL of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako S.A., France). After one hour of incubation followed by washing, 100 μL of O-phenylenediamine dihydrochloride (OPD) (Sigma, France) dissolved in phosphate citrate buffer, pH5, activated with H_2O_2 were added as a substrate. Colour development was stopped after 20 minutes by adding 30 μL of 4 N H_2SO_4 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, peroxydase conjugated goat anti-mouse IgG1

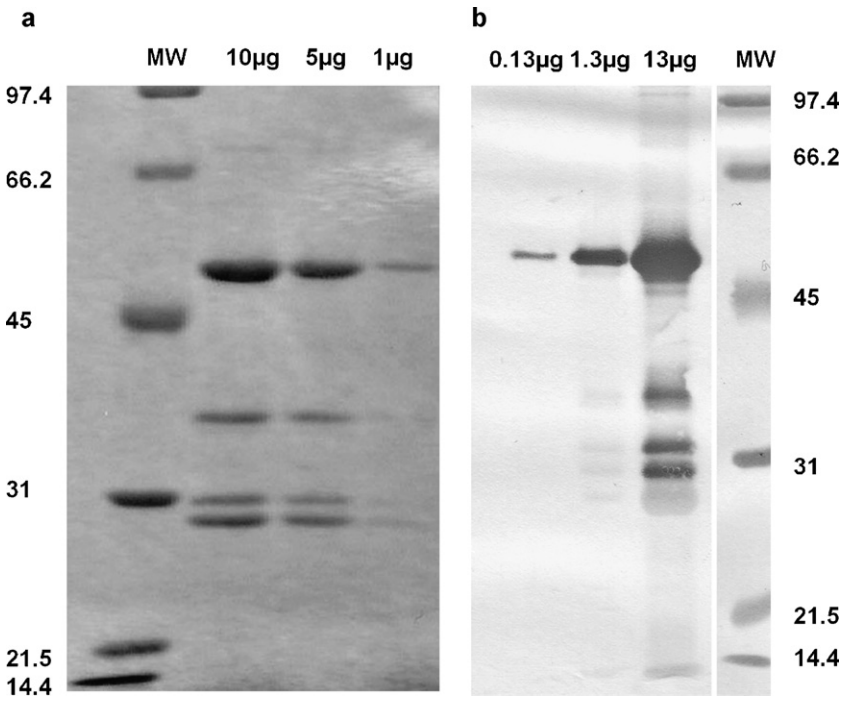

Fig. 1. Characterization of affinity purified GST-$Nef_{SIVmac\ 251}$. Produced and purified GST-$Nef_{SIVmac\ 251}$ (1 μg, 5 μg and 10 μg) was analyzed by SDS-PAGE (a) and by WB using an anti-Nef antibodies (b).

or IgG2a (Santa Cruz, USA) were substituted for the anti-mouse immunoglobulins described above.

Antibody titers were expressed as the reciprocal of the highest dilution which gave an absorbance ratio of 0.2.

2.7. Western blots

The equivalent of 1 to 10 μg of GST-Nef protein were heated at 100 °C for 3 min in the presence of 10% SDS and 1% β-mercaptoethanol before running sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer, the nitrocellulose membrane was blocked with 5% dried milk for 2 h at room temperature. GST-Nef was characterized by incubating the nitrocellulose membrane with rabbit anti GST-Nef antibodies. After intensive washing, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin (Dako S.A, France) for 1 h at room temperature. Staining was detected with PBS containing diaminobenzedine and 0.1% H_2O_2.

3. Results

3.1. Production and purification of GST-$Nef_{SIVmac\ 251}$

The recombinant protein was produced as a nonmyristoylated molecule in *E.coli* transformed with the *GST-$nef_{SIVmac\ 251}$* gene. GST-Nef protein was purified by affinity chromatography on glutathione agarose. Three major proteins remained bound to the solid phase and were eluted after adding reduced glutathione (Fig. 1). The first band, migrating at 55 kDa, corresponds to GST-Nef that at 38 kDa was a derivative of GST-Nef and the double band at 27 kDa corresponds to two GST isomers.

3.2. Characterization of formulated GST-Nef protein

The formulation of GST-Nef protein was evaluated by SDS-PAGE and Western blot. Results presented in Fig 2, clearly show the presence of GST-Nef (Fig. 2 lane 1). The fact to find a similar panel of GST-Nef formulated in particles as that obtained with the purified protein (Fig. 1 lane 2) indicated that the procedure of formulation did not cause protein alterations such as oligomerization or degradation. It is also interesting to note that formulated GST-Nef remained stable at least during 3 months when stored at 4 °C (data not shown).

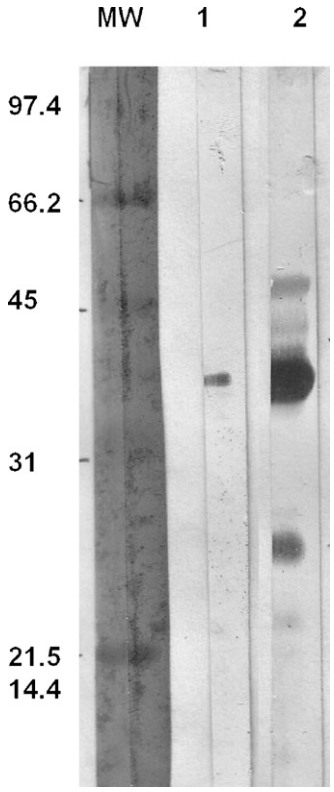

Fig. 2. Characterization of nanoglycolipidic particles associated with GST-Nef. Nanoglycolipidic particles associated with GST-Nef (1 μg) were analyzed by WB using an anti-GST antibody (1) or anti-Nef antibody (2).

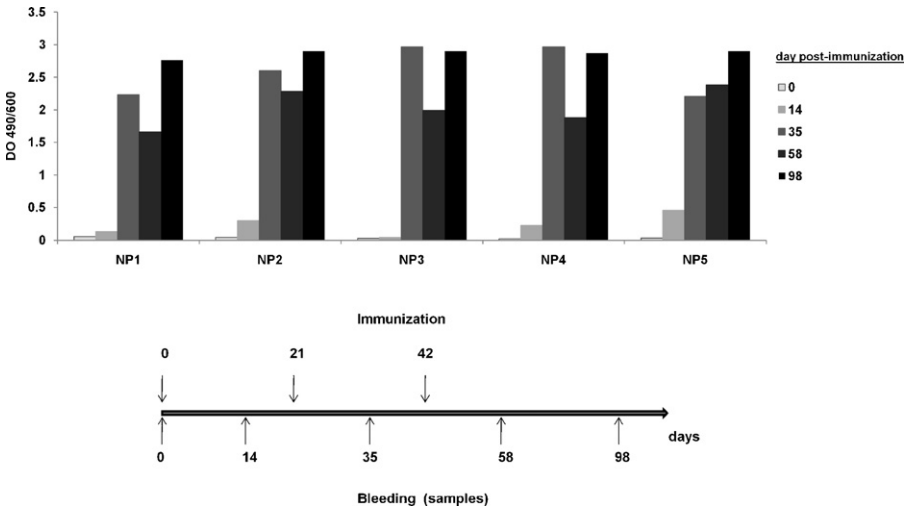

Fig. 3. Titration of anti-GST-Nef sera. Sera obtained at days 0, 14, 35, 58, 98 post-immunization from mice immunized with GST-Nef associated with nanoglycolipidic particles (NP1–NP5) were tested by ELISA against GST-Nef.

3.3. Adjuvant effects of the nanoglycolipidic particles

In order to test the adjuvant effect of the nanoglycolipidic particles the formulated GST-Nef was injected subcutaneously to a group of 5 BALB/c mice. The kinetics of anti-Nef antibodies production was evaluated by testing the presence of anti-GST-Nef antibodies in the sera obtained at days 14, 35, 58 and 89. As expected, no antibodies were detected in the sera obtained before immunization (pre immune sera). Anti GST-Nef antibodies became detectable in all five mice from the first immunization with GST-Nef associated NP. The titers increased over the course of GST-Nef injections (Fig. 3). The highest titers were reached after the second injection. These results clearly underline the great adjuvant effect of these nanoglycolipidic particles. It is also interesting to note the safety of these vectors. Indeed no clinical manifestations of toxicity or reactions at the injection site were observed in mice after administration of formulated GST-Nef.

One limitation of the use of vectors in the development of vaccines or in gene therapy is related to their capacity to stimulate a specific immune response against the vector. To explore this eventuality, sera obtained from mice after the third injection were tested by ELISA against the vector alone or formulated with GST-Nef. In this case, no significant positive reactions were observed against the vector alone in the five sera tested (Fig. 5). These results indicated that glycolipidic parts of the nanoglycolipidic particles are not immunogens. In parallel we tested the immune reactivity of antibodies produced against GST-Nef associated with nanoglycolipidic particles by ELISA against GST or GST-Nef. The sera of the five immunized mice were titrated by testing various dilutions. The obtained results showed preferential response against the Nef part of the fusion protein with a titer at least ten times higher than that obtained against GST (Fig. 4).

3.4. Comparison of the adjuvant effect of the glycolipidic nanoglycolipidic particles and Freund's adjuvant or muramyl dipeptide adjuvant

In order to compare the adjuvant effect of the glycolipidic nanoglycolipidic particles, with the more known adjuvant: CFA, IFA in association with MDP or MDP alone, we immunized three other groups of mice with the same amount of GST-Nef in association with the cited adjuvants. The kinetics and anti-GST-Nef titers were analyzed as described above. At a kinetic level, it is interesting to note that no significant positive reaction except in one mouse was observed in the sera of these three groups of mice (Fig. 6), while a clear positive response was observed after the first injection in the 5 out of 5 sera from mice immunized with GST-Nef associated with the nanoglycolipidic particles (Fig. 3). These results indicated the efficient adjuvant effect of the nanoglycolipidic particles. However, after the second injection a strong response comparable to that obtained with the nanoglycolipidic particles was observed in the two groups of mice immunized with GST-Nef in association with CFA or IFA + MDP (data not shown). In contrast, only a weak response was obtained in mice immunized with GST-Nef in association with MDP alone (Fig. 6).

3.5. Analysis of anti-GST-Nef antibody isotypes

We analyzed the IgG1 and IgG2a isotypes of specific antibodies produced in mice immunized with SIV GST-Nef associated with NP, CFA, MDP or a mixture of MDP and IFA. Sera obtained after the last injection were tested by ELISA using recombinant GST-Nef protein to coat the wells and peroxidase-conjugated anti-IgG1 or IgG2a for the visualization. The results depicted in Fig. 7 showed the predominance of IgG1 antibody isotypes in all animal groups. These protocols of immunization lead to IgG1/IgG2a ratios of 8.8, 3.6, 4.5 and 10.5 in animals immunized with Nef associated with NP, CFA, MDP or a mixture of MDP and IFA respectively. These data suggest the predominance of a Th2 type immune response.

4. Discussion

The need to develop an efficient HIV-1 vaccine remains a high priority of this century. In fact, the majority of approaches aimed to induce neutralizing antibodies and/or cellular cytotoxic cells against HIV/SIV gave modest efficacy (Rerks-Ngarm et al., 2009; Tatsis et al., 2009; Buchbinder et al., 2008). However, it is interesting to note that a recent study using a fragment of gp41 HIV-1 grafted on virosomes as immunogen and delivered by mucosal route to monkeys (*Macaca mulatta*) induced mucosal antibodies able to protect against vaginal SHIV challenges (Bomsel et al., 2011). This latter study underlines the importance of the design of novel immunogens and vectors.

In addition, the success of traditional vaccines based on killed viruses or bacteria is mainly due to the presence of ligands capable of stimulating innate immunity, which in turn contributes to the development of specific immunity. This intrinsic adjuvant effect is mainly related to the capacity of some components, such as viral

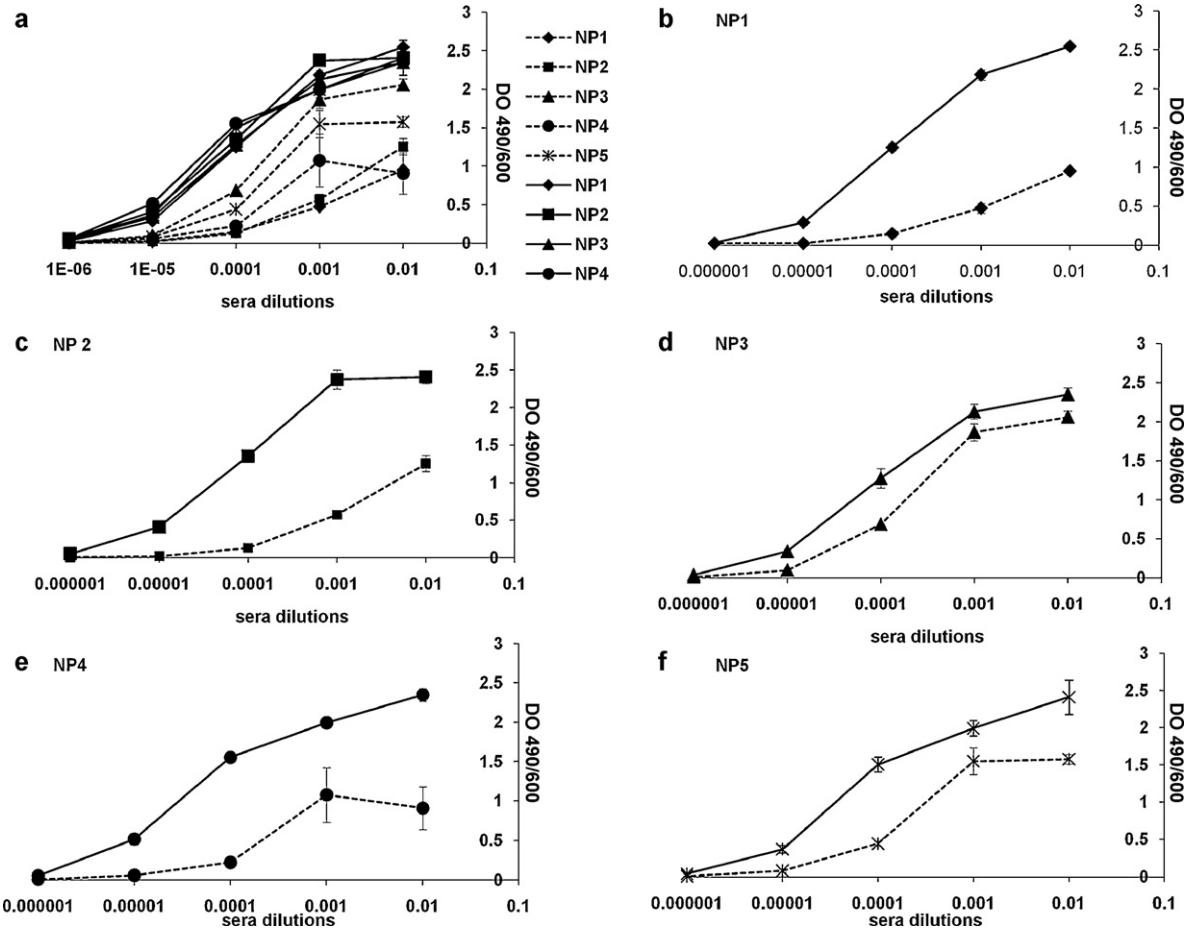

Fig. 4. Immunoreactivity of anti-GST-Nef antibodies against GST-Nef and GST. Sera from mice immunized with GST-Nef associated with nanoglycolipidic particles (NP1–NP5) were tested by ELISA against GST-Nef (solid line) and against GST alone (dashed line). The results of all mice are presented in (a), the results for mouse NP1 in (b) mouse NP2 in (c) mouse NP3 in (d), mouse NP4 in (e) and mouse NP5 in (f).

single strand or double strand RNA to stimulate TLR7/8 and TLR3 respectively, or bacterial CpG, LPS and other glycolipidic molecules to stimulate a large panel of TLR pathways including TLR2, 4, 5 and 9 (Kawai and Akira, 2010; Takeuchi and Akira, 2010). However, thanks to the development of genetic engineering and the inability to culture some viruses *in vitro*, such as the Hepatitis B virus (Tiollais and Chen, 2010), vaccines based on soluble recombinants proteins were emerged. One great limitation of this approach is the weak or the absence of immunogenicity of the soluble proteins to stimulate the innate immunity (McElrath and Haynes, 2010), in addition to their poor stability in vivo. To circumvent these limitations, different strategies have been used for the formulation of soluble proteins either by their encapsulation in lipidic or glycolipidic particles including liposomes (Heurtault et al., 2010), virosomes (Buonaguro et al., 2010), ISCOMs or by their association with adjuvant such as complete Freund's adjuvant, aluminum hydroxide and several other adjuvants based on mineral oil (Coffman et al., 2010).

In this study we showed that the adsorption of GST-Nef from SIVmac251 on the nanoglycolipidic particles enhances the immunogenicity of this soluble protein. This enhancement is manifested by the rapid detection of anti-Nef antibodies. One injection seems to be sufficient to generate detectable amount of anti-Nef antibodies. Whiles at least two injections, except in one mouse from five in the group immunized with Nef in association with IFA/MDP,

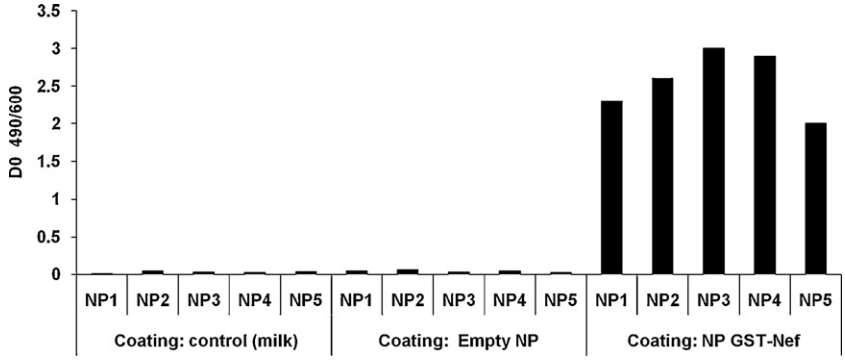

Fig. 5. Nanoparticles alone are not immunogens. Sera of the last injection of mice immunized with GST-Nef associated with nanoglycolipidic (NP1–NP5) were tested by ELISA against control (milk), empty nanoglycolipidic particles or GST-Nef associated with nanoglycolipidic particles.

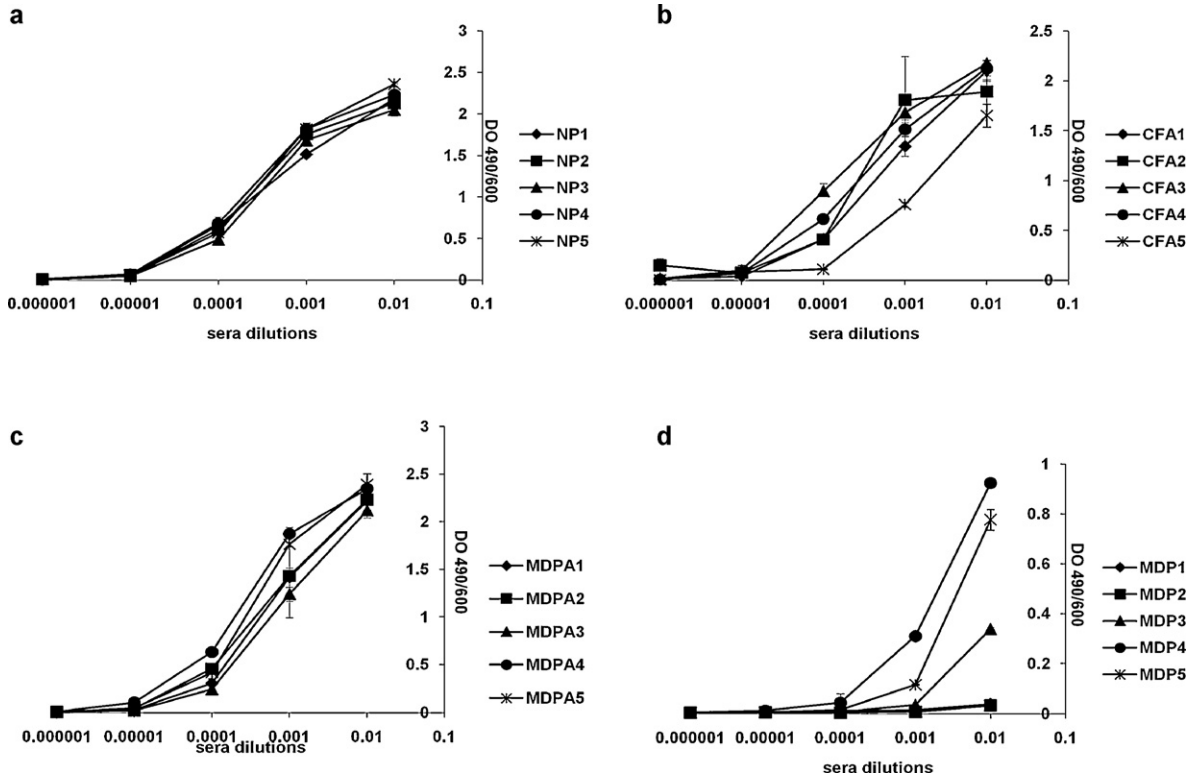

Fig. 6. Comparison of the adjuvant effect of the nanoparticles and CFA, MDP and IFA/MDP. Sera from mice immunized with GST-Nef associated with nanoglycolipidic particles (NP1–NP2) (a), GST-Nef formulated with CFA (CFA1–CFA5) (b), with a mixture of MDP and IFA (MDPA1–MDPA5) (c) and MDP (MDP1–MDP2) (d) were tested against GST-Nef by ELISA.

are needed in the groups receiving CFA, mixture of IFA and MDP or MDP as adjuvant. The protocols in this study using three injections at 3 weeks intervals were chosen because it leads to strong humoral responses with immunogens emulsified in CFA, one of the most powerful adjuvant. Due to its toxicity this adjuvant is not allowed in humans. The analysis of the kinetics of the anti-GST-Nef humoral response showed that the highest titer of anti-GST-Nef antibodies was reached since the second injection. While a third injection seems to be needed to reach this highest titer in the presence of CFA or the mixture of IFA and MDP. However when the muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine), a synthetic peptidoglycan, was used as adjuvant only a moderate humoral immune response was observed as shown by the weak titers reached after the third injection. This weak immunogenicity may be related to the rapid degradation of the GST-Nef when administered in soluble mixture with MDP. In contrast, in the three other protocols, the immunogens are associated with the nanoparticles or entrapped in the oil emulsion. As a consequence the immunogens are protected from rapid protease degradation and are probably released gradually over a large period of time. This latter explanation is consistent with the long lasting persistence of the anti-Nef titers. Indeed, three months after the last injection the anti-Nef antibody titers remained stable in the group of mice immunized in the presence of nanoglycolipidic particles and CFA or IFA in mixture with MDP.

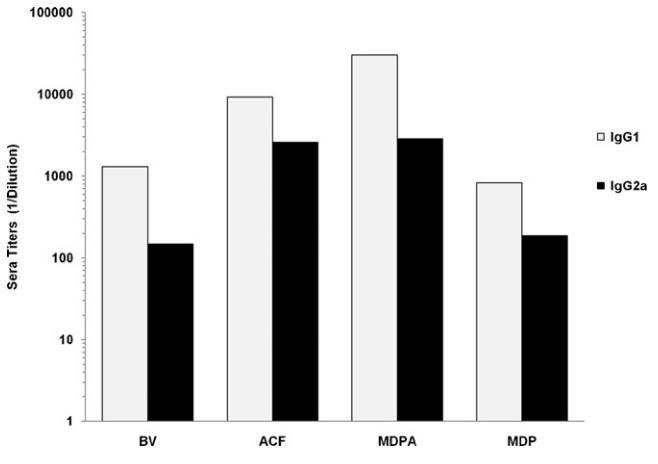

Fig. 7. Analysis of the anti-GST-Nef antibody isotypes. Sera from mice immunized with GST-Nef associated with nanoglycolipidic particles (NP), or mixed with CFA (CFA), or a MDP and IFA (MDPA) (c) or MDP alone (MDP) were tested against GST-Nef by ELISA. The IgG isotypes were measured by using peroxidase-conjugated anti-mouse IgG1 or IgG2a immunoglobilins.

When we compared the immunogenicity related to the GST part to that of the Nef, the obtained data showed the strongest response against the Nef part. This phenomenon is observed only in the group of mice immunized with GST-Nef associated with the nanoglycolipidic particles. This effect may be related to the mode of formulation and presentation of GST-Nef in the nanoglycolipidic particles. It is possible that the sequence of Nef, located at the C-terminal part of fusion protein, is more accessible at the surface the nanoglycolipidic particles, or is more processed and efficiently presented par MHC-class II antigens when released in the endosome/lysosome. It is also possible that the formulation of GST-Nef into the nanoglycolipidic particles is homogeneously oriented.

The primary benefit of this formulation is the presentation of the immunogen to APCs. Such targeting is facilitated in part by the size of the particles. Interestingly, the particle size is similar to that of retroviruses (100 nm) and allows its internalization by endocytosis (Berton et al., 1997; Chang et al., 2009; Dombu et al., 2010). This targeting is facilitating by the electrostatic interaction between the cationic charge of the nanoglycolipidic particles and the negative charges of the cell membrane. In addition to the protection of the antigen from rapid degradation, its formulation in

these nanoglycolipidic particles allowed its release in the endosome where it associates with MHC class II antigens. It is important also to note that in contrast to the majority of adjuvants tested in animal models, only few adjuvants are allowed to be administered to humans. The nanoglycolipidic particles used in this assay are showed to be safe in mice and human, when administered in the noses of human volunteers (von Hoegen, 2001). In addition it was demonstrated that in contrast to the more used adjuvant of the mucosal immunity, including the detoxified LT and CT toxins, these types of nanoparticles are able to stimulate the mucosal immunity without the side effects due to the inflammation and the cytotoxicity (van Ginkel et al., 2000). Indeed when used as adjuvant, LT and CT detoxified toxins target the nervous system via the olfactory bulb when administered by intranasal route and stimulate the production of inflammatory cytokines including TNF-α and IL-1β.

5. Conclusion

Taken together, these results demonstrated the effectiveness of the nanoglycolipidic particles to deliver GST-Nef protein to induce a strong and long lasting immune response. Indeed the nanoglycolipidic particles could overcome the limitations of other adjuvants and enhance the immunological activities and efficiency of vaccine proteins.

Acknowledgments

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