Nasal Immunization with a Recombinant HIV gp120 and Nanoemulsion Adjuvant Produces Th1 Polarized Responses and Neutralizing Antibodies to Primary HIV Type 1 Isolates

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ABSTRACT

Epidemiological and experimental data suggest that both robust neutralizing antibodies and potent cellular responses play important roles in controlling primary HIV-1 infection. In this study we have investigated the induction of systemic and mucosal immune responses to HIV gp120 monomer immunogen administered intranasally in a novel, oil-in-water nanoemulsion (NE) adjuvant. Mice and guinea pigs intranasally immunized by the application of recombinant HIV gp120 antigen mixed in NE demonstrated robust serum anti-gp120 IgG, as well as bronchial, vaginal, and serum anti-gp120 IgA in mice. The serum of these animals demonstrated antibodies that cross-reacted with heterologous serotypes of gp120 and had significant neutralizing activity against two clade-B laboratory strains of HIV (HIVBaL and HIVSF162) and five primary HIV-1 isolates. The analysis of gp120-specific CTL proliferation, INF-γ induction, and prevalence of anti-gp120 IgG2 subclass antibodies indicated that nasal vaccination in NE also induced systemic, Th1-polarized cellular immune responses. This study suggests that NE should be evaluated as a mucosal adjuvant for multivalent HIV vaccines.

INTRODUCTION

The unique immunobiology of HIV, the diversity of virus isolates, variables in the immunogenicity of viral antigens, and the lack of clear correlates of protection with specific aspects of anti-HIV immunity have all raised fundamental questions about the potential for protective HIV vaccination.1–3 Despite this, multiple approaches for the development of a vaccine to prevent HIV infection or control virus-associated disease have been attempted. The HIV envelope glycoprotein (Env) is a major target for neutralizing antibodies.4 Because of its central role in mediating virus binding to cellular CD4 and facilitating coreceptor interaction, the gp120 component of Env, in both monomeric and native oligomeric forms, has been used as a principal HIV vaccine immunogen.5,6 Unfortunately, initial attempts to employ gp120 as a traditional vaccine immunogen failed.7–9 Because epidemiological and immunological evidence suggests that an effective HIV vaccine will need to elicit both virus-specific neutralizing antibodies and cytotoxic T cell responses against HIV-1,10–16 recent HIV vaccines explored various combinations of viral and/or DNA vectors and recombinant Env proteins.3,17–21 While some of these approaches may work, many are complex and it is clear new approaches are still needed.22–24

Primary HIV-1 infection typically occurs via mucosal surfaces turning attention to vaccines that could induce anti-HIV mucosal immunity. Intranasal immunizations have been reported to induce both mucosal and systemic immune responses.19,25,26 Several experimental and commercial human nasal vaccines for mucosally transmitted viral and bacterial pathogens have been pursued in recent years.27–30 In contrast, initial candidate HIV vaccines have predominantly used traditional human adjuvants not associated with mucosal immunity and have been administered by subcutaneous injection.3,14 A few recent HIV vaccine approaches have attempted mucosal immunization through multiple strategies, including inactivated HIV-1,31,32 live-attenuated viral vectors,33,34 bacterial vectors,35,36 virus-like particles (VLPs),37 naked DNA,38,39 toxins,
and cytokines.\(^{40-42}\) However, both safety concerns regarding attenuated pathogens and side effects from mucosal bacterial toxin-containing adjuvants have limited the use of these approaches for human mucosal vaccines.\(^{43-45}\) Therefore, the lack of safe and effective mucosal adjuvants capable of eliciting both neutralizing antibodies and cytotoxic T lymphocyte (CTL)-mediated responses limits development of mucosal vaccines, especially for HIV.\(^{46-48}\)

The current studies evaluate an oil-in-water nanoemulsion (NE) as a mucosal adjuvant for a recombinant gp120 HIV vaccine. Previous studies have demonstrated that NEs have broad antimicrobial activity\(^{49,50}\) and are safe and effective noninflammatory mucosal adjuvants for influenza and recombinant anthrax protective antigen vaccines.\(^{51,52}\) In the present work, the nanoemulsion was simply mixed with recombinant gp120 and applied to the nares of mice and guinea pigs. We then assessed the induction of both mucosal and systemic anti-gp120 antibodies and the cross-reactivity of these antibodies with different gp120 serotypes, and also evaluated the animal’s sera for antibodies and the cross-reactivity of these antibodies with different gp120 serotypes. In addition, we characterized the systemic gp120-specific cellular response induced by mucosal immunization with NE adjuvant. Our results suggest that this type of NE deserves further evaluation as adjuvant for an HIV vaccine.

**MATERIALS AND METHODS**

**Animals**

Pathogen-free, female BALB/c mice (5–6 weeks old) and Hartley guinea pigs (females, 250 g) were purchased from Charles River Laboratories (Wilmington, MA). The mice and guinea pigs were cared for in accordance with the American Association for Accreditation of Laboratory Animal Care standards. All procedures involving animals were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

**Reagents**

Recombinant HIV gp120\(_{\text{Bal}}\) and gp120\(_{\text{SF162}}\) serotype proteins produced in yeast were obtained from Quantum Biological, Inc., through The NIAID Vaccine Research Resource. The 5 mg/ml aliquots of the protein solutions in a sterile saline were stored at \(-80^\circ\)C until used. The synthetic V3 loop peptide (Bal) was obtained as a gift from Dr. Steven King (University of Michigan). The 20-mer oligonucleotide (ODN) 5’-TCC ATG ACG TTC CGT ACG TT -3’,\(^{53}\) containing nonmethylated CpG repeats, was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The *Salmonella minnesota* monophosphoryl lipid A (MPL A, \#L-6638), phytohemagglutinin protein (PHA-P), bovine serum albumin (BSA), dithiothreitol (DTT), and other chemicals used in buffers were purchased from Sigma-Aldrich Corporation (St. Louis, MO). The saline solution, phosphate-buffered saline (PBS), cell culture media, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY) and HyClone (Logan, UT), respectively. The alkaline phosphatase (AP)-conjugated antibodies, goat antimouse IgG, and goat antimouse IgA (\(\alpha\) chain specific) were purchased from Sigma. The secondary antibodies, AP conjugates rabbit antimouse IgG1, IgG2a, IgG2b, and IgG3 and rabbit anti-guinea pig IgG, were bought from Rockland Immunochemicals, Inc.

**Preparation of the gp120/adjuvant formulations**

The oil-in-water NE used in these studies was supplied by NanoBio Corporation (Ann Arbor, MI). NE was produced by the emulsification of cetylpyridinium chloride (CPC, 1%), a nonionic surfactant (5%), and ethanol (8%) in water with hot-pressed soybean oil (64%), using a high-speed emulsifier.\(^{49}\) Except for the CPC, this nanoemulsion is formulated with surfactant and food substances considered “Generally Recognized as Safe” (GRAS) by the Food and Drug Administration (FDA). NE mean droplet size (approximately 350 nm in diameter) was determined by dynamic light scattering (DLS) using the NICOMP 380 ZLS (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA).

All gp120/NE formulations were prepared by mixing gp120 protein solution with NE, using pyrogen-free saline as a diluent. Murine immunization studies were performed with a 20 \(\mu\)g dose of gp120 mixed with 0.1%, 0.5%, and 1% NE concentrations. For immunization with additional immunostimulants, either 5 \(\mu\)g of MPL A or 10 \(\mu\)g CpG ODN was added to 20 \(\mu\)g gp120 in 1% NE or to 20 \(\mu\)g gp120 in saline. A guinea pig immunization study was performed using a 50 \(\mu\)g dose of gp120 mixed with 1% NE and normal saline as diluent.

**Immunization procedures**

BALB/c mice (five animals per group) were immunized with two, and on one occasion with three, intranasal administrations of gp120/NE formulation at 3 weeks apart. The immunizations were performed by slowly applying gp120/NE mixes (10 \(\mu\)l per nare) to the nares of isoflurane-anesthetized mice. During administration, animals were held in the inverted position until the droplets were completely inhaled. Control mice were immunized with gp120 in saline, with NE alone or saline. Intramuscular immunization was performed with two doses, 3 weeks apart, of 20 \(\mu\)g gp120 injected in 50 \(\mu\)l of either saline or 1% NE. Hartley guinea pigs (three animals per group) were anesthetized with the ketamine injection (40 mg/kg) and immunized intranasally with two intranasal administrations of gp120/NE mix (50 \(\mu\)l per nare) at 3 weeks apart.

**Collection of blood, bronchial alveolar lavage, vaginal washes, and splenocytes**

Blood samples were obtained before and 3 weeks after final immunizations either from the saphenous vein, at various time points during the course of the experiment, or by cardiac puncture from euthanized premorbid mice. Serum was obtained from coagulated blood (30–60 min at room temperature) by centrifugation at 1500 \(\times\) g for 5 min. Collected serum samples were heat inactivated at 56°C for 1 h and stored at \(-20^\circ\)C until analyzed.

Mouse bronchial alveolar lavage (BAL) fluid was obtained from animals euthanized by inhalation of isoflurane. The lung was infused twice with 0.5 ml of PBS with 10 \(\mu\)M DTT and 0.5 mg/ml aprotinin and approximately 1 ml of aspirate was recovered. BAL samples were stored at \(-20^\circ\)C until analyzed. Vaginal wash samples were collected from anesthetized mice.
by infusion of vaginal cavities with 100 μl of PBS with 10 μM DTT and 0.5 mg/ml apronitin. The samples were centrifuged at 10,000 × g for 5 min at 4°C, and the supernatants were stored at −20°C until analyzed.

Murine splenocytes were mechanically isolated to obtain single cell suspension in PBS. The red blood cells (RBC) were removed by lysis with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), and the remaining cells were washed twice in PBS. For the antigen-specific proliferation or cytokine expression assays, splenocytes (2–4 × 10⁶ cells/ml) were resuspended in RPMI 1640 medium, supplemented with 2% FBS, l-glutamine, and penicillin/streptomycin (100 U/ml and 100 μg/ml).

Determination of anti-gp120 IgG and IgA antibodies

Mouse anti-gp120-specific IgG and IgA levels were determined by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (MaxiSorp; Nalge Nunc International, Rochester, NY) were coated with 5 μg/ml (100 μl) of either gp120Bal or gp120SF162 serotype envelope protein in the coating buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. After the protein solution was removed, plates were blocked for 30 min at 37°C with PBS–1% dry milk solution. The blocking solution was aspirated and plates were used immediately or stored sealed at 4°C until needed. Serum and BAL samples were serially diluted in 0.1% BSA in PBS, and 100 μl/well aliquots were incubated in gp120-coated plates for 1 h at 37°C. The plates were washed three times with PBS–0.05% Tween 20, followed by 1 h incubation with either antimouse IgG or antimouse IgA AP-conjugated antibodies, then washed three times and incubated with AP substrate SigmaFast (Sigma, St. Louis, MO) according to the manufacturer’s protocol. Spectrophotometric readouts were performed using the Spectra Max 340 ELISA reader (Molecular Devices, Sunnyvale, CA) at 405 nm and reference wavelength of 690 nm. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 405 nm was greater than two times absorbance above negative control. Guinea pig anti-gp120 IgG was determined by the same method, except that rabbit anti-guinea pig IgG AP conjugate (Rockland) was used for detection. Antibody concentrations are presented as the mean ± standard deviation (SD) of endpoint titers.

HIV-1 single-round neutralization assay

The panel of eight strains of clade B HIV-1 used in this study consists of the laboratory strains Bal, SF162, and MN, and primary HIV-1 isolates SS1196.11, BG1168.1, QH0692.42, 3988.25, and 5768.04.54 Virus neutralization was measured as a function of the reductions in luciferase reporter gene expression after a single round of virus infection in TZM-bl cells as described previously.55 The TZM-bl cells are engineered to express CD4 and CCR5 and contain integrated reporter genes for firefly luciferase and Escherichia coli β-galactosidase under control of an HIV-1 LTR. Primary HIV-1 isolates (TCID₅₀, 100–200) were incubated in triplicate with serial dilutions of sera for 1 h at 37°C. Subsequently virus/serum mixtures were added to the 96-well flat-bottom culture plate containing adherent TZM-bl cells. Control consisted of cells plus virus (virus control) and cells only (background control). Bioluminescence was measured after 48 h using Bright Glo substrate solution per the supplier’s protocol (Promega, Madison, WI). Neutralization titers (NT₅₀) are the dilutions at which relative light units (RLUs) were reduced by 50% compared to those of virus control wells after subtraction of background RLUs.

Proliferation assay

The proliferation of mouse splenocytes was measured by 5-bromo-2-deoxyuridine (BrDU) incorporation using a commercial kit (Cell Proliferation ELISA, Roche Molecular Biochemicals, Mannheim, Germany). To assess antigen-specific proliferation, cells (2 × 10⁶ cell/ml) were incubated with and without gp120Bal (5 μg/ml), and as a positive control PHA-P (2 μg/ml, for 48 h, then pulsed with BrdU for 24 h). Cell proliferation was measured according to the manufacturer’s instructions using a Spectra Max 340 ELISA Reader (Molecular Devices, Sunnyvale, CA) at 370 nm and reference wavelength of 492 nm.

Analysis of cytokine expression in vitro

Mouse splenocytes were seeded at 4 × 10⁶ cells/ml (RPMI 1640, 2% FBS) and incubated with either gp120Bal, gp120SF162 (5 μg/ml), or with V3 loop peptide (20 nM) for 72 h at 37°C. Cell culture supernatants were harvested and analyzed for the presence of cytokines. The cytokine assays were performed using Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis of the results was performed using ANOVA, and Student’s t-test for the determination of the p value.

RESULTS

Nasal immunization with recombinant HIV gp120 protein mixed with nanoemulsion induces anti-gp120 IgG antibodies in serum

BALB/c mice were intranasally immunized with either gp120Bal or gp120SF162 serotypes to assess whether NE has adjuvant activity in mucosal immunization with recombinant gp120 protein. Immunizations were carried out using 20 μg of gp120Bal in either saline or mixed with 0.1%, 0.5%, and 1% NE concentrations. Blood samples collected at 6 weeks after prime immunization (in animals boosted only at 3 weeks) and at 12 weeks after prime immunization (in animals boosted at 3 and 6 weeks) were analyzed for gp120-specific antibodies by ELISA. All mice immunized with either of the gp120Bal/NE preparations were seropositive after only two immunizations (Fig. 1). The anti-gp120Bal IgG response showed a concentration-dependent effect of NE, with the lowest titers in gp120Bal/0.1% NE and highest in gp120Bal/1% NE immunization groups (mean titers of 1.3 × 10⁴ and 2.6 × 10⁵, respectively, Fig. 1A). In contrast, mice immunized with gp120Bal in saline did not have detectable anti-gp120Bal antibodies even after three immunizations.
FIG. 1. Antibody response in mice intranasally vaccinated with two serotypes of recombinant gp120 and the nanoemulsion adjuvant. (A) Induction of serum anti-gp120BaL IgG in mice immunized with gp120BaL in 0.1%, 0.5%, and 1% NE. Anti-gp120BaL blood samples collected at 6 weeks after prime immunization (in animals boosted only at 3 weeks) and at 12 weeks after prime immunization (in animals boosted at 3 and 6 weeks) were analyzed for gp120-specific antibodies by ELISA. Intranasal (i.n.) and intramuscular (i.m.) routes of immunization are indicated in the Figure. (B) Induction of anti-gp120SF162 IgG in mice i.n. immunized with two doses of gp120SF162 in 1% NE alone or with addition of CpG or MPL A. Anti-gp120 IgG antibodies were measured at 6 weeks after prime immunization (in animals boosted at 3 weeks). Anti-gp120 antibody levels are presented as a mean of endpoint titers (± SD) in serum of individual animals. Cross-reactivity of the anti-gp120 antibodies. Serum IgG from mice immunized with either gp120BaL (C) or gp120SF162 (D) reacts with both gp120 serotypes. Data are presented as titration curves of pooled serum from all immunized animals binding to either gp120SF162 or gp120BaL. (E) Anti-gp120 IgG subclass pattern in mice immunized i.n. with gp120/NE. The results are presented as a titer ratio of the specific subclass IgG to the overall IgG titer.
titers after intranasal immunization with either 0.5% or 1% NE were comparable with the antibody response obtained after two intramuscular injections with gp120BaL in saline or mixed with 1% NE, and the addition of a third intranasal immunization did not significantly increase antibody titers (Fig. 1A). These results indicate that two intranasal gp120BaL/NE immunizations produce a systemic immune response in mice.

Additional studies examining the NE immunizations were carried out with a second gp120 serotype, gp120SF162. Similarly to immunization with the gp120BaL, all mice immunized with gp120SF162/NE responded with high anti-gp120 SF162 IgG titers. NE-produced immune responses were not enhanced significantly by the addition of unmethylated CpG ODN and MPL A (Fig. 1B). To investigate the effect of combining the NE with other immunostimulants, mice were immunized with the gp120SF162/NE with the addition of either CpG (gp120SF162/NE + CpG) or MPL A (gp120SF162/NE + MPL A). Combining NE with MPL A (but not with CpG) resulted in a modest increase in mean antibody titer of 2- to 3-fold over immunization with gp120SF162/NE alone; however, this difference was not statistically significant (p > 0.05). In contrast, nasal immunization with gp120SF162 antigen mixed with either CpG or MPL A alone produced minimal immune responses (Fig. 1B).

Intranasal immunization with either serotype of gp120 protein in NE produced cross-reacting IgG antibodies to the other serotype (Fig. 1C and D). Serum IgG antibodies raised against either gp120BaL or gp120SF162 reacted with the other serotype with activity comparable to the immunizing envelope protein and similar endpoint titers. This suggested that NE adjuvant produced a repertoire of IgG capable of recognizing both variable and conserved epitopes of the gp120 immunogen, which could be of importance for protective immunity against different serotypes of HIV-1. The subclass analysis of the anti-gp120 IgG antibodies indicated a prevalence of IgG2b and IgG2a over IgG1 antibodies, thus suggesting a Th1 polarization of the immune response in mice immunized with gp120/NE (Fig. 1E). The absence of antibody response to intranasal immunization with gp120 antigen alone did not allow for the determination of IgG subclass without NE adjuvant.

**Induction of secretary mucosal anti-gp120 IgA and IgG**

BAL fluids, vaginal washes, and sera were analyzed for evidence of mucosal response. Mice nasally immunized with gp120SF162/NE had significant levels of gp120SF162-specific secretory IgA and IgG antibodies in BAL fluid (Fig. 2A and B). Similar to serum IgG, both mucosal IgA and IgG responses demonstrate cross-reactivity with the gp120BaL immunogen. Anti-gp120BaL IgA was also detected in the serum and importantly in secretions from distant (vaginal) mucosa after intranasal NE immunization (Fig. 2C). In contrast, immunization with either type of gp120 in saline failed to produce any type of mucosal IgA and IgG response.

**Cell-mediated immune responses**

Cellular immune responses were assessed in vitro by antigen-specific T cell proliferation and the pattern of cytokine production. Antigen-specific proliferative responses were detected only in splenic lymphocytes from animals immunized with the gp120BaL/NE and were absent in either mice immunized with either gp120BaL/saline or with control animals treated with saline or NE alone (Fig. 3A). Intranasal immunization with gp120BaL/NE also produced systemic, cross-reacting, cell-mediated immunity as demonstrated by significant interferon (IFN)-γ production after in vitro stimulation with either gp120BaL or gp120SF162 in cells harvested from animals immunized with gp120BaL (Fig. 3B). A substantial induction of the IFN-γ was also obtained in these spleen cells with an oligopeptide fragment of the V3 loop, thus indicating the presence of cells recognizing the dominant epitope associated with virus binding and neutralization. In contrast, there was no antigen-specific induction of interleukin (IL)-4 expression (not shown), suggesting a Th1 polarization of the cellular immune response. In addition, no significant cytokine response was stimulated by gp120 in splenocytes from either control, non-immune mice, or from mice immunized with gp120BaL in saline.

**Immunization with gp120/NE induces antibodies that neutralize HIV-1**

To investigate the ability of gp120-specific antibodies induced by NE nasal immunization to neutralize HIV, guinea pigs were immunized with two doses of gp120SF162 in 1% NE. Immunization again produced significant levels of serum anti-gp120 IgG antibodies in all animals (Fig. 4A) and as observed with the murine immunizations the serum anti-gp120 IgG cross-reacted with the gp120BaL immunogen. The immune sera from guinea pigs were then tested for neutralization activity against HIV-1. The breadth of the neutralizing response was evaluated using a panel of eight viruses, including three laboratory strains and five primary HIV-1 isolates. Figure 4B documents that the highest NT50 occurred in a single animal with the immunizing serotype bearing M-tropic strain of HIV SF162 with an NT50 = 225. Neutralizing activity was also present, albeit at lower levels (NT50 > 50), in the two other animals immunized with this antigen despite lower anti-gp120 IgG levels. Of interest, significant neutralization was also observed with six of the seven other HIV isolates (including all five primary isolates), with all three guinea pigs generating an NT50 of at least 50 and exceeding 100 with two of the primary isolates (Fig. 4B). No neutralization was observed with the laboratory strain of T-tropic HIVMN virus.

**DISCUSSION**

Mucosal vaccines have the unique ability to induce both mucosal and systemic immunity. This is potentially important in the prevention of respiratory or sexually transmitted infections. However, antigens delivered via mucosal routes are usually not immunogenic and require adjuvants to enhance immunogenicity. In this study we have demonstrated that intranasal immunization with recombinant gp120 administered in a nanoemulsion adjuvant induces potent systemic and mucosal immune responses. In contrast to other mucosal HIV vaccines in development, which reportedly require three or four immunizations, the NE adjuvanted material produced high titers of anti-gp120 antibodies after only two administrations.
FIG. 2. Nasal immunization with gp120/NE induces mucosal IgA and IgG antibodies. Secretory anti-gp120 IgA (A) and IgG (B) in bronchial lavage (BAL). The IgA and IgG antibodies recognize both autologous SF162 and heterologous BaL serotypes as indicated. Assays were performed with undiluted and 1:10 diluted BAL fluids for IgA and IgG, respectively. (C) Anti-gp120 IgA in serum and in the vaginal washes of mice vaccinated with gp120BaL and NE adjuvant. Assays were performed with undiluted vaginal washes and with 1:50 diluted serum. Anti-gp120 IgA and IgG concentrations are presented as mean absorbance (OD 405 nm ± SD) obtained in ELISA. A statistically significant difference was observed between gp120/saline and all gp120/NE groups (p < 0.05), but no significant differences were observed in various NE concentrations (p > 0.05).
In addition, serum IgG gp120 titers were comparable to those reported for intramuscular immunization and those obtained when we intramuscularly immunized mice (Fig. 1). The response to NE-based nasal immunization also compared favorably with responses achieved through parenteral, bimodal vaccine regimens of multiple DNA injections (three or four) followed by subsequent boosts with recombinant antigen or adenoviral vector.

Nasal immunization with gp120/NE also induced significant antigen-specific IgA and IgG antibodies in BAL fluid and vaginal secretions. The IgA in these compartments may result from local IgA production, which is supported by finding these antibodies in both bronchial alveolar and vaginal mucosal secretions. These results support the concept that nasal mucosa immunization can produce specific antibody responses in distant mucosal sites. The presence of anti-HIV antibodies in the genital tract could be useful in the prevention of HIV-1 infection.

A major goal in the design of the HIV vaccine is to generate high titers of antibodies that can neutralize many different isolates of HIV. Multiple studies and clinical trials with gp120-based vaccines document that despite high titers of anti-gp120 antibodies there is often limited neutralizing activity against naturally occurring HIV-1 isolates. Mucosal immunization with gp120/NE induced highly cross-reactive IgG and IgA antibodies against two diverse serotypes of gp120, BaL and SF162, in both mice and guinea pigs. The relevance of this result was further illustrated in the HIV-1 neutralization analysis. When tested for neutralizing activity against a panel of clade B HIV-1 strains, sera from guinea pigs nasally immunized with gp20SF162/NE demonstrated broad, neutralizing activity against seven of eight strains and against all five primary HIV-1 isolates. This included neutralization of the phylogenetically diverse clones BG1168.1, SS1196.11, QH0692.42, 3988.25, and 5768.4. These isolates were chosen because of their inclusion in the recently proposed list of clade B HIV-1 clones for the
standardized assessment of vaccine-induced neutralizing antibodies.\textsuperscript{54,72,73} Sequence and glycosylation site analysis indicated a wide spectrum of genetic diversity, as expected for virus strains obtained from independent infections. The reason for this extensive cross-neutralization activity may involve stabilization of the gp120 protein conformation, especially as a trimer, by NE (data not shown). These data suggest a mucosal vaccine consisting of multiple serotypes of recombinant HIV-1 proteins and NE adjuvant could potentially produce even broader-spectrum HIV-1 neutralizing activity.

Interestingly, no significant neutralizing activity was found against another clade B laboratory strain—HIV\textsubscript{MN}. It is not clear if this insensitivity may be related to the diversity of V3 loop epitope, which is linked to T-tropism of this viral isolate.\textsuperscript{74} In earlier studies and in vaccine trials, gp120\textsubscript{MN} and gp160\textsubscript{MN} failed to produce neutralizing antibodies to the majority of HIV-1 isolates,\textsuperscript{75,76} so there may be something uniquely non-immunogenic about this strain.

While NE nasal immunization induced potent systemic and mucosal antibodies, it also induced significant cellular immune responses, as documented by antigen-specific proliferation and IFN-\gamma production in splenic lymphocytes. The induction of hallmark Th1 cytokine IFN-\gamma indicates activation of CD4\textsuperscript{+} and CD8\textsuperscript{+} CTLs with both autologous gp120\textsubscript{Bal} and heterologous gp120\textsubscript{SF162} and with 35 aa V3 loop peptide.\textsuperscript{77,78} The pattern of cytokine induction with significant INF-\gamma and absence of IL-4 suggests that NE alone can produce the Th1 polarization cellular response.\textsuperscript{79} The cytokine data are supported by the anal-

\begin{figure}[h]
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\caption{(A) Nasal immunization of guinea pigs. Hartly guinea pigs (GP) were nasally vaccinated with 50 \(\mu\)g gp120\textsubscript{SF162} in 1% NE then boosted i.n. with the same dose 3 weeks later. The serum IgG antibody response toward gp120\textsubscript{SF162} and gp120\textsubscript{Bal} serotypes was then measured at 6 weeks after initial immunization. Anti-gp120 IgGs are presented as OD 405 nm \(\pm\) SD obtained in an ELISA using 1:200 dilutions of serum and either gp120\textsubscript{SF162} or gp120\textsubscript{Bal} as the coating antigen. (B) Neutralizing antibody produced by i.n. immunization with gp120\textsubscript{SF162}/NE. The neutralization of the laboratory strains and the primary isolates of HIV was performed in the TZM-bl cell system. NT\textsubscript{50} values represent the serum dilution at which relative luminescence units (RLU) were reduced 50\% compared to virus control. Individual preimmune sera from the same animals were used to determine background antiviral activity.}
\end{figure}
Polarized Responses and Neutralizing Antibodies to HIV-1

The anti-gp120 IgG isotype contribution to overall antibody response. A significant prevalence of IgG2 (b and a) over IgG1 subclass antibodies clearly indicates a Th1 bias produced by NE vaccination.90 In contrast, combining NE adjuvant with adjuvants such as CpG or MPL A did not have a significant effect on the response to immunization. The lack of CpG effect could be due to a nonoptimized oligonucleotide sequence and to the relatively low dose of CpG used in the study.21,82–84 The addition of MPL A resulted in only a minimal, nonsignificant increase in serum antibody titers, which is in accordance with the findings of VanCott et al.83 where intranasal immunization was performed with gp160/liposome/MPL A. It is a possibility that mixing MPL A with lipids can lower activity as reported with liposome formulations.84 In any case, it appears that this mucosal adjuvant activity is unique and requires the presence of NE.

In summary, data presented in these studies illustrate that nasal gp120/NE immunization produces a systemic and mucosal antibody response with significant, cross-reactive neutralizing immunity and a Th1 polarized CTL response. The mucosal adjuvant capability could be helpful in the design of novel HIV vaccines.

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References


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