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

ANNA U. BIELINSKA,[1] KATARZYNA W. JANCZAK,[1] JEFFREY J. LANDERS,[1]
DAVID M. MARKOVITZ,[2] DAVID C. MONTEFIORI,[3] AND JAMES R. BAKER, JR.[1]

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 (HIV_{BaL} and HIV_{SF162}) 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,

[1]Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS), University of Michigan, Ann Arbor, Michigan 48109.
[2]Internal Medicine, Infectious Diseases, University of Michigan, Ann Arbor, Michigan 48109.
[3]Department of Surgery, Laboratory for AIDS Vaccine Research and Development, Duke University Medical Center, Durham, North Carolina 27706.

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 HIV neutralization activity. 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 $gp120_{BaL}$ and $gp120_{SF162}$ 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°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 (α 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 μg dose of gp120 mixed with 0.1%, 0.5%, and 1% NE concentrations. For immunization with additional immunostimulants, either 5 μg of MPL A or 10 μg CpG ODN was added to 20 μg gp120 in 1% NE or to 20 μg gp120 in saline. A guinea pig immunization study was performed using a 50 μg 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 μl 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 μg gp120 injected in 50 μl 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 μl 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°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 μM DTT and 0.5 mg/ml aprotinin and approximately 1 ml of aspirate was recovered. BAL samples were stored at −20°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 aprotinin. The samples were centrifugated at 10,000 $\times$ *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 NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA), and the remaining cells were washed twice in PBS. For the antigen-specific proliferation or cytokine expression assays, splenocytes (2–4 $\times$ 10^6 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 $gp120_{BaL}$ or $gp120_{SF162}$ 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 ($TCID_{50}$, 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 (NT_{50}) 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 $\times$ 10^6 cell/ml) were incubated with and without $gp120_{BaL}$ (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 $\times$ 10^6 cells/ml (RPMI 1640, 2% FBS) and incubated with either $gp120_{BaL}$, $gp120_{SF162}$ (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 preformed 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 $gp120_{BaL}$ or $gp120_{SF162}$ serotypes to assess whether NE has adjuvant activity in mucosal immunization with recombinant gp120 protein. Immunizations were carried out using 20 μg of $gp120_{BaL}$ 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 $gp120_{BaL}$/NE preparations were seropositive after only two immunizations (Fig. 1). The anti-$gp120_{BaL}$ IgG response showed a concentration-dependent effect of NE, with the lowest titers in $gp120_{BaL}$/0.1% NE and highest in $gp120_{BaL}$/1% NE immunization groups (mean titers of 1.3 $\times$ 10^4 and 2.6 $\times$ 10^5, respectively, Fig. 1A). In contrast, mice immunized with $gp120_{BaL}$ in saline did not have detectable anti-$gp120_{BaL}$ antibodies even after three immunizations. Serum anti-gp120 IgG

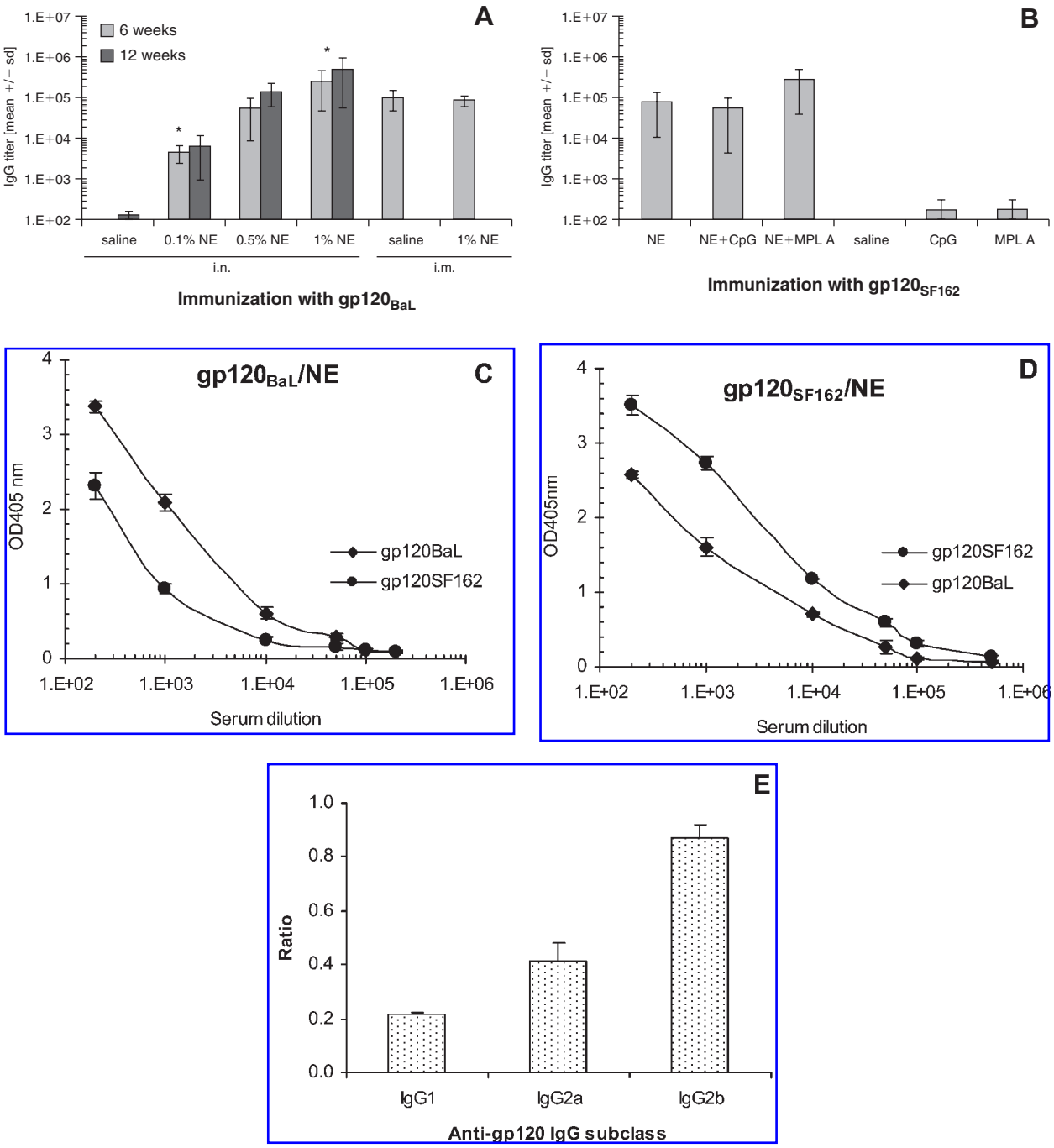

FIG. 1. Antibody response in mice intranasally vaccinated with two serotypes of recombinant gp120 and the nanoemulsion adjuvant. (**A**) Induction of serum anti-$gp120_{BaL}$ IgG in mice immunized with $gp120_{BaL}$ in 0.1%, 0.5%, and 1% NE. Anti-$gp120_{BaL}$ blood samples collected at 6 weeks after 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-$gp120_{SF162}$ IgG in mice i.n. immunized with two doses of $gp120_{SF162}$ 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 $gp120_{BaL}$ (**C**) or $gp120_{SF162}$ (**D**) reacts with both gp120 serotypes. Data are presented as titration curves of pooled serum from all immunized animals binding to either $gp120_{SF162}$ or 120_{BaL}. (**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 $gp120_{BaL}$ 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 $gp120_{BaL}$/NE immunizations produce a systemic immune response in mice.

Additional studies examining the NE immunizations were carried out with a second gp120 serotype, $gp120_{SF162}$. Similarly to immunization with the $gp120_{BaL}$, all mice immunized with $gp120_{SF162}$/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 $gp120_{SF162}$/NE with the addition of either CpG ($gp120_{SF162}$/NE + CpG) or MPL A ($gp120_{SF162}$/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 $gp120_{SF162}$/NE alone; however, this difference was not statistically significant ($p > 0.05$). In contrast, nasal immunization with $gp120_{SF162}$ 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 $gp120_{BaL}$ or $gp120_{SF162}$ 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.[56] The subclass analysis of the anti-gp120 IgG antibodies indicated a prevalence of IgG2b and IgG2a over IgG1 antibodies, thus suggesting 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 adjuvation.

Induction of secretory mucosal anti-gp120 IgA and IgG

BAL fluids, vaginal washes, and sera were analyzed for evidence of mucosal response. Mice nasally immunized with $gp120_{SF162}$/NE had significant levels of $gp120_{SF162}$-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 $gp120_{BaL}$ immunogen. Anti-$gp120_{BaL}$ 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 $gp120_{BaL}$/NE and were absent in either mice immunized with either $gp120_{BaL}$/saline or with control animals treated with saline or NE alone (Fig. 3A). Intranasal immunization with $gp120_{BaL}$/NE also produced systemic, cross-reacting, cell-mediated immunity as demonstrated by significant interferon (IFN)-γ production after *in vitro* stimulation with either $gp120_{BaL}$ or $gp120_{SF162}$ in cells harvested from animals immunized with $gp120_{BaL}$ (Fig. 3B). A substantial induction of the IFN-γ was also obtained in these splenic 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.[9,57] 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, nonimmune mice, or from mice immunized with $gp120_{BaL}$ 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 $gp120_{SF162}$ 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 $gp120_{BaL}$ immunogen. The immune sera from guinea pigs were then tested for neutralizing 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 NT_{50} occurred in a single animal with the immunizing serotype bearing M-tropic strain of HIV_{SF162} with an $NT_{50} = 225$. Neutralizing activity was also present, albeit at lower levels ($NT_{50} > 50$), 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 NT_{50} 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 HIV_{MN} 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.[26,58–62] 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,[41,59,60,62,63] the NE adjuvanted material produced high titers of anti-gp120 antibodies after only two administrations.

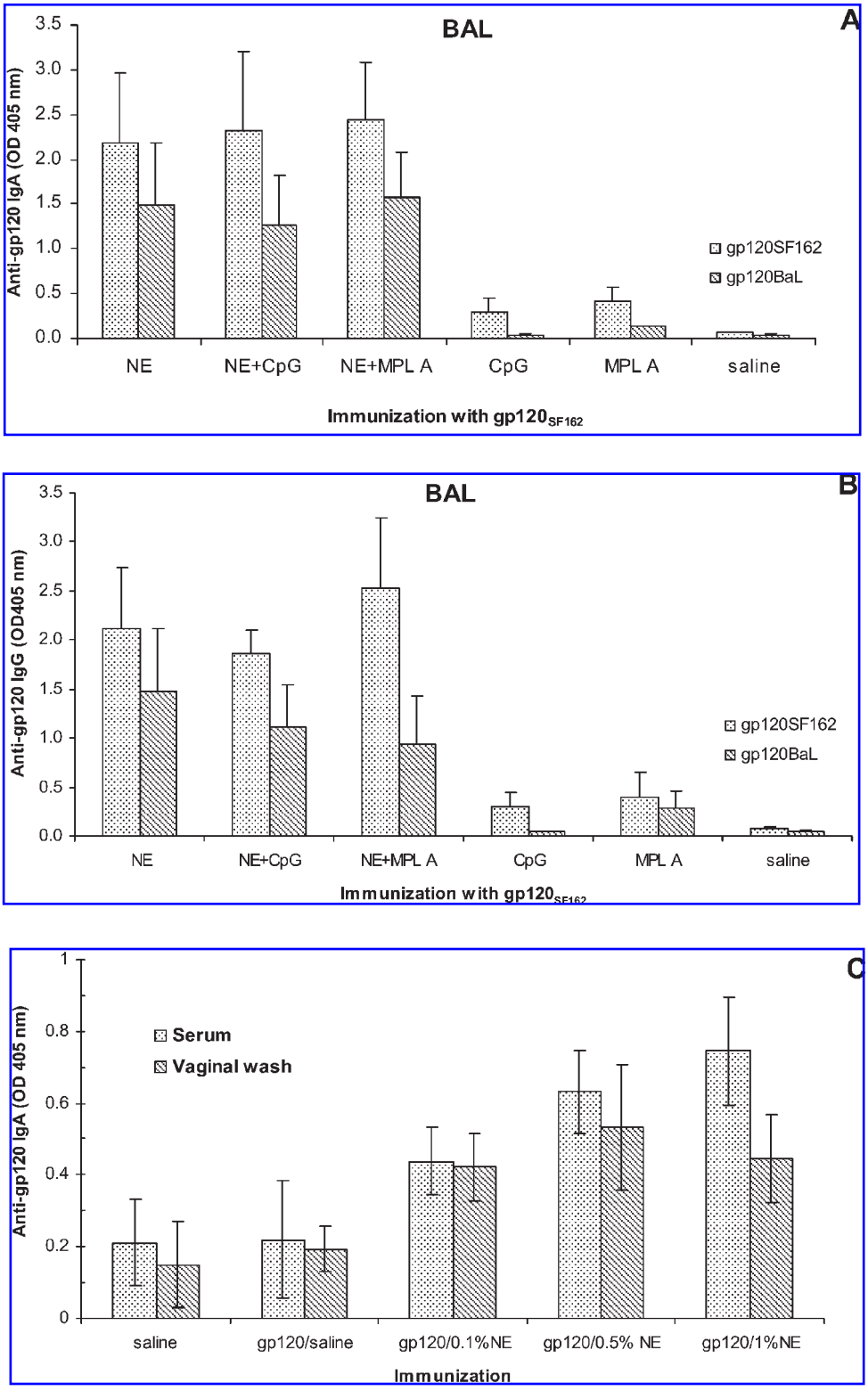

FIG. 2. Nasal immunization with gp120/NE induces mucosal IgA and IgG antibodies. Secretory anti-gp120 IgA (**A**) and IgG (**B**) in bronchial lavage (BAL). The IgA and IgG antibodies recognize both autologous SF162 and heterologous BaL serotypes as indicated. Assays were performed with undiluted and 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 $gp120_{BaL}$ 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 ($p > 0.05$).

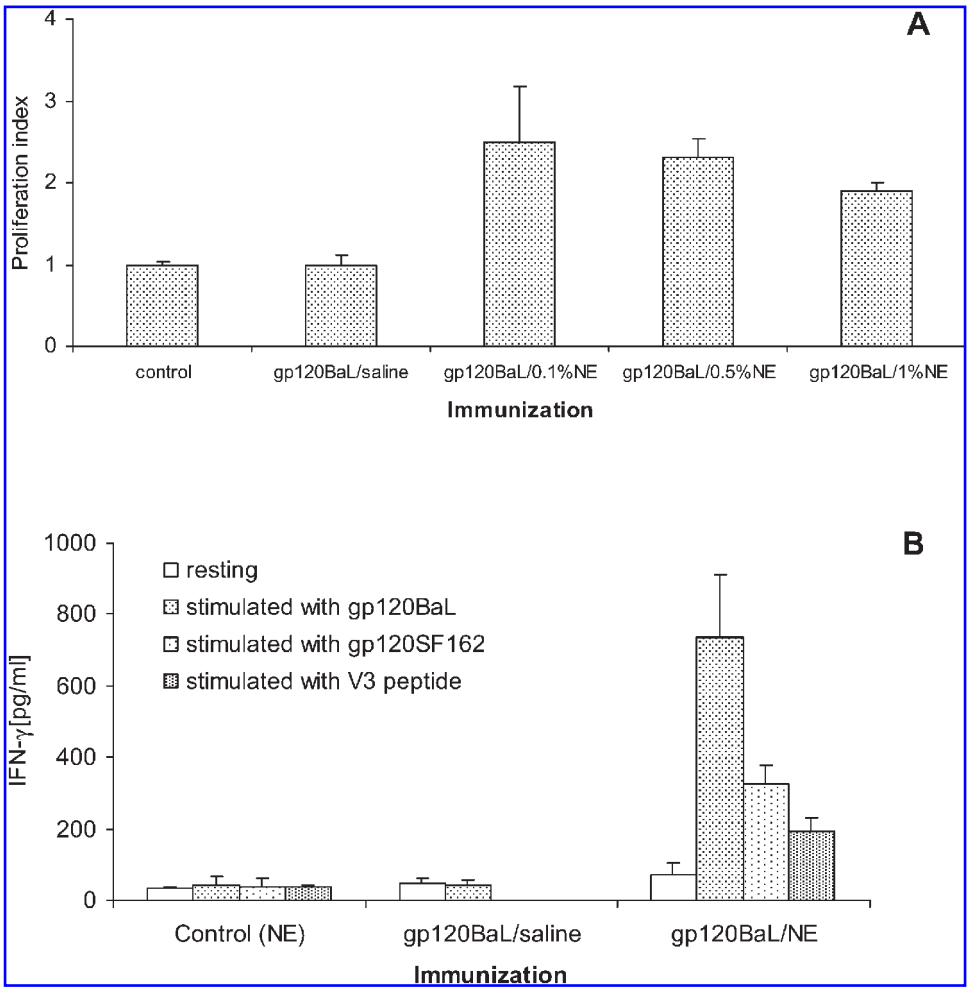

FIG. 3. (**A**) Antigen-specific splenocyte proliferation. Splenocytes from animals immunized with $gp120_{BaL}$ were stimulated *in vitro* with 2 μg/ml of this anitgen. Cell proliferation was normalized to controls and presented as mean ± SD of individual proliferation indexes. The differences between the $gp120_{BaL}$/saline and the $gp120_{BaL}$/NE groups were all statistically significant ($p < 0.05$). (**B**) Antigen-specific activation of cytokine production in splenocytes *in vitro.* Splenocytes from mice immunized with $gp120_{BaL}$ were activated with 5 μg/ml of either $gp120_{BaL}$ or $gp120_{SF162}$, and with 20 nM of the V3 loop peptide. IFN-γ in cell supernatants was determined by ELISA and concentrations are presented as the mean of individual samples ± SD.

In addition, serum IgG gp120 titers were comparable to those reported for intramuscular immunization[59,60] 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 regiments of multiple DNA injections (three or four) followed by subsequent boosts with recombinant antigen or adenoviral vector.[21,64,65]

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.[40,66–68] The presence of anti-HIV antibodies in the genital tract could be useful in the prevention of HIV-1 infection.[2,69–71]

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.[20] 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.[6,56] 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 $gp20_{SF162}$/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

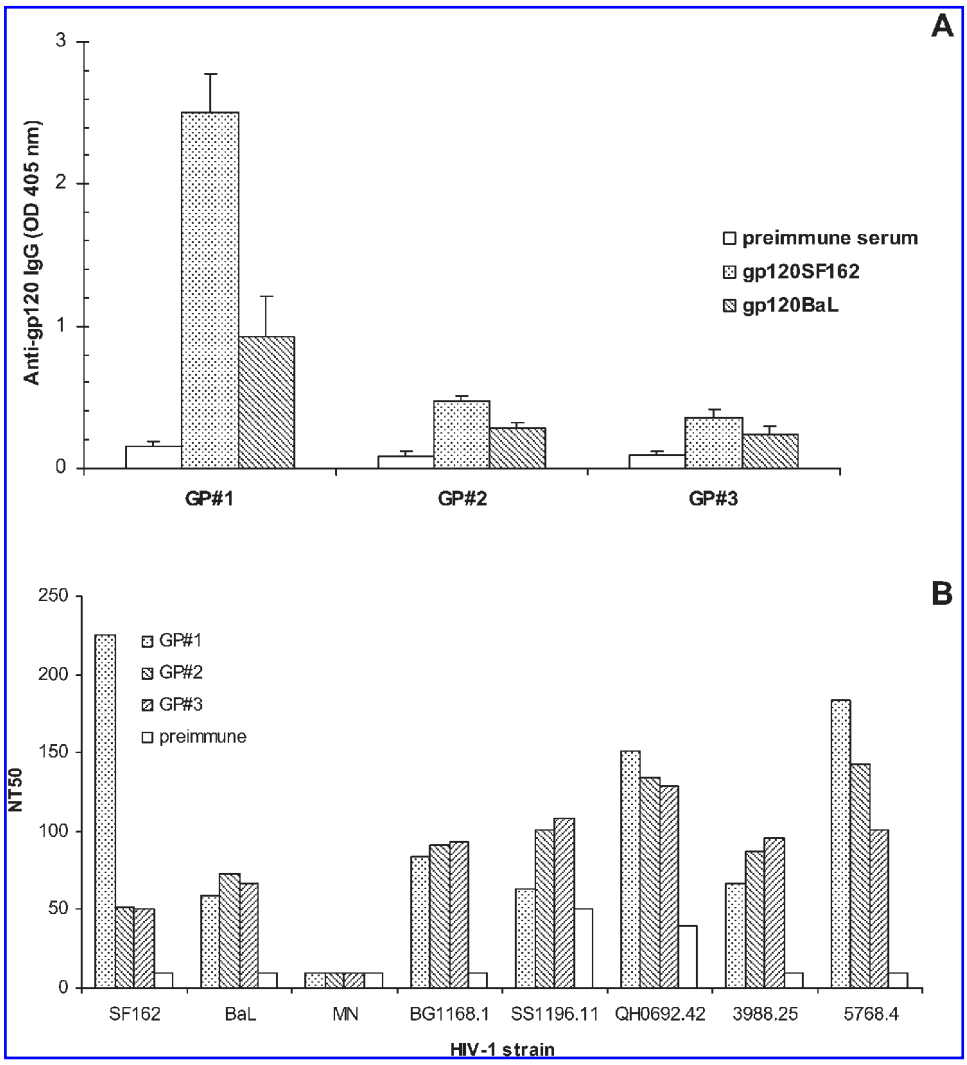

FIG. 4. (**A**) Nasal immunization of guinea pigs. Hartly guinea pigs (GP) were nasally vaccinated with 50 μg $gp120_{SF162}$ in 1% NE then boosted i.n. with the same dose 3 weeks later. The serum IgG antibody response toward $gp120_{SF162}$ and $gp120_{BaL}$ serotypes was then measured at 6 weeks after initial immunization. Anti-gp120 IgGs are presented as OD 405 nm ± SD obtained in an ELISA using 1:200 dilutions of serum and either $gp120_{SF162}$ or $gp120_{BaL}$ as the coating antigen. (**B**) Neutralizing antibody produced by i.n. immunization with $gp120_{SF162}$/NE. The neutralization of the laboratory strains and the primary isolates of HIV was performed in the TZM-bl cell system. NT_{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.

standardized assessment of vaccine-induced neutralizing antibodies.[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_{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.[74] In earlier studies and in vaccine trials, $gp120_{MN}$ and $gp160_{MN}$ failed to produce neutralizing antibodies to the majority of HIV-1 isolates,[75,76] so there may be something uniquely nonimmunogenic 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-γ production in splenic lymphocytes. The induction of hallmark Th1 cytokine IFN-γ indicates activation of $CD4^+$ and $CD8^+$ CTLs with both autologous $gp120_{BaL}$ and heterologous $gp120_{SF162}$ and with 35 aa V3 loop peptide.[77,78] The pattern of cytokine induction with significant INF-γ and absence of IL-4 suggests that NE alone can produce the Th1 polarization cellular response.[79] The cytokine data are supported by the anal-

ysis of 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.[80] In contrast, combining NE adjuvant with adjuvants such as CpG or MPL A[58,81] 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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Address reprint request to:
James R. Baker, Jr.
University of Michigan-MNIMBS
1150 West Medical Center Drive
9220 MSRB III
Ann Arbor, Michigan 48109

E-mail: jbakerjr@umich.edu

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