Proteosomes, Emulsomes, and Cholera Toxin B Improve Nasal Immunogenicity of Human Immunodeficiency Virus gp160 in Mice: Induction of Serum, Intestinal, Vaginal, and Lung IgA and IgG

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Intranasal immunization of mice with human immunodeficiency virus (HIV) rgp160 complexed to proteosomes improved anti-gp160 serum IgA and IgG titers, increased the number of gp160 peptides recognized, and stimulated anti-gp160 intestinal IgA compared with immunization with uncomplexed rgp160 in saline. These enhanced responses were especially evident when either a bioadhesive nanoemulsion (emulsomes) or cholera toxin B subunit (CTB) was added to the proteosome-rgp160 vaccine. Furthermore, anti-gp160 IgG and IgA in vaginal secretions and fecal extracts were induced after intranasal immunization with proteosome-rgp160 delivered either in saline or with emulsomes. Formulation of uncomplexed rgp160 with emulsomes or CTB also enhanced serum and selected mucosal IgA responses. Induction of serum, vaginal, bronchial, intestinal, and fecal IgA and IgG by intranasal proteosome-rgp160 vaccines delivered in saline or with emulsomes or CTB is encouraging for mucosal vaccine development to help control the spread of HIV transmission and AIDS.

Vaccines designed to control human immunodeficiency virus (HIV) transmission will be expedited by the advanced development of mucosal vaccine delivery systems and adjuvants that improve immunity at intestinal and vaginal mucosal surfaces. The strategy of this approach is to confer protection against HIV by limiting viral infection and dissemination at the portals of entry. For HIV, transmission occurs predominantly at vaginal or rectal sites during intercourse [1, 2]. Because of the dichotomy of the systemic and secretory immune systems, mucosal immunization at gastrointestinal or respiratory sites is far more effective than parenteral immunization in inducing mucosal responses [3–5]. Moreover, the concept of the “common mucosal immune system” indicates that appropriately formulated mucosal vaccines can stimulate mucosal immune responses at secretory sites other than the site of vaccine administration [3–5]. When necessary to confer protection, an effective mucosal vaccine should also elicit serum antibodies, particularly when pathogens can be transmitted directly to the systemic circulation, for example, as occurs with HIV in the intravenous drug user population.

Bacteria [6, 7] and viruses [8, 9] that replicate or initiate abortive infections at the mucosal level have been studied as potential vectors for live mucosal vaccines designed to stimulate mucosal and systemic immune responses to heterologous antigens. The nonliving, subunit approach to mucosal vaccines has focused on four categories of delivery systems or adjuvants to enhance mucosal immunogenicity: microspheres (DI-lactide-co-glycolide polymers) [10–13], outer membrane protein proteosomes [14–20], lipids in the form of liposomes [21–25] or lipid matrix coacervates [26], and cholera toxin (CT) [27] and CT analogues. These include a nontoxic recombinant construct of Escherichia coli heat-labile toxin (LT) [28] and CT and LT B subunits (CTB and LTB, respectively), which have been used without conjugation [29–31] or after chemical [31, 32] or genetic [33] linkage to antigens. We investigated intranasal immunization with subunit vaccines using examples from three of these four categories: proteosomes, lipids, and CTB.

Intranasal administration was selected because of proteosome-Shigella vaccine studies, at least 10-fold less vaccine induced comparable or better immune responses and protection.
these preparations were delivered in saline or with CTB or bioadhesive emulsomes. gated in sera, in vaginal, intestinal, and bronchial secretions, and peptide immune responses in sera [38].

rgp 160 intrinsically formulates with submicron lipid emulsions, and the emulsions enhanced epitope-specific anti-rgp 160 protein or (especially) HIV rgp160 plus the combination of proteosomes immunized the HIV envelope. We previously showed that mice or rabbits readily lends itself to proteosome vaccines, since the hydrophobic core of liposomes is a lipid, whereas the internal core in liposomes is an aqueous compartment. By using specific combinations of lipids for the emulsion core, proprietary emulsification and manufacturing techniques, the formation of stable lipid particles, 50–250 nm in diameter, has been achieved [34]. In addition, a bioadhesive polymer in an amount sufficient to confer mucosal adhesion properties to the lipoidal particles has been incorporated with the emulsome preparations to further improve the delivery and attachment of vaccine antigens to target mucosal surfaces.

HIV recombinant envelope protein rgp160, given intramuscularly (im) adsorbed to alum, is currently in clinical vaccine trials [35], as are several other recombinant HIV envelope proteins (reviewed in [36, 37]). Proteosome vaccines consist of virus-sized, multimolecular, membranous vesicles that form from noncovalent complexes between neisserial outer membrane proteins and selected amphiphilic antigens designed to bind to the multi-molecular proteins via their hydrophobic moieties. HIV gp160 readily lends itself to proteosome vaccines, since the hydrophobic gp41 portion of gp160 can serve as the hydrophobic anchor to complex with proteosomes, just as it does in anchoring to the HIV envelope. We previously showed that mice or rabbits immunized im with HIV rgp160 plus proteosomes alone, HIV rgp160 intrinsically formulates with submicron lipid emulsions, or (especially) HIV rgp160 plus the combination of proteosomes and the emulsions enhanced epitope-specific anti-rgp160 protein and peptide immune responses in sera [38].

In this report, anti-HIV IgG and IgA responses were investigated in sera, in vaginal, intestinal, and bronchial secretions, and in fecal extracts. Mice were immunized with either uncomplexed HIV rgp160 or rgp160 complexed to proteosomes, and these preparations were delivered in saline or with CTB or bioadhesive emulsomes.

Materials and Methods

Vaccine Delivery and Adjuvant Components

rgp160. HIV recombinant envelope protein rgp160 was prepared by MicroGeneSys as described [35].

Proteosomes. Outer membrane protein proteosome preparations were purified as described [14, 16, 18] from group B type 2 Neisseria meningitidis by extraction of phenol-killed bacterial paste with a solution of 6% Empigen BB (EBB; Albright and Wilson, Whitehaven, UK) in 1 M calcium chloride followed by precipitation with ethanol, solubilization in 1% EBB-TRIS/EDTA-saline, and then precipitation with ammonium sulfate. The precipitates were resolubilized in the 1% EBB buffer, dialyzed, and stored in 0.1% EBB at -70°C.

Emulsomes. Mucoadhesive emulsome particles were prepared at Pharmos (Rehovot, Israel). As previously described [34], a 1:1 (wt/wt) fat/phospholipid mixture was dissolved in chloroform. The organic solvent was evaporated to complete dryness under reduced pressure using a rotary evaporator (Heidolph, Germany). To the dry lipid film, an aqueous solution containing 0.1% EDTA was added, and the mixture was then hydrated by shaking for 30 min using a multistirrer shaker (Labline, Maywood, NJ) until all the lipids were homogenously dispersed in the aqueous phase. The dispersion was homogenized using a Microrat 70 Gaulin homogenizer (5 cycles at 800 bar). The resultant emulsome particles were determined by use of an particle size analyzer (N4MD; Coulter Electronics, Hialeah, FL) to have a mean diameter of 140 ± 50 nm. Carbocol 934 (BF Goodrich, Atlanta) was added (0.1% final concentration) to enhance mucoadhesive properties of the emulsome preparation. Emulsomes were vigorously mixed with equal parts of rgp160 or proteosome-rgp160 preparations to result in a 2.5% lipid concentration in the emulsome vaccines.

CTB. CTB was purchased from Sigma (St. Louis), and a 1 mg/mL solution was added to appropriate vaccine preparations so that each mouse would receive 10 μg.

Preparation of Proteosome-rgp160 Vaccine

A portion of the stock rgp160 was formulated with proteosomes using dialysis as originally described for noncovalent complexing of proteosomes to peptides [39–42]. Before noncovalent formulation with proteosomes, rgp160 was dialyzed against a TRIS-saline buffer across a 50,000 molecular weight (MW) cutoff membrane for 48 h to remove Tween detergent used in the preparation of rgp160. This rgp160 was then mixed with proteosomes at a 1:1 (wt/wt) ratio in PBS containing 1% Empigen. The mixture was dialyzed across a dialysis membrane with a 1000-MW cutoff (SpectraPor 7; Spectrum Medical Industries, Los Angeles) against 0.01 M TRIS–0.15 M NaCl (TNS) buffer for 10 days at 4°C, exchanging 2 L of TNS buffer each day, and stored at 4°C before and during the immunizations.

Nasal Immunizations

Female BALB/c mice (Frederick Cancer Research Center, Frederick, MD) were used throughout the experiment. Mice (5/group/experiment) were nasally immunized on weeks 0, 3, and 6 with uncomplexed rgp160 or proteosome-rgp160 vaccines containing
All incubations were done in a humid chamber. After aspiration plates (Immulon 2; Dynatech, Chantilly, VA) were coated with a solution of rgp160 (8–10 μg/mL) and incubated at 37°C for 1 h. Debris before storage of the supernatants at −20°C. Serum. Blood samples were collected 7–14 days after the second and third immunization and stored individually at −70°C. Assays were done on pooled samples using equivalent volumes of freshly defrosted samples of sera from each of the 5 mice in the group.

Vaginal secretions. Samples were collected as described [43] using wicks (Polyfiltronic Group, Rockland, MA) inserted before sacrifice, 10–14 days after the third immunization. The day of wick insertion was timed to coincide with the estimated time of ovulation, which had been previously synchronized by placing a male mouse in a nearby cage on the appropriate day. Briefly, the wicks were inserted after instillation of 25 μL of PBS intravaginally and allowed to adsorb secretions for 30–60 s, after which the wick was removed, an additional 25 μL of PBS was instilled, and the opposite end of the wick was inserted into the vagina for another 30–60 s. The wick was transferred to a microfuge tube, immediately frozen with dry ice, and stored individually at −70°C. Secretions from each mouse were individually eluted from the wicks by adding 0.8 mL of a solution of 0.5% each of bovine serum albumin (BSA) and casein plus protease inhibitors to the tube with the wick. The tube was then centrifuged at 10,000 rpm for 15 min before sampling for the ELISA.

Intestinal and lung lavage fluids. Secretions were collected at sacrifice as described [14, 18], 14 days after the third immunization. Briefly, for bronchial lavage samples, immediately after sacrifice by CO2 suffocation, the lungs were surgically exposed, a cannula was inserted in the trachea, and, using a three-way stopcock and PBS containing 0.1% BSA, 2 lung lavage samples (1 mL each) were collected and combined. Intestinal lavage samples were then collected as described [14, 18] by passing 2 mL of PBS containing 0.1% BSA, 50 mM EDTA, and 1 mg/mL soybean trypsin inhibitor through a 20- to 25-cm section of small intestine. Lavage fluids from each mouse were vortexed and centrifuged to remove cell debris and then individually stored at −70°C.

Fecal extracts. Fecal pellets (25–30) were collected and pooled from groups of 5 mice 1 week after the last immunization. Each collected pool of pellets was weighed, and PBS containing 0.1% sodium azide was added to the pellets at 1 mL/0.1 g of fecal pellets. The samples were vortexed vigorously for 15 min and then centrifuged in a microfuge at 14,000 rpm for 15 min to remove debris before storage of the supernatants at −20°C.

Antibody Detection

ELISA. An ELISA was done as described [18], using rgp160 as the detecting antigen. Briefly, 96-well round-bottom microtiter plates (Immulon 2; Dynatech, Chantilly, VA) were coated with a solution of rgp160 (8–10 μg/mL) and incubated at 37°C for 1 h. All incubations were done in a humid chamber. After aspiration of the antigen by use of a plate washer (Skatron, Sterling, VA), plates were washed once with PBS containing 0.05% Tween (PBS-T) and incubated with blocking solution containing 0.5% each casein and BSA (IgG- and fatty acid–free) for 60–90 min at 37°C. After the blocking solution was aspirated and washed twice with PBS-T, duplicate samples of sera, intestinal lavage fluids, bronchial lavage fluids, vaginal secretions, or fecal extracts, serially diluted 2-fold in blocking solution, were added, and the plates were incubated overnight at 37°C. After being washed four times with PBS-T, TMB solution (Bio-Rad Laboratories, Richmond, CA) was added, and plates were incubated at room temperature overnight. After the samples were aspirated and washed twice with PBS-T, absorbance values at 630 nm by use of a microtiter ELISA plate reader (Molecular Devices, Menlo Park, CA).

Absorbance values (shown in figures 1–5) were determined after 1 h of incubation for serum samples and after 10 min of incubation for intestinal and bronchial lavage samples; plates with vaginal samples were stopped with 0.1 M NaOH after 30 min of incubation and then read. For data shown in the table, assays of each of the biologic samples (sera, intestinal lavage fluids, lung lavage fluids, vaginal secretion collections, and fecal extractions) were made on duplicate samples pooled from the 5 mice in each group, ELISA plates were read after 15 min of incubation, and all data were expressed as the highest dilutions that resulted in an OD >0.5. Accordingly, determinations recorded for each of the ELISAs reflect titers measured at the linear portion of the ELISA titration curve and not end-point titers.

Quantitative Western blots. Immunoblots on serum pooled from the 5 mice in each group were done as previously described [38, 44], using seven well-characterized regions spanning gp120 and gp41 in gp160. The amino acids in detecting peptide antigens represented gp160 as follows: C1 (48–128), C2 (254–274), and C3 (342–405), the constant regions 1, 2, and 3, respectively; V3 (290–338), variable region 3 with the principal neutralizing epitope; C448 (453–518), the C-terminus of gp120; C41 (579–605), the gp41 immunodominant region; and CK (735–752), in gp41.

Statistics

Significance of differences of ELISA results were determined by the Mann-Whitney test, using Minitab for Windows version 10.5 software (Minitab, State College, PA).

Results

Serum IgG and IgA responses recognizing rgp160 protein as measured by ELISA (figure 1). Each of the groups of mice (5 mice per group) that were nasally immunized with rgp160 complexed to proteosomes elicited anti-rgp160 protein responses of IgG and IgA classes in pooled sera that were 10- to 100-fold and 100- to 10,000-fold higher, respectively, than those elicited by uncomplexed rgp160 in saline. Enhanced immunogenicity of these proteosome-rgp160 vaccines, delivered either in saline or with emulsomes or CTB, was clearly shown.
after either two or three immunizations, especially when immunizing three times with proteosome-rgp160 plus emulsomes. The capacity of emulsomes or CTB to enhance rgp160 immunogenicity without proteosomes was most evident in serum IgA responses, in which strong anamnestic responses, comparable to those attained by the proteosome-gp160 vaccine in saline, were elicited after three immunizations.

**Serum IgG responses recognizing gp160 peptides as measured by quantitative Western blot analyses (figure 2).** As measured in sera (pooled from the 5 mice in each group) by immunoblot analyses, even after three immunizations, the only peptide recognized by nasal immunization with rgp160 alone in saline was peptide ID (amino acids [aa] 579–605), the known immunodominant peptide in gp41. In marked contrast, nasal immunization with either of the three proteosome-rgp160 preparations not only enhanced the response to peptide ID compared with the response after immunizing with rgp160 in saline but also clearly increased the breadth of the immune response. Proteosome-gp160 elicited antibodies against all seven peptides tested when formulated with either emulsomes or CTB, while five peptides were recognized after immunizing with proteosome-gp160 in saline.

Formulating rgp160 with either emulsomes or CTB without proteosomes elicited intermediate responses compared with those elicited using the proteosome-gp160 vaccine with these adjuvants. Specifically, emulsomes improved the response to ID and induced responses to two other peptides, C448 (aa 453–518) and CK (aa 735–752), and although adding CTB to rgp160 did not improve anti-ID levels, it did elicit strong responses to three other peptides (C1 [aa 48–128], V3 [aa 290–338], and C448) and a weak response to C3 (aa 342–405). These data are consistent with and delineate the specificity of the increases in serum IgG ELISA responses induced by immunizing with rgp160 plus emulsomes or CTB without proteosomes (figure 1).

**Anti-rgp160 intestinal IgA responses (figure 3).** Nasal administration of rgp160 delivered in saline or with CTB but without proteosomes induced minimal levels of anti-gp160 IgA in intestinal lavage fluids. Immunization with proteosome-rgp160 in saline or free rgp160 plus emulsomes elicited intestinal IgA responses with geometric mean levels that appeared to be higher than those elicited with rgp160 alone, but these differences were not statistically significant \( (P < .18) \), since only half of the mice showed strong responses. In contrast, combining proteosome-rgp160 with either emulsomes or CTB induced anti-rgp160 intestinal IgA levels that were 15- to 60-fold higher than vaccines containing rgp160 plus only one of these components and 150- to 300-fold higher \( (P < .01 \) for either group) than the minimal levels induced by rgp160 alone.
Figure 2. Mean serum IgG responses to rgp160 peptides as measured by quantitative Western blot (immunoblot) analyses of duplicate samples of pooled sera (from 5 mice/group) obtained after 3 intranasal immunizations with rgp160 or proteosome-rgp160 administered either in saline or with emulsomes or cholera toxin B subunit (CTB). Immunoblots were done using 7 well-characterized regions spanning both gp120 and gp41 portions of gp160.

**Anti-rgp160 protein vaginal IgA and IgG responses (figure 4).** Nasal administration of rgp160 in saline without proteosomes did not elicit any detectable anti-rgp160 IgA or IgG in collections of vaginal secretory fluids. In contrast, proteosome-rgp160 formulated in either saline or with emulsomes induced substantial amounts of anti-rgp160 of both IgA and IgG classes (P < .007 for either subclass using either vaccine vs. rgp160 in saline). Immunization with rgp160 plus CTB also elicited more anti-gp160 IgG and IgA (P < .01 and P < .027 for IgG and IgA, respectively) than did rgp160 alone, whereas formulation of rgp160 with emulsomes improved vaginal IgG (P < .045) but not vaginal IgA responses.

**Anti-rgp160 protein bronchial IgA responses (figure 5).** Comparable amounts of anti-rgp160 IgA in lung lavage fluids were induced after immunization with each of the formulations of rgp160 without proteosomes (in saline or with emulsomes or CTB) and with proteosome-rgp160 in saline. In contrast, proteosome-rgp160 formulated with either emulsomes or CTB induced at least 10-fold higher anti-rgp160 lung IgA responses than did rgp160 in saline (P < .014 for either group).

**Anti-rgp160 protein IgG and IgA responses in fecal extracts, sera, and mucosal fluids.** To examine IgG and IgA responses in extracts of mouse feces (as a measure of antibodies in intestinal and rectal secretions) and in mucosal fluids (vaginal, intestinal, and bronchial) and sera, 2 additional groups of mice were nasally immunized three times with proteosome-rgp160 formulated either in saline or with emulsomes. As shown in table 1, both groups elicited IgG and IgA anti-rgp160 antibodies that were readily detectable in pooled samples of fecal extracts, in vaginal, intestinal, and bronchial secretions, and in sera.

**Discussion**

Stimulation of intestinal, fecal, and vaginal IgA and IgG (figures 3, 4; table 1) and serum antibodies (figures 1, 2) by nasal immunization with proteosome-rgp160 vaccines fulfilled the prime goals of this study, since HIV infection is transmitted primarily at vaginal or rectal sites during intercourse [1, 2] or directly into the blood, as in the intravenous drug user population. Nasal immunization with proteosome-rgp160 vaccines formulated with emulsomes or CTB also elicited high levels of anti-rgp160 IgA in bronchial lavage fluids, although this was less dramatic since immunization with the other rgp160 vaccines, including uncomplexed rgp160 in saline, also induced significant lung antibody levels (figure 5). The ability of rgp160 alone to elicit lung antibodies may reflect the fact that in mice, a portion of the nasal vaccine (using 30-μL volumes) directly stimulates bronchial mucosa when the mouse inhales, thereby facilitating local bronchial antibody production. For nasal immunization to consistently elicit antibodies at nonrespiratory sites, such as in intestinal or vaginal secretions, however, immunization with enhanced rgp160 formulations was needed to activate the common mucosal immune system. Thus, nasal immunization with proteosome-rgp160 plus emulsomes or
CTB induced the strongest intestinal responses, and proteosome-rgp160 in saline or with emulsomes elicited high levels of vaginal and fecal IgA and IgG. Similarly, addition of CTB to proteosome *Shigella* vaccines improved intestinal responses after respiratory immunization and respiratory responses after orogastric immunization [15].

Other researchers have used various combinations of immunization routes to induce anti-simian immunodeficiency virus (SIV) or anti-HIV antibodies in vaginal, intestinal, or salivary secretions: Microsphere-encapsulated formalin-treated whole SIV elicited vaginal IgG and IgA in monkeys after priming three times im and boosting four times either intratracheally or, to a lesser extent, orally, but not when immunizing only orally [12]. SIV gag p27 virus-like particles elicited vaginal or rectal antibodies in macaques after priming two to four times vaginally or rectally, respectively, and then boosting two or three times orally with the virus-like particles linked to CTB [45, 46]. Rectal antibodies were also elicited by priming twice orally and then boosting four times rectally with these CTB-linked SIV virus-like particles [47]. Anti-HIV salivary IgA was induced by a liposome vaccine in mice after oral priming followed by im boosts [24]. Unlike these reports, the present study shows that by formulating the antigen with proteosomes, vaginal and intestinal antibodies can be induced using only nasal immunization.

Nasal immunization with the proteosome-rgp160 vaccine plus either emulsomes or CTB was optimal for eliciting both IgG and IgA responses in sera, although adding only one of these components to rgp160 also improved immunogenicity compared with that from rgp160 in saline alone (figure 1). The increases in anti-rgp160 protein antibodies were supported by immunoblot data showing that the enhanced vaccine formulations not only increased the intensity of the anti-peptide ID response—the only peptide recognized by sera from mice immunized with uncomplexed rgp160 in saline—but also increased the number of gp160 peptides recognized by serum antibodies to include the hypervariable V3 loop region and each of the other gp120 and gp41 peptides tested (figure 2). Such broadening of the immune response by formulating antigens with enhanced vaccine delivery systems may be especially significant when the antigen alone elicits a response limited to an immunodominant region that does not recognize desired critical peptide epitopes of the protein.

Respiratory immunization has also been shown to be advantageous with other vaccines. Linking CTB to *Streptococcus mutans* protein antigen I/II induced vaginal IgA (when free CT was added) and stronger serum and salivary antibody responses (with or without free CT) in mice after nasal compared with intragastric immunization [31]. Free CTB plus unconjugated protein antigen I/II was immunogenic nasally in mice [31] but not intragastrically [32], and CTB plus influenza virus hemagglutinin was more efficient when delivered nasally than orally to mice [30]. Similarly, liposomes containing influenza virus [25] or polysaccharide [21] antigens were more effective in mice via nasal than by im or oral immunization, respectively. Last, microsphere-encapsulated *Bordetella pertussis* filamentous hemagglutinin was immunogenic and protective in mice after nasal but not oral immunization [13], and intratracheal priming or boosting in monkeys with microsphere-encapsulated SEB toxoid was effective, whereas oral delivery was not [11] (although this vaccine was immunogenic orally in mice [10]).

Several mechanisms may have contributed to the enhancement of mucosal immunogenicity generated by the formulations used in this study, especially when two vaccine delivery/adjuvant components were used. The function of the lipid-core emulsomes may be analogous to that of liposomes, which have been postulated to improve antigen processing by facilitating fusion of antigen-lipid complexes with the lipid membranes of antigen-presenting cells [21]. The bioadhesive component added to the emulsomes used in this study may also have enhanced immunogenicity by promoting “stickiness” of the vaccine, thereby retarding rapid clearance from the respiratory tract and facilitating antigen uptake by mucosal cells.

It is well established that the ability of CTB to bind to mucosal cell surface GM1 ganglioside promotes mucosal IgA responses [27, 29–33]. In addition, commercial CTB prepara-
Figure 4. Anti-gp160 IgG and IgA responses measured by ELISA in vaginal secretions collected after 3 intranasal immunizations with gp160 or proteosome-gp160 preparations administered either in saline or with emulsomes or CTB. Results are geometric mean titers and SEs of individually measured samples of vaginal secretions collected from groups of 5 mice. Titers represent highest dilutions of fluids with adsorbed vaginal secretions with OD >0.5 and hence reflect titers measured at linear portion of ELISA titration curve and not end-point titers.

...tions similar to that used in this study stimulate transepithelial flux of antigens from the nasal cavity into the mucosal tissue [48] and activate antigen-presenting cells [49]. In this regard, Tamura et al. [50] reported that CTB from the commercial source used in this study and in previously reported studies from their laboratories [29, 30, 48, 49] generally contains 0.01%–0.1% CT and that this trace amount of CT contributes synergistically with CTB to the mucosal adjuvanticity of such 99.9% pure CTB preparations. It is posited that the CT in the CTB preparations may provide immunostimulatory activities, such as up-regulation of macrophage major histocompatibility complex class II expression [51] and stimulation of B cell differentiation toward IgG1 and IgA [52], which complement the enhanced uptake mediated by CTB. From a practical vaccine development perspective, we concur with the suggestion of Tamura et al. [50] that CTB or LTB containing 0.1% holotoxin may be a suitable adjuvant for human use, since Levine et al. [53] have shown that such small amounts of CT can safely be given to people. The recent clinical study by Hashigucci et al. [54] suggests that a nasal influenza-LTB vaccine with trace amounts of LT may be promising, although these authors conclude that further studies will be necessary to reduce local and systemic side effects and increase immunogenicity of the vaccine.

Proteosome vaccines contain outer membrane proteins of N. meningitidis, bacteria known to naturally colonize respiratory mucosa. Mucosal epithelial M cells specialize in antigen uptake and transport to antigen-presenting cells in the subepithelium by virtue of a variety of putative glycoconjugate receptors for lectins and lectin-like microbial surface proteins on their surface [55]. In this manner, M cells are thought to selectively deliver bacteria and bacterial antigens to the mucosal immune system for antigenic processing [55]. We speculate that proteosomes may enhance mucosal immunogenicity of the antigens noncovalently associated with them by facilitating M cell-mediated antigen uptake and processing of the complexes via their constituent meningococcal outer membrane proteins.

Immunopotentiation by proteosomes may also be related to the capacity of neisserial outer membrane proteins to recognize and up-regulate major histocompatibility complex class II and
B7.2 costimulatory ligands on B cells [56] and other antigen-presenting cells and to mitogenically stimulate B cell differentiation [42]. The report [57] that alveolar macrophages present antigen inefficiently to CD4 T cells due to defective expression of B7 costimulatory cell surface molecules suggests that proteosomes may enhance respiratory immunogenicity by overcoming this deficiency.

The data in this study indicate that mucosal vaccines using the systems described here should be tested in nonhuman primates to help select the optimal regimen before clinical trials. The recent studies showing that respiratory delivery of proteosome vaccines containing SEB [19] or Shigella [20] antigens enhance mucosal and serum antibodies in nonhuman primates after either two nasal immunizations [20] or booster immunizations after a primary im immunization [19] are encouraging. Furthermore, the potential of nasal proteosome vaccines for human use is being demonstrated in safety and immunogenicity proteosome-Shigella phase I vaccine trials [58]. Indeed, the safety and immunogenicity of the meningococcal outer membrane proteins in proteosome vaccines [59] is one of the compelling reasons advancing proteosome vaccine development.

Table 1. Anti-rgp160 ELISA titers in fecal extracts, mucosal fluids, and sera of mice after intranasal immunization with proteosome-rgp160 or proteosome-rgp160–emulsome.

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<th>Proteosome-rgp160</th>
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<td></td>
<td>IgG</td>
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<tr>
<td>Fecal extracts</td>
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<tr>
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NOTE. ELISAs were done on duplicate samples pooled from 5 mice/group; titers recorded are highest dilutions that demonstrated OD >0.5 after 15 min of incubation with substrate and hence reflect values measured at linear portion of ELISA titration curve and not end-point titers.

The extent to which the enhancement generated by proteosomes, emulsomes, and CTB is further developed in vaccines that are tested on humans will also depend, to a large extent, on the identification of HIV antigens that show significant potential to induce immune responses that neutralize native virus strains. The induction of mucosal IgA by nasal vaccines as described here is significant, since HIV can infect vaginal [60] and rectal [61] epithelial cells, and it is now known that mucosal IgA not only can prevent adherence and absorption of pathogens [4, 62] but also can neutralize intracellular viruses directly within epithelial cells [62]. Neutralizing antibodies were not measured in this study, but it has been reported that IgA from the sera of HIV-infected patients can neutralize HIV [63]. It is anticipated that analyses of neutralizing antibodies will be most fruitful when the delivery systems described in this report are applied to a recently isolated conformationally correct oligomeric form of HIV gp160 [64]. The data described here suggest that application of the proteosome and emulsome or CTB systems to such HIV antigens may further the development of mucosal vaccines designed to induce secretory antibodies that interfere with mucosal transmission of HIV and thereby help limit the spread of AIDS.

Acknowledgments

We thank Anthony Johnson, T. Moseley, Wallace Baze, and Donald Burke for their support and encouragement for this study, Ann-Marie Smith, Jennifer Hatch, Shameera Deane, and Michael Kaminski for skilled laboratory assistance, and Jared Minsk, Steve Gordon, and especially Jerome Smith for their computer program expertise.

References


