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Induction of mucosal HIV-specific B and T cell responses after oral immunization with live coxsackievirus B4 recombinants

Rui Gu[#], Cristy Stagnar[#], Lesya Zaichenko, and Arlene I. Ramsingh^{*}

Wadsworth Center, New York State Department of Health, 120 New Scotland Avenue, Albany, NY 12208

Abstract

Given the limited success of clinical HIV vaccine trials, new vaccine strategies are needed for the HIV pipeline. The present study explored the novel concept that a live enteric virus, with limited disease potential, is a suitable vaccine vector to elicit HIV-specific immune responses in the gut mucosa of immunized mice. Two coxsackievirus B4 (CVB4) vaccine vectors were designed to induce HIV-specific B or T cell responses. A B cell immunogen, CVB4/gp41(2F5), was constructed by expressing an epitope from the membrane proximal external region (MPER) of gp41 as a structural peptide within a surface loop of a capsid protein of CVB4. The T cell immunogen, CVB4/p24(73₃), was constructed previously by expressing a gag p24 sequence as a non-structural peptide at the amino-terminus of the CVB4 polyprotein. The CVB4/gp41(2F5) recombinant was antigenic in mice and elicited anti-gp41 antibodies in both the mucosal and systemic compartments. The route of immunization affected the antibody response since oral delivery of CVB4/gp41(2F5) induced anti-gp41 antibodies in the mucosal but not in the systemic compartment while parenteral delivery induced anti-gp41 antibodies in both compartments. In contrast, oral immunization with CVB4/p24(73₃) elicited both mucosal and systemic gag p24-specific T cell responses. Since coxsackieviruses are ubiquitous in the human population, a key question is whether pre-existing vector immunity will inhibit the ability of a CVB4-based vaccine to induce HIV-specific immune responses. We show that pre-existing vector immunity did not preclude the development of mucosal anti-gp41 antibodies or gag p24-specific T cell responses after oral immunization with the CVB4/HIV recombinants. We suggest that the CVB4/HIV recombinants have the potential to be a viable vaccine product because of ease of delivery, safety, immunogenicity, ease of large-scale production, and storage conditions requiring cold-chain temperatures provided by refrigeration.

Keywords

coxsackievirus; vaccine; gp41; gag p24; gut mucosa; anti-gp41 antibodies; p24-specific T cell responses

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^{*}To whom correspondence should be addressed: FAX: 518-486-7971, Telephone: 518-474-8634, ramsingh@wadsworth.org.

[#]The two authors contributed equally to the study.

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1. Introduction

The development of an effective HIV/AIDS vaccine has proven to be elusive [1–3]. A major barrier in the development of an HIV vaccine is that the immune correlates of protection are not fully understood. The modest success reported for the RV144 efficacy trial in Thailand [4] provides tantalizing evidence that an HIV vaccine capable of preventing systemic infection will halt the HIV epidemic. The outcome of the RV144 trial mirrors that observed with other vaccine platforms in the SIV/macaque model for AIDS [5;6]. To date, four vaccine platforms, SIVmac239 delta nef [7;8], poliovirus/SIV recombinants [9], a DNA vaccine [6], and CMV/SIV recombinants [5], have demonstrated significant protection after challenge of macaques. Of note is that four out of seven macaques immunized using a particle-mediated (PMED) DNA vaccine were completely protected from systemic infection after mucosal challenge with a heterologous SIV [6]. Interestingly, as was observed in the RV144 trial, the DNA vaccine appeared to impart control at the mucosal interface because the plasma virus burden in the unprotected vaccinates was no different from that found in the controls. The data indicate that T cell responses present at the mucosal portals of entry are sufficient to block sexual transmission.

Additional studies have shown that broadly neutralizing antibodies can protect against mucosal SHIV challenge in macaques [10]. Three broadly neutralizing antibodies, 2F5, 4E10, and Z13, recognize linear epitopes in the membrane proximal external region (MPER) of gp41 [11]. Passive administration of either 2F5 or 4E10 provides protection against SHIV challenge at moderate serum neutralizing titers [10]. In addition to neutralizing antibodies, non-neutralizing antibodies with antiviral effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI) play a role in modulating HIV infection [12]. In the macaque model, a vaccination regimen consisting of replication-competent Ad5 prime and a gp140 envelope protein boost elicits non-neutralizing antibody activities that correlate with improved acute- and chronic-phase viremic control following intravenous SHIV challenge [13]. In the RV144 vaccine trial, the immunogens also elicited non-neutralizing antibodies [4]; highlighting the role of non-neutralizing antibodies in protection from HIV infection. The combined data from nonhuman primate studies and human vaccine trials indicate that new vaccine strategies should target the induction of B and T cell responses in both the mucosa and in the systemic circulation.

Given that the gastrointestinal mucosa is the primary reservoir for HIV replication [14;15], vaccine strategies must be able to target the induction of immune responses in the gut. We have been developing a novel HIV vaccine platform using a live coxsackievirus B4 (CVB4) vector [16–18] with the goal of inducing mucosal responses in the gut after oral delivery. Like the polioviruses, coxsackieviruses are small RNA viruses belonging to the enterovirus genus of the Picornaviridae family [19]. Since the group B coxsackieviruses (CVBs) generally cause asymptomatic infections [20], CVB vaccines have not been developed. The CVBs normally enter the body via the oral route, survive the acidic environment of the stomach, establish transient infection in the gut, and induce mucosal and systemic immune responses. Enteroviruses are therefore ideal candidates for development as oral vaccine vectors for the induction of mucosal immunity [21].

We have developed two CVB4 vaccine vectors for inducing HIV-specific T or B cell responses. One vaccine vector is designed to elicit T cell responses [17;18]. This is accomplished by expressing a foreign sequence as an intracellular, non-structural peptide which is processed through the MHC class I pathway, thereby allowing presentation of the peptide to CD8 T cells. Uptake of extracellular antigens from cellular debris by antigen presenting cells (APCs) is expected to allow processing through the MHC class II pathway,

thereby allowing presentation of the peptide to CD4 T cells [22]. We have shown that a recombinant virus, CVB4/p24(73₃), expressing seventy-three amino acids of gag p24 as a non-structural peptide, induces systemic gag p24-specific CD8 and CD4 T cell responses after oral delivery in mice [18]. The other vaccine vector is designed to elicit B cell responses. This is accomplished by expressing a foreign sequence as a structural peptide within a surface loop of a capsid protein that is an immunogenic region, containing both B and T cell epitopes [16;23]. We have chosen an epitope from the MPER of gp41, 2F5, for expression within the DE-loop of the VP1 capsid of the CVB4 vector. In this first report of the construction and characterization of the CVB4/gp41(2F5) recombinant, we also test the hypothesis that oral immunization of mice with the CVB4 recombinants will induce HIV-specific mucosal B and T cell responses. We show that (a) the CVB4/gp41(2F5) recombinant was antigenic in mice, (b) oral immunization with the CVB4/gp41(2F5) recombinant induced mucosal anti-gp41 antibodies, (c) oral immunization with the CVB4/p24(73₃) recombinant induced mucosal and systemic gag p24-specific T cell responses, and (d) pre-existing vector immunity did not preclude the development of mucosal B or T cell responses in immunized mice.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and immunized at the age of 6 to 8 weeks with CVB4 recombinants. Immunization was by intraperitoneal (IP) injection or by oral gavage. The immunizing dose consisted of 5×10^4 plaque-forming units (pfu) of virus followed by a boosting dose of 10^6 pfu of virus, administered four weeks later. Tissues were harvested five days after boosting with the CVB4/gp41(2F5) recombinant and eleven days after boosting with the CVB4/p24(73₃) recombinant. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

2.2. Construction of the CVB4/gp41(2F5) recombinant

An oligonucleotide sequence corresponding to the 2F5 B cell epitope, ELDKWA, was cloned into the CVB4 capsid cassette vector as previously described [17]. Recombinants were screened by restriction enzyme analysis and clones were verified by DNA sequencing. RNA transcripts, synthesized from recombinant cDNA clones with T3 RNA polymerase, were used to transfect monkey kidney cells (LLC-MK2(D)) by electroporation using a GenePulserTM electroporator (Bio-Rad, Hercules, CA). Virus was harvested when transfected cells exhibited 80–100% cytopathic effect (CPE). Recombinant viruses were plaque-purified prior to preparing large-scale viral stocks in LLC-MK2(D) cells. Limited sequence analysis was done on viral RNA obtained from plaque-purified virus and on RNA obtained from infected cells to confirm the presence of the MPER epitope in the CVB4 recombinant.

2.3. Viral infectivity assays

Organs were weighed and homogenates were prepared in phosphate balanced saline (PBS) using a Mini-BeadBeater and 1.0 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) as previously described [18]. Homogenates were prepared from the Peyer's patches, intestine, pancreas, spleen, and mesenteric lymph nodes (MLN). To detect low levels of infectious virus, HeLa cells, grown in 6-well plates, were infected with 0.2 ml of organ homogenate and monitored for the development of CPE. For both the thermal stability studies and the viral replication studies, infectivity was measured using a standard plaque assay with LLC-MK2(D) cells.

2.4. Isolation of small intestinal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL)

The detailed protocol for preparing IEL and LPL was kindly provided by Dr. Leo Lefrancois at the University of Connecticut Health Center (Farmington, CT) (personal communication). After flushing the intestine with cold calcium and magnesium-free Hank's balanced salt solution (HBSS) (supplemented with 1 mM HEPES, 2.5 mM NaHCO₃, 2% fetal bovine serum (FBS), pH7.3) (CMF), Peyer's patches were removed and the intestine was cut into pieces and washed in CMF containing 2% FCS. Tissues were pooled from two mice and IEL were released from the intestine by mechanical disruption. Tissue pieces in CMF containing 10% FBS and 1 mM dithioerythritol (Sigma) were stirred and vortexed briefly to release the IEL into the supernatant. After washing in RPMI-1640, the IEL preparation was resuspended in RPMI-1640, filtered through a 40 μ m cell strainer, and purified on a Percoll gradient. The IEL preparation at the interface of the gradient was collected and washed with cold RPMI-1640. The yield of the IEL preparation from two mice was $4\text{--}5 \times 10^6$ cells. The purity of the IEL preparation was evaluated by staining with anti-CD4 and anti-CD8 antibodies. CD4 T cells comprised 3–5% of the IEL preparation while CD8 T cells comprised 61–64% of the cell preparation.

LPL were then isolated from the intestinal pieces from which the IEL had already been harvested. Tissue pieces were incubated in calcium and magnesium-free HBSS containing 1.3 mM EDTA for 30 minutes at 37°C and subsequently digested with 100 Units/ml collagenase (Gibco), 1 mM CaCl₂, 1 mM MgCl, and 5% FCS for 30 minutes at 37°C. Cells released into the supernatant were harvested and tissue pieces were subjected to a repeat digestion with collagenase. After washing in PBS and 5% FBS, the LPL preparation was purified on a Percoll gradient. Cells were harvested and washed with cold RPMI-1640. The yield of the LPL preparation from two mice was $6\text{--}8 \times 10^6$ cells. The purity of the LPL preparation was evaluated by staining with anti-CD4 and anti-CD8 antibodies. CD4 T cells comprised 20–25% of the LPL preparation while CD8 T cells comprised 5–8% of the cell preparation.

2.5. Isolation of lymphocytes from Peyer's patches

Peyer's patches were excised from the small intestine after flushing the intestine with cold CMF. Six-to-eight nodules were obtained per mouse. The tissue was digested with 100 Units/ml collagenase (Gibco) for 60 minutes at 37°C. The cell suspension was filtered through a 40 μ m cell strainer, washed in CMF and resuspended in RPMI-1640. The average cell yield from one mouse was $5\text{--}10 \times 10^6$ cells. Antibody-secreting cells in the Peyer's patches were detected using the B cell ELISPOT assay (section 2.7). In addition, lymphocytes (4×10^6) from the Peyer's patches were seeded in a T25 flask and stimulated with lipopolysaccharide (LPS) (10 μ g/ml) during a five-day incubation at 37°C. The culture supernatant was then harvested, concentrated using a centrifugal filter unit (Millipore), and tested for the presence of antibodies by ELISA (section 2.6).

2.6. Measurement of anti-gp41 antibodies by ELISA

Anti-gp41 antibodies in sera of immunized mice and in supernatants of LPS-stimulated lymphocytes were measured by ELISA [24]. ELISA Maxisorp (Nunc) 96-well plates were coated with 50 μ g/ml of a gp41 peptide (T-20, fusion inhibitor from the NIH AIDS Research and Reference Reagent Program) in PBS and incubated at 4°C overnight. Purified CVB4 (0.25 μ g/well) was used as the bound antigen for the positive control. Plates were washed three times with PBS, 0.25% bovine serum albumin (BSA), and 0.05% Tween 20 and blocked with PBS, 0.25% BSA, 0.05% Tween 20, and 1 mM EDTA for 30 min at room temperature. Serial-fold dilutions of sera in blocking buffer or concentrated supernatants from cultured cells (section 2.5) were loaded into duplicate wells. Samples added to wells

without bound antigen served as negative controls. After two hours at room temperature, plates were washed three times and blocked for one hour. Plates were washed three times and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) or goat anti-mouse IgA (eBiosciences, San Diego, CA) was added to the wells. Plates were incubated for two hours at room temperature and then washed three times. A p-nitrophenyl phosphate substrate solution (Sigma) was added to each well and optical densities were measured using an ELISA plate reader Sunrise (Tecan) at 405 nm. Serum antibody titer is defined as the reciprocal of the lowest serum dilution whose absorbance is greater than two-fold the absorbance of the corresponding dilution of control serum.

2.7. Detection of anti-gp41 antibody-producing lymphocytes by an ELISPOT assay

Anti-gp41 antibody-producing cells in the Peyer's patches of immunized mice were detected using an ELISPOT assay [24]. Briefly, 96-well nitrocellulose-based plates were coated with 50 µg/ml of a gp41 peptide (T-20, fusion inhibitor from the NIH AIDS Research and Reference Reagent Program) in PBS and incubated at 4°C overnight. Purified CVB4 (0.25 µg/well) was used as the bound antigen for the positive control. Plates were washed in PBS and blocked with 5% BSA. Lymphocytes (2.0×10^7 cells/ml) from the Peyer's patches of immunized mice were added to the wells and incubated at 37°C overnight. Cultures were done in duplicate. After washing with PBS and 0.05% Tween 20, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) or goat anti-mouse IgA (eBiosciences, San Diego, CA) was added to the wells and bound antibodies were detected as described (section 2.6). Spot-forming cells (SFC) were counted using a CTL ImmunoSpot S5 UV Analyzer and CTL ImmunoSpot6 software (Cellular Technology Ltd, shaker Heights, OH). Negative controls in which lymphocytes were added to wells without bound antigen allowed us to define a negative response as less than 50 SFC per well.

2.8. Detection of IFN-γ producing lymphocytes by an ELISPOT assay

The ELISPOT assay [25] was used to enumerate the number of individual IFN-γ secreting cells in the lamina propria, IEL compartment, mesenteric lymph nodes, and spleens of immunized mice as previously described [18]. Antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA) or eBiosciences (San Diego, CA). Briefly, 96-well nitrocellulose-based plates were coated with rat anti-mouse IFN-γ antibody (10 µg/ml), incubated overnight at 4°C, washed with PBS, and blocked with 5% BSA. Lymphocytes were cultured in the presence of an HIV-1 gag p24 peptide (2 µg/ml) for 24 hr at 37°C. The two gag p24 peptides used in the study are p24(10–30), an H-2^d restricted T helper cell (TH) epitope, and p24(65–73), an H-2^d restricted CTL epitope [26]. The p24(10–30) peptide was used at a final concentration of 5 µg/ml while the p24(65–73) peptide was used at a final concentration of 2 µg/ml. Cultures were done in duplicate. Positive controls consisted of lymphocytes cultured with heat-inactivated CVB4 (1×10^6 pfu/well) or a combination of anti-mouse CD3 antibody and anti-mouse CD28 antibody. Negative controls consisted of lymphocytes cultured in the absence of antigen. After washing with PBS, biotinylated rat anti-mouse IFN-γ was added to each well and the bound antibody was detected using a streptavidin-alkaline phosphatase conjugate. SFC were counted as described (section 2.7). Negative controls allowed us to define a negative response as less than 50 SFC per well.

3. Results

3.1. Expression of the 2F5 epitope of gp41 within a capsid protein of CVB4 yields a replication-competent recombinant virus

The ELDKWA sequence in the MPER of gp41 was inserted into the DE surface loop of the VP1 capsid protein of CVB4 (Figure 1A). Since the DE-loop of VP1 plays a role in maintaining the physical stability of the virion [27], disruption of the loop might have

altered viral stability and viral replication. The physical stability of the recombinant was assessed by thermal inactivation. The parental CVB4 vector is thermostable since overall infectivity decreased 20-fold after heating at 44° for 1 hr. The CVB4/gp41(2F5) recombinant displayed a moderately thermostable phenotype since infectivity decreased 200-fold after heating at 44° for 1 hr (Fig. 2A). To determine whether insertion of the 2F5 epitope into the VP1 capsid protein affected viral replication, we assessed the growth kinetics of the recombinant in cell culture, under single-step conditions (Fig. 2B). After 16 hr, the CVB4/gp41(2F5) recombinant replicated as well as the parental CVB4 vector. However, the recombinant showed a longer lag time and replicated to lower titers during the exponential phase of growth than the parental virus. Although the insertion of the 2F5 epitope into the VP1 capsid of CVB4 affected viral stability and viral replication, the CVB4/gp41(2F5) recombinant retains a moderately thermostable phenotype and is replication competent. The CVB4/p24(73₃) recombinant was constructed by inserting the gag p24 sequence (HXB2) corresponding to amino acids 3 to 75 at the amino-terminus of the CVB4 polyprotein [18] (Fig. 1B). We showed that the CVB4/p24(73₃) recombinant shares the thermostable phenotype of the parental virus and is also replication competent [18].

3.2. Route of immunization influences pathogenicity

Viral pathogenicity was assessed after administration of the CVB4/gp41(2F5) recombinant via the oral or intraperitoneal route by measuring viral loads in target tissues and monitoring tissue damage in the pancreas. After oral delivery, infectious virus was detected in the gastrointestinal tract but not in the mesenteric lymph nodes, pancreas or spleen (Table 1). Histological assessment of pancreatic tissues revealed a characteristic pancreatitis in mice immunized via the intraperitoneal route (Fig. 3, panels A and B). Pancreatitis was characterized by exocrine tissue loss, acinar cell necrosis, focal inflammation, and edema. In contrast, pancreatic tissues in mice immunized via the oral route appeared healthy (Fig. 3, panels C and D). Pancreatic acinar cells were intact and well-granulated and showed no evidence of damage. The combined data indicate that after oral delivery, the CVB4/gp41(2F5) recombinant induces a short-lived, localized infection of the gut without systemic spread similar to that observed with the CVB4/p24(73₃) recombinant [18].

3.3. Immunization with the CVB4/gp41(2F5) recombinant elicits serum anti-gp41 antibodies

To determine whether the ELDKWA sequence of gp41, expressed in a surface loop of CVB4, was antigenic, mice were immunized with the CVB4/gp41(2F5) recombinant and serum samples were assayed by ELISA for anti-gp41 antibodies. Two doses of the recombinant were administered in each of three immunization regimens: IP prime and IP boost, oral prime and IP boost, and oral prime and oral boost. Mice immunized via the IP route displayed the highest anti-gp41 antibody titers (Fig. 4). Mice immunized via the oral/IP route developed moderate anti-gp41 antibody titers. In contrast, anti-gp41 antibodies were not detected in mice immunized via the oral route. Sera from mice immunized via the oral route were also assayed for anti-CVB4 antibodies. Mice immunized with the CVB4/gp41(2F5) recombinant had anti-CVB4 antibody titers ranging from 25 to 200 while mice immunized with the empty vector had antibody titers ranging from 100 to 500 (data not shown). The lower anti-CVB4 antibody titers observed in mice immunized with the recombinant probably reflects the altered antigenicity of the recombinant due to disruption of a major neutralization antigenic site in the VP1 capsid by insertion of the gp41 sequence.

3.4. Oral immunization with the CVB4/gp41(2F5) recombinant induces anti-gp41 antibody responses in Peyer's patches

Antibody responses were assessed by (a) determining the frequency of gp41-specific antibody-producing cells in the Peyer's patches of immunized mice by ELISPOT and (b) measuring antibodies in the supernatant of LPS-stimulated lymphocytes from Peyer's

patches of immunized mice by ELISA. Lymphocytes from the Peyer's patches of vector-naïve mice displayed a robust gp41-specific IgA recall response (mean value of 496 SFC) while lymphocytes from vector-immune mice displayed a more modest response (mean value of 142 SFC) (Fig. 5A). Lymphocytes from the Peyer's patches of control mice immunized with the CVB4 vector alone exhibited background responses (< 50 SFC) (data not shown). A gp41-specific IgG response was not detected in lymphocytes from the Peyer's patches of immunized mice (data not shown). The frequencies of gp41-specific IgA producing B cells were determined using the mean SFC values and were 0.02% and 0.01% for vector-naïve and vector-immune mice, respectively. The yield of lymphocytes from the Peyer's patches was insufficient to carry out both ELISPOT and ELISA studies on individual mice. To overcome this problem, we carried out ELISA studies on a pooled sample of lymphocytes from the vector-naïve mice (Fig. 5B) and on individual samples from a subsequent immunization study of vector-naïve mice (Fig. 5C). Lymphocytes from the Peyer's patches were stimulated with LPS in culture for 5 days and the culture supernatant was tested for gp41-specific antibodies by ELISA. The culture supernatant of the pooled lymphocytes from the Peyer's patches of vector naïve mice contained gp41-specific IgA but not IgG thereby confirming the results of the ELISPOT assay (Fig. 5B). Using a standard curve for the IgA antibody, the concentration of gp41-specific IgA in the culture supernatant of the pooled lymphocytes was determined to be 40 ng/ml. All of the samples from the Peyer's patches of individual mice were also shown to contain gp41-specific IgA antibodies (Fig. 5C).

3.5. Oral immunization with the CVB4/p24(73₃) recombinant induces mucosal gag p24-specific T cell responses

The CVB4/p24(73₃) recombinant contains two H-2^d restricted T cell epitopes, a CTL epitope, p24(65–73) and a TH epitope, p24(10–30) [18]. Gag p24-specific T cell responses were monitored by assaying IFN- γ production by ELISPOT after stimulation of lymphocytes with the p24 peptides. Mucosal recall responses were measured in the IEL, LPL, and MLN compartments. Both the IEL and LPL from vector naïve mice produced IFN- γ in response to stimulation with the p24 CTL peptide (Fig. 6A). (IEL and LPL were not stimulated with the p24 TH peptide due to insufficient cell yield). Lymphocytes from the MLN of both vector naïve mice and vector-immune mice produced similar IFN- γ recall responses after stimulation with either the p24 CTL peptide or the p24 TH peptide (Fig. 6A, 6B). A comparison of the IFN- γ recall response in the mucosal and systemic compartments of vector-immune mice showed that while the response to the p24 CTL peptide was similar in the MLN and the spleen, the response to the p24 TH peptide was higher in the MLN than in the spleen (Fig. 6B).

4. Discussion

Several studies show that HIV-1 targets CD4 effector memory T (T_{EM}) cells in the gastrointestinal mucosa [14;15;28;29]. Infection of target cells results in depletion of T_{EM} cells within a few weeks after HIV infection. An effective HIV-1 vaccine should therefore target the induction of mucosal immune responses in the gut. Our reasoning is that a live enteric virus with limited disease potential would be an ideal vaccine vector to elicit mucosal immune responses in the gut. Of note, is that none of the more common viral vectors (adenovirus 5, canarypox, modified vaccinia Ankara, cytomegalovirus) under development as HIV vaccines are enteric viruses. In this study, we showed that oral immunization of mice with live CVB4 recombinants induced HIV-specific B and T cell responses in the gut mucosa. The mucosal B cell response was assessed in the Peyer's patches, an IgA inductive site. Others have shown that antigen-specific B cell responses in the Peyer's patches of mice, immunized orally with tetanus toxoid and cholera toxin, were

10-fold less than that observed in the lamina propria, an IgA effector site [30]. The gp41-specific mucosal B cell response in this study may have, therefore, been underestimated.

Since coxsackieviruses are ubiquitous in the human population [31], a key question is whether pre-existing vector immunity inhibits the ability of a CVB4-based vaccine to induce HIV-specific immune responses. In the present study, we show that pre-existing vector immunity did not abolish the development of anti-gp41 antibodies and gag p24-specific T cell responses in the gut mucosa of immunized mice. Oral immunization of vector naïve mice with the CVB4/gp41(2F5) recombinant resulted in the induction of robust mucosal anti-gp41 antibody recall responses while immunization with the CVB4/p24(73₃) recombinant induced modest mucosal gag p24-specific T cell responses. Interestingly, although pre-existing vector immunity resulted in a reduction of mucosal anti-gp41 antibody responses in mice immunized with CVB4/gp41(2F5), pre-existing vector immunity did not result in a reduction of mucosal gag p24-specific responses in mice immunized with CVB4/p24(73₃). These results are similar to those obtained with a poliovirus vaccine vector. Immunization with a higher dose of a poliovirus recombinant was able to overcome pre-existing vector immunity in a mouse model [32]. In our studies, the dose of the vaccine boost was 20-fold higher than the dose used to induce vector immunity, and may explain, in part, the ability of the CVB4 recombinants to overcome CVB4-induced immunity. Additional evidence that pre-existing immunity does not abolish recall responses after administration of a vaccine boost comes from studies with the oral polio vaccine. Fifty percent of individuals previously immunized with oral polio vaccine show at least a 4-fold increase in antibody titers after administration of an oral polio vaccine boost [21].

Recent work highlights the role of host responses in mucosal tissues in the dissemination of HIV/SIV [33] and several studies have been undertaken to elicit vaccine-induced immune responses in various mucosal compartments. PMED HIV and SIV DNA vaccines have been shown to induce CD8 T cells and TH responses in rhesus macaques [6;34–38]. Challenge studies have shown that PMED DNA vaccines alone or in combination with a viral vector boost afford complete or partial protection against challenges with AIDS viruses [6;38–42]. Additional studies have shown that PMED DNA vaccination is especially effective for inducing mucosal immune responses in the gut that have afforded complete protection against a disseminated infection following rectal challenge with a primary isolate of SIV that is heterologous to the vaccine [6]. Recent work with RhCMV/SIV vectors suggest that the protection observed from intrarectal challenge (repeated limiting dose) with a homologous virus, SIV_{MAC239}, is related to the high frequency of CD8, and possibly CD4, T_{EM}-biased T cell responses that are elicited and maintained both in the mucosal portals of entry and at potential sites of distant viral spread [43]. Studies with recombinant adenoviruses have used different routes of immunization to induce mucosal immune responses in mice and macaques. Intranasal [44–46], oral [47;48], or ileal [48] priming with recombinant adenoviruses has been shown to induce antibody or cellular immunity in the intestinal mucosa.

In the present study, oral immunization of vector naïve mice with the CVB4 recombinant expressing the 2F5 epitope as a structural peptide resulted in the induction of robust mucosal anti-gp41 antibody recall responses and undetectable antibody responses in the systemic circulation. In contrast, the CVB4 recombinant expressing the gag p24 sequence as a non-structural peptide in infected cells induced both mucosal and systemic [18] gag p24-specific T cell responses after oral delivery. Since oral immunization with either recombinant induced a transient infection of the gut, the ability of CVB4/gp41(2F5) to induce mucosal anti-gp41 antibodies after oral delivery and of CVB4/p24(73₃) to induce both mucosal and systemic gag p24-specific T cell responses is likely due to the design of the two constructs. The CVB4 vector has multiple neutralization antigenic sites but the 2F5 epitope is expressed

in only one of the antigenic sites [23]. On the other hand, the p24 sequence is expressed within the CVB4 polyprotein vector and should be expressed at the same level as all CVB4 T cell epitopes.

Given that the MPER of gp41 is an attractive vaccine target, multiple strategies have been used to express MPER epitopes, such as the 2F5 epitope, in various bacterial and viral vector systems with the goal of eliciting neutralizing antibody responses (summarized in [49]). However, these strategies have had limited success. While some recombinants induced antibodies to the 2F5 peptide, none of the recombinants elicited neutralizing antibodies. Only a recombinant rhinovirus, expressing the 2F5 epitope in a surface loop of a capsid protein, has yielded modest neutralizing activity [49] suggesting that the context in which the peptide epitope is expressed is critical. Interestingly, a study published over twenty years ago showed that a poliovirus recombinant, expressing an epitope from the transmembrane region of gp41 in a major neutralization antigenic site (the BC-loop of VP1) of the vector, elicited broadly neutralizing antibodies against HIV-1 [50]. The result provides additional support for the concept that the context of expression of a peptide epitope in a viral vector is important for eliciting HIV-1 neutralizing antibodies. In the present study, the 2F5 epitope was expressed in a neutralization antigenic site of CVB4. To date, we have not assessed the effector functions (neutralization, ADCC, or ADCVI) of antibodies elicited by the CVB4/gp41(2F5) recombinant.

For any live vector system that is being developed for use as an HIV vaccine, a critical question is safety. Historically, live vaccines provide the most effective protection against disease while safer, inactivated or subunit vaccines induce immunity of more limited duration [51]. Vaccine development, therefore, requires balancing safety and efficacy. The HIV vaccine field has focused primarily on the development of replication-defective vaccines and/or subunit vaccines. Given the limited success with those approaches, the field might benefit from including the development of live vaccine candidates such as CVB4 recombinants. The most common result of CVB infection is asymptomatic infection, an undifferentiated febrile illness, or mild upper respiratory symptoms [20]. As a result, a vaccine has not been developed for the group B coxsackieviruses. On rare occasions, CVB infection can result in chronic inflammatory disease of the pancreas, heart or central nervous system. The population most at risk of illness from CVB infection is neonates [31] and individuals with deficiencies in antibody production [52]. When antibody production is defective, coxsackieviruses can establish persistent, productive infections. Persistent infection does not occur in immunocompetent hosts. Therefore, a live CVB-based vaccine is expected to be safe in immunocompetent and T-cell deficient people but not in pregnant women and B-cell deficient individuals. Studies of animal models suggest links between CVB infections and chronic diseases of the heart [53] and pancreas [54;55]. Experimental models of CVB-induced disease utilize an intraperitoneal route of delivery to cause systemic disease. We have shown that pathogenicity is influenced by the route of infection. Oral delivery of CVB4/HIV recombinants results in a short-lived localized infection of the gut without systemic spread while intraperitoneal delivery results in systemic infection and the development of a transient pancreatitis [18;20].

In summary, we have explored the concept that a live enteric virus with limited disease potential is a suitable vaccine vector to elicit HIV-specific antibody responses and cellular immunity in the gut mucosa of immunized mice. We have shown that oral immunization with a B cell immunogen, CVB4/gp41(2F5), was safe and elicited anti-gp41 antibodies in the gut mucosa. Future studies of mucosal immunity will include analysis of anti-gp41 and anti-CVB4 antibodies in extracted feces and vaginal washes. We have also shown that oral immunization with a T cell immunogen, CVB4/gag p24(73₃), was safe and elicited gag p24-specific T cell responses in both the mucosal and systemic compartments. Finally, pre-

existing vector immunity did not preclude the development of HIV-specific mucosal B cell responses, mucosal T cell responses or systemic T cell responses. We suggest that the CVB4/HIV recombinants have the potential to be a viable vaccine product because of ease of delivery, safety, immunogenicity, ease of large-scale production, and storage conditions requiring cold-chain temperatures provided by refrigeration. Additional studies will assess whether oral immunization of macaques with CVB4 recombinants will protect against challenge with a pathogenic SIV.

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Highlights

- Oral immunization with a CVB4 recombinant induced anti-gp41 antibodies
- Oral immunization with a CVB4 recombinant induced gag p24-specific T cell responses
- CVB4 recombinants induced HIV-specific immunity in the gut mucosa
- Pre-existing vector immunity did not prevent induction of HIV-specific responses

Figure 1.

Schematic representation of the viral genomes of two CVB4 recombinants expressing HIV sequences. (A) An oligonucleotide sequence corresponding to the 2F5 B cell epitope, ELDKWA, was cloned into the VP1 sequence at a site encoding a surface loop that is an immunogenic region. The CVB4/gp41(2F5) recombinant expresses the 2F5 B cell epitope as a structural peptide in the VP1 capsid. (B) A partial gag p24 sequence (HXB2) corresponding to residues 3 to 75 was inserted between the 5' untranslated region (UTR) and the start of the VP4 sequence [18]. An artificial 3C protease site, downstream of the gag p24 sequence, results in cleavage of the p24 sequence from the CVB4 polypeptide. The design of the CVB4/p24(73₃) recombinant results in the expression of the p24 sequence as an intracellular non-structural peptide.

Figure 2.

The CVB4/gp41(2F5) recombinant is thermostable and replication-competent. (A) Kinetics of heat-inactivation for the CVB4/gp41(2F5) recombinant and the CVB4 parental vector show that the CVB4/gp41(2F5) recombinant is moderately thermostable. Virus was heated at 44°C for different time intervals and the residual infectivity was determined by plaque assay. Experiments were done twice and both inactivation profiles for each virus are shown. (B) Kinetics of viral replication for the CVB4/gp41(2F5) recombinant and the CVB4 parental vector show that the CVB4/gp41(2F5) recombinant is replication competent. Viral replication was assessed in LLC-MK2(D) cells under single-step conditions. Viral infectivity at different time intervals was determined by plaque assay. Experiments were done twice and both growth curves for each virus are shown. ○, ●: CVB4 vector; △, ▲: CVB4/gp41(2F5).

Figure 3.

The CVB4/gp41(2F5) recombinant induces a transient pancreatitis after IP administration but not after oral delivery. Mice (n=4) were infected with 5×10^4 pfu via the IP route or with 10^6 pfu via the oral route. Pancreas was harvested at 7 or 14 days post infection (dpi), processed for routine histology, and stained with hematoxylin and eosin. Representative sections are shown. (A) 7 dpi, IP infection depicting pancreatitis. (B) 14 dpi, IP infection depicting pancreatitis. (C) 7 dpi, oral infection depicting a healthy, intact pancreas. (D) 14 dpi, oral infection depicting a healthy, intact pancreas. A, acinus (core unit of the exocrine pancreas containing 8–12 acinar cells); IL, islet of Langerhans; an, acinar cell necrosis; f, fat replacement. Original magnification, X250.

Figure 4.

The CVB4/gp41(2F5) recombinant elicits serum anti-gp41 antibodies after IP immunization but not after oral immunization. Mice were immunized with two doses of the recombinant in each of three immunization protocols: IP prime and IP boost, oral prime and IP boost, and oral prime and oral boost. Sera harvested 10 days after the boost were tested by ELISA for anti-gp41 antibodies. Antibody titer is defined as the reciprocal of the lowest serum dilution whose absorbance is greater than two-fold the absorbance of the corresponding dilution of control serum from mock-immunized mice. Each symbol represents the result from one mouse. ND: not detected.

Figure 5.

Oral immunization with the CVB4/gp41(2F5) recombinant induces a gp41-specific antibody recall response in the gut mucosa. Mice were immunized orally with two doses of the recombinant. Peyer's patches were harvested ten days after the boost and the frequency of gp41-specific antibody-producing cells was determined by an ELISPOT assay. In addition, gp41-specific antibodies in the supernatant of LPS-stimulated lymphocytes from Peyer's patches were measured by ELISA. (A) A gp41-specific IgA recall response, as measured by ELISPOT, was observed in the Peyer's patches of immunized mice. The recall response was greater in vector naïve mice than in vector-immune mice. Each symbol depicts the result from one mouse. Statistical analysis was done using the t-test. (B) Gp41-specific IgA antibodies, as measured by ELISA, were observed in the concentrated supernatant (6-fold) of a pooled sample of LPS-stimulated lymphocytes from the Peyer's patches of vector naïve mice. Background absorbance defined by the negative controls is indicated by a dashed line. ■, IgA; □, IgG. (C) Gp41-specific IgA antibodies, as measured by ELISA, were observed in the concentrated supernatants (100 μ l) of LPS-stimulated lymphocytes from the Peyer's patches of individual vector naïve mice. Each bar shows the result from one mouse. Each supernatant was concentrated 5-fold to allow a comparison among mice. Background absorbance defined by the negative controls is indicated by a dashed line.

Figure 6.

Oral immunization with the CVB4/p24(73₃) recombinant induces mucosal gag p24-specific T cell responses. Vector naïve or vector-immune mice were immunized orally with two doses of the recombinant. Tissues were harvested 10 days after the boost and mucosal immune responses were measured using an IFN- γ ELISPOT assay in lymphocytes from the IEL, LPL, and MLN. (A) Gag p24-specific recall responses were observed in the IEL, LPL, and MLN compartments of vector naïve mice. The magnitude of the recall responses in the three compartments was similar. IFN- γ responses were observed after stimulation with either the p24 CTL peptide or the p24 TH peptide. (B) Gag p24-specific recall responses were observed in lymphocytes from both the mucosal and systemic compartments of vector-immune mice. The IFN- γ response in the MLN of vector-immune mice was greater than that in the spleen after stimulation with the p24 TH peptide. Negative controls defined a negative response as less than 50 SFC per well, indicated by dashed lines. Statistical analysis was done using the t-test.

The CVB4/gp41(2F5) recombinant induced a transient infection in the gut after oral delivery.

Table 1

Time p.i. (hrs)	No. of organs containing infectious virus			
	Intestine	Peyer's patches ¹	MLN ²	Spleen
16	3/3	1/3	0/3	0/3
48	2/3	0/3	0/3	0/3

Organ homogenates (0.2 ml) were used to infect HeLa cells and infectivity was evaluated by the development of CPE.

¹ 5 or 6 Peyer's patches harvested per mouse;
² 5 or 6 MLN harvested per mouse.