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Oral Immunization of Rhesus Macaques with Adenoviral HIV Vaccines Using Enteric-coated Capsules

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Abstract

Targeted delivery of vaccine candidates to the gastrointestinal (GI) tract holds potential for mucosal immunization, particularly against mucosal pathogens like the human immunodeficiency virus (HIV). Among the different strategies for achieving targeted release in the GI tract, namely the small intestine, pH sensitive enteric coating polymers have been shown to protect solid oral dosage forms from the harsh digestive environment of the stomach and dissolve relatively rapidly in the small intestine by taking advantage of the luminal pH gradient. We developed an enteric polymethacrylate formulation for coating hydroxy-propyl-methyl-cellulose (HPMC) capsules containing lyophilized Adenoviral type 5 (Ad5) vectors expressing HIV-1 gag and a string of six highly-conserved HIV-1 envelope peptides representing broadly cross-reactive CD4⁺ and CD8⁺ T cell epitopes. Oral immunization of rhesus macaques with these capsules primed antigen-specific mucosal and systemic immune responses and subsequent intranasal delivery of the envelope peptide cocktail using a mutant cholera toxin adjuvant boosted cellular immune responses including, antigen-specific intracellular IFN- γ -producing CD4⁺ and CD8⁺ effector memory T cells in the intestine. These results suggest that the combination of oral adenoviral vector priming followed by intranasal protein/peptide boosting may be an effective mucosal HIV vaccination strategy for targeting viral antigens to the GI tract and priming systemic and mucosal immunity.

Keywords

oral delivery; enteric coatings; adenoviral vectors; HIV-1 peptides; mucosal vaccination

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INTRODUCTION

Adenoviral type 5 (Ad5) vectors are potent gene delivery vehicles that are capable of stimulating systemic and mucosal immune responses [1]. Mucosal responses, including secretory IgA (sIgA) and mucosal-homing T cells have been shown to be important for protection of mucosal surfaces [2–4]. In fact, mucosal immunization is superior for generating long-lasting mucosal immunity [4–6]. However, studies have shown that oral mucosal vaccination produces much lower CD8⁺ T cell responses than systemic delivery [7] and requires much higher viral particle numbers for eliciting strong responses [8,9]. Moreover, oral vaccination studies with Ad vectors have shown that some animals do not mount an immune response, indicating that intestinal mucosal surfaces may not be reliably infected [8,10]. The difficulty of oral vaccination is not surprising considering that Ad5 is a respiratory pathogen and thus not adapted for survival in the intestinal tract. Moreover, the low pH and digestive enzymes of gastric fluid have been attributed to inactivation of Ad vectors *in vitro* [11]. A change in pH from 8 to 5 can decrease viral titer by 10⁷-fold [12]. Since stomach acids can reach a pH as low as 1.2, there is strong rationale for the enteric protection of Ad vectors for oral delivery.

Previous studies have successfully used acid-resistant, biodegradable enteric polymer coatings to protect antigens for oral immunization [13–15]. Additionally, enteric coatings have been used with lyophilized Ad vaccines and recombinant Ad vectors for oral immunization [16–19]. These pH-dependent enteric polymers have proven effective for controlling drug delivery to the small intestine and the proximal colon [20–23]. The efficacy of any oral vaccine may depend on the delivery to regions where large aggregates of lymphocytes are located in the distal mammalian small intestine, the jejunum and ileum [24]. Therefore, we hypothesized that enteric-coated lyophilized Ad vaccine vectors could be protected from the harsh conditions of the digestive tract and controlled to release in immunologically relevant portions of the intestinal tract.

While there is prior art for applying enteric-coated adenoviruses, much of this work has been performed in an industrial setting where few methodological details were provided to guide other investigators to test the approach in small scale in a laboratory setting. Towards this end, we developed an enteric polymethacrylate formulation for coating hydroxy-propyl-methyl-cellulose (HPMC) capsules containing lyophilized Ad5 using a simplified coating apparatus. We verified the integrity of the coated capsules at varying pH and the activity of the lyophilized Ad vectors after capsule dissolution. A first generation recombinant Ad5 vector expressing HIV-1 gag (Ad-gag) and Ad5 expressing a string of six conserved CD4⁺ and CD8⁺ T cell epitopes of HIV-1 envelope (Ad-env-peptide) were lyophilized in HPMC capsules, coated using a non-industrial simplified tray technique with anionic hydrophilic 1:1 and 1:2 methacrylic acid and methylmethacrylate co-polymers (Eudragit®). Given commercial capsules are too large for testing in mice, the enteric-coated capsules were tested in a pilot study using rhesus macaques. Three adult rhesus macaques were immunized by oral priming with the enteric-coated capsules containing the Ad-gag and Ad-env-peptide constructs, and induction of antigen-specific humoral and cellular immune responses were observed. The peptide-specific cellular responses were boosted by intranasal delivery of the peptide-cocktail using a mutant cholera toxin that was effective as a mucosal adjuvant in our earlier murine studies [25,26]. These results in a non-human primate model provide proof of principle for a prime-boost HIV vaccination strategy employing Ad vectored antigens for priming and peptides/proteins in combination with the CT2* adjuvant for boosting in order to generate mucosal and systemic protective immunity.

MATERIALS & METHODS

Cells and Viruses

The human cervical carcinoma cell line (HeLa) and the human embryonic kidney cell line (293A) were maintained in Dulbecco's modified Eagle's Medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% antibiotic-antimycotic (Gibco, Gaithersburg, MD).

Ad vectors used in this study are based on the AdEasy system (Q-BIOgene) and carry the full E1- and E3-deleted Ad5 genome. The stability studies used an Ad vector containing the firefly luciferase gene, an internal ribosome entry site, and the humanized *Renilla* GFP expressed from a cytomegalovirus (CMV) immediate-early promoter in the E1 region (Ad-Luc-IRES-hrGFP). The immunization studies used two Ad vectors containing two separate antigen genes expressed from a cytomegalovirus (CMV) immediate-early promoter in the E1 region. The first vector, Ad-gag, was generated by cloning a codon-optimized HIV-1 gag gene [27] downstream of a chimeric intron obtained from the pCI vector (Promega, Madison, WI). The second vector, Ad-env-peptide, was generated by gene building a synthetic construct (5'-CTCCTGAATTCAGATCTGACCATGGTACCGTCCTCCGTGTCCTGGGGCATCCTCC TGCTGGCCGGCCTGTGCTGCCTGGTCCCCGTCTCCCTGGCGGTACCCAAGCTGTG GGTACCCTACTACGGGGTGCCCGTGTGGAAGGAGGCCATTATCAGCCTGTG GGACCAGTCCCTCAAGCCCTGCGTCAAGCTCACCCCCCTGTGCGTGTCCCTGTCC GTGATACCCAAGCCTGTCCCTAAGGTGAGTTTCGAGGGAACCGGGCCCTGTACC AACGTGTCTACCGTGCAGTGCACCCACGGTATCAAGCCGGTGC GGATCCAGCGC GGACCAGGCAGGACATTCGTAACGATCGGCAAATTTCTGGGCTTCCTCGGCGCC GCCGGGAGCACGATGGGCGCGGCAAGCCTGACTCTTACTGTCCAGGCTAGACA GTATTTGCGCGACCAGCAGCTGCTGGGCATCTGGGGCTGCGGACCGGTGTAAGC GGCC GCCTCGAGTCTAGATGAGTGAG-3') from 14 overlapping 60-mer oligonucleotides. This construct expresses the six conserved HIV-1 envelope peptides, 104, 111, 113, 116, 63, and 61 as a "string of beads" fused to the c-terminus of the α -1 antitrypsin secretory leader and a linker to facilitate secretion and MHC II display. Virus was propagated, purified by CsCl gradient centrifugation, and viral particles (v.p.) were quantitated as described [28].

Animals

Three adult female rhesus macaques (*Macaca mulatta*) of Indian origin between the ages of 8–17 years, from the specific pathogen-free breeding colony at the Michael Keeling Center for comparative medicine and research of The University of Texas MD Anderson Cancer Center, Bastrop TX, were used in the study. The monkeys were maintained in animal facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and the study was conducted according to National Institute of Health Guidelines on Care and Use of Laboratory Animals.

Peptides

A group of six HIV-1 envelope peptides, demonstrated in our previous studies to be effective antigens/immunogens for priming HIV-specific cellular immune responses in multiple animal models and humans, were employed [29–34]. The peptides were prepared in the institutional antigen-core facility utilizing FMOC solid phase chemistry on a PTI Symphony Peptide Synthesizer (Protein Technologies Inc., Tucson, AZ) and the purity of the peptides was determined to be >95% by high-pressure liquid chromatography and was validated by mass spectrometry.

Lyophilization of Adenoviral Vectors

A maximum of 20 μl per capsule of Ad vectors dissolved in potassium phosphate buffered saline (KPBS) with 1.0 M sucrose [35] were flash frozen in clear Quali-V[®] HPMC capsules (Size 2, Qualicaps Inc., Whitsett, NC) on dry ice, placed in 1.5-ml microcentrifuge tubes, wrapped with weighing paper, and immediately placed in a pre-chilled LabConco 300-ml flask. The contents were freeze-dried using a FreezeZone[®] 4.5 L Benchtop Freeze Dry System 77500 (LabConco, Kansas City, MO) for 2 hr at -50°C with an initial vacuum of 133×10^{-3} mBar and a final vacuum of 3×10^{-3} mBar. Capsules were immediately stored desiccated at 4°C .

Enteric Coating of HPMC Capsules

Prior to usage, HPMC capsules were evaluated for uniformity of weight and material integrity by light microscopy. Disintegration time of uncoated capsules was also evaluated in ddH₂O. Under constant stirring in a 500-ml sterile beaker, talc ($\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$, Sigma, St. Louis, MO) was added slowly and gradually to an aqueous solution of isopropyl alcohol (95.6% v/v). A 4:1 ratio (8.8% w/w) of Eudragit[®] L100 and S100 methacrylic acid co-polymers (Degussa Röhm Pharma Polymers, Darmstadt, Germany) was added very slowly to the talc suspension. The plasticizer triethyl citrate (TEC, Sigma) was added drop-wise to the suspension, covered with Parafilm, and mixed for an additional hour to ensure homogeneity. Both sides of the capsules were coated with three wet coats of the enteric polymer dispersion using a ProCoater (Torpac Inc., Fairfield, NJ).

Adenoviral Vector Stability Assay

Ad-Luc-IRES-hrGFP (5.0×10^9 v.p.) was lyophilized in HPMC capsules, enteric-coated, and stored desiccated at 4°C . Preparations were dissolved in triplicate at room temperature (RT) in 5 ml of phosphate-buffered saline (PBS, pH 7.2) in a 6-well plate until complete dissolution was observed. Next, HeLa cells were infected with reconstituted solution at 100 v.p./cell, assuming a maximum theoretical reconstituted concentration of 10^6 v.p./ μl . After incubation at 37°C for 24 hr, cells were lysed, and luciferase activity (in lumens) was measured as described [36]. Capsule preparations were measured at 24 hr, 48 hr, and each week for four weeks post-lyophilization, and luciferase activity was compared at each time point to a stability control of freshly-diluted stock virus at 100 v.p./cell. Gene expression was also monitored by green fluorescence microscopy.

Integrity Test of Coated HPMC capsules

Prior to each dissolution test and oral delivery into rhesus macaques, enteric-coated HPMC capsules ($n=6$) were placed in a 0.1 N HCl solution at 37°C in a water bath with shaking for 2 hr. Afterwards, the capsules were dried at 25°C for 45 min., weighed, and visually observed by light microscopy.

In Vitro Capsule Dissolution and Stability Test

HPMC capsules were loaded with 100 mg of bromophenol blue sodium salt (Sigma) and enteric-coated as described. Capsules ($n=6$) were dissolved with shaking in sodium phosphate buffers of varying pH (6.0, 6.5, 7.0, and 7.5) and small samples were taken at varying time points until the capsules were completely dissolved. The absorbance was read and analyzed at 590 nm using an HTS 7000 Bio Assay Reader (PerkinElmer, Wellesley, MA). *For Ad release, 5×10^9 vp of virus were lyophilized and coated as described above. Capsules were exposed to buffer at pH 6 or 7.5 and samples of the buffer were assayed by real time PCR of hexon. 5 μl sample of each virus was diluted 1/20 in PBS. 1 μl of Qiagen Proteinase K was added to the sample, and it was incubated at 55°C for 1hr and the Proteinase K was heat inactivated at 95°C for 20 minutes. The sample was then subjected to real-time PCR and viral genomes were calculated based on a standard curve of Ad virions.*

Immunization of Rhesus Macaques

In a pilot study funded by the Center for AIDS Research (CFAR) at Baylor College of Medicine, 1.0×10^{11} v.p. of Ad-gag and 1.5×10^{10} v.p. of Ad-env-peptide, were lyophilized and enteric coated 24 hr prior to oral administration to rhesus monkeys. Macaques were anesthetized and enteric-coated capsules were inserted down the esophagus in to the stomach using a feeding tube. This procedure was repeated at 4, 8, and 12 weeks, making a total of four oral immunizations. At weeks 28 and 32, the macaques received intranasal immunizations with a mixture of the six HIV-1 envelope peptides (each at 100 µg/dose/monkey) along with the mucosal adjuvant CT2* (10 µg/dose/monkey) in 600 µl sterile PBS. Given the limited budget of the pilot study, control macaques were not included in the test. Instead, pre-immunization samples and historical comparisons were used to assess immunogenicity. Given that these macaques were specific-pathogen-free (SPF) and had not been manipulated previously, pre-immunization controls all generated only background levels of antibody and cellular responses.

Collection of Samples

All samples were collected at *the indicated time points just prior to immunization*. Blood samples were collected in sodium heparin from the femoral vein. Before the separation of peripheral blood mononuclear cells (PBMC) from the blood samples, plasma was collected and stored immediately at -80°C until analyzed. The PBMC prepared from the blood samples by the standard ficoll-hypaque density-gradient centrifugation were used for various immune assays. Using a 3 cc syringe, saliva was collected from the pharyngeal area or in the cheek pouch. Vaginal wash samples were obtained from anesthetized animals by applying approximately 1 ml of sterile PBS into the vagina and aspirating the released fluid. Soon after collections, the various biological fluids were subjected to a short centrifugation, mixed with protease inhibitors and kept at -80°C until assayed. At week 36, the study was terminated and various tissues were obtained from the three monkeys at necropsy for various immunological and immunohistochemistry analyses.

Anti-gag Enzyme-linked Immunosorbent Assay

Nunc-Immuno™ MaxiSorp™ (Nalge Nunc International, Rochester, NY) enzyme-linked immunosorbent assay (ELISA) plates (Corning Inc., Corning, N.Y.) were coated with 100 µl/well of 1 µg/ml of HIV-1 p55 (LAV/SF2 mix, NIH AIDS Research and Reference Reagent Program) in Plate Coating Solution (Hybridoma Subtyping Kit, Calbiochem, San Diego, CA), washed thoroughly with PBS with 1% Tween (PBST) and then blocked with 5% milk in PBST at RT for 2 hr. Plates were then washed with PBST, and saliva and vaginal washes were diluted 1:2 in PBS. Diluted samples were incubated in triplicate at 100 µl/well at RT for 1 hr. Plates were washed with PBST and peroxidase-conjugated anti-monkey IgA goat antibody (Rockland Immunochemicals, Gilbertsville, PA) was incubated at RT for 1 hr. Plates were then washed with PBST, and bound antibody was detected by a colorimetric reaction with tetramethylbenzidine (Calbiochem). Reactions were stopped with 1M sulfuric acid, and absorbance was read at 450 nm. The oral (saliva) and vaginal responses were normalized by subtracting the background absorbance taken prior to immunization (week 0) and plotted against the number of weeks post-oral priming. Statistical significance was verified for each time point against the background using a Student's paired *t* test and a *p* value < 0.05 .

Assay for Neutralizing Antibodies Against Adenovirus

Samples were incubated in triplicate at the indicated dilutions with Ad5 expressing luciferase for 1 hour at 37°C . The resulting solution was then added to 293 cells at 250 viral particles per cell for 24 hours and luciferase activity was measured. Data is expressed as geometric mean titers that reduced Ad luciferase activity 50%.

Proliferation Assay

PBMC samples were obtained from animals at the indicated time points before each immunization. The proliferation of PBMC samples from the monkeys obtained at different time points during the study were determined by the standard [³H] thymidine incorporation assay, using the six HIV-1 envelope peptides (each at 10 µg/ml final concentration) as a single mixture (designated as pep-mix) as well as the peptides corresponding to the gp41 and gp120 portions of the envelope as separate mixtures (designated as gp41 peptides and gp120 peptides, respectively), recombinant HIV-1 gag protein p55 (0.5 µg/ml), heat-inactivated purified preparation of the HIV-1_{III}B (equivalent to 20,000 cpm of reverse transcriptase activity), and heat-inactivated control Ad vector (0.5 µg/ml). The culture medium and Con A (5 µg/ml) served as negative and positive controls, respectively. Additionally, in some experiments an unrelated control peptide (from human papilloma virus, HPV) at 10 µg/ml final concentration was used as negative control, and no differences were observed compared to culture medium as a negative control. Aliquots of the PBMC (10⁵/well) were seeded in triplicate wells of 96-well plates. The proliferative response in terms of stimulation index (SI) was calculated as fold-increase in the radioactivity over that of the cells cultured in medium alone. The responses to antigens were considered positive when the SI values were 2.0.

ELISPOT Assay for Detecting Antigen-specific IFN-γ-producing Cells

Freshly-prepared PBMC were stimulated with the different HIV antigens as described above for the proliferation assay to determine the numbers of IFN-γ-producing cells by the ELISPOT assay using the methodology reported earlier [33,37,38]. Briefly, aliquots of PBMC (10⁵/well) were seeded in triplicate wells of 96-well plates (polyvinylidene difluoride backed plates, MAIP S 45, Millipore, Bedford, MA) pre-coated with the primary IFN-γ antibody. After incubation for 36 hr at 37°C, the cells were removed and the wells were thoroughly washed with PBS. Subsequently, 100 µl of biotinylated secondary antibody to IFN-γ (detection antibody) was added to the wells for 3 hr at 37°C followed by avidin-peroxidase treatment for another 30 min. Purple colored spots representing individual cells secreting IFN-γ were developed using freshly-prepared substrate (0.3 mg/ml of 3-amino-9-ethyl-carbazole) in 0.1 M sodium acetate buffer, containing 0.015% hydrogen peroxide. Plates were washed to stop color development, and spots were counted by an independent agency (Zellnet Consulting, New Jersey, NJ) using the KS-ELISPOT automatic system (Carl Zeiss, Inc. Thornwood, NY) for the quantitative analysis of the number of IFN-γ spot forming cells (SFC) for 10⁵ input PBMC. Responses were considered positive when the numbers of spot forming cells (SFC) with the test antigen were at least five and also were five above the background control values from cells cultured in the medium alone or with the negative control peptide.

Isolation of Intestinal Lymphocytes

The intraepithelial lymphocytes (IEL) from the jejunal sections of the macaques were isolated by using modifications of previously described procedures [39,40]. Briefly, samples of jejunum 10- to 20-cm long were collected within 30 min of necropsy. The tissue was cut into 0.5-cm² pieces and rinsed in cold PBS. The mucosa were removed from the underlying muscularis layer and the tissue strips were treated with dithiothreitol (DTT, 1.5 mg/ml) in Hank's balanced salt solution (HBSS) for 30 min at RT with agitation to remove intestinal mucus. The tissue strips were then treated with 0.75 mM EDTA in Ca²⁺- and Mg²⁺-free HBSS at RT for 60 min with stirring. After the treatment, the supernatants containing epithelial cells were removed and digested with 15 U of collagenase D and DNase I in complete RPMI 1640 medium containing 100 U of penicillin and 100 U of streptomycin per ml, 5 ml L-glutamine, 5 ml HEPES buffer, and 10% FCS at 37°C with rapid shaking for 2 hr. The resulting cell suspensions were passed through stainless steel screen cups to remove the residual tissue fragments. The cells were washed and re-suspended in complete RPMI 1640 medium and enriched for lymphocytes by

isotonic discontinuous Percoll (Sigma) density gradients (35 and 60% [vol/vol]) at 1,000 *g* for 20 min at 4°C. The lymphocyte band at the interface between the 35 and 60% Percoll layers was collected. Viability of lymphocytes was greater than 95% as determined by trypan blue exclusion staining. Aliquots of freshly-isolated cells were stained with fluorescently-labeled antibodies for flow cytometric analysis, while the remaining cells were washed in PBS and cryopreserved.

Assay for intracellular cytokine production

Lymphocyte populations were isolated from various tissues as previously described [41]. Cells were cultured in RPMI with 10% FBS, HEPES, and antibiotics at a density of 10⁶ cells/ml in a 24-well dish at 37°C. To stimulate antigen-specific T cells, cultures were treated with or without pep-mix (each peptide at 1 µg/ml) along with the costimulatory mAb, anti-human CD49d (9F10, 0.5 µg/ml). Golgistop (BD) was added to un-stimulated and stimulated cultures at a concentration of 1 µl/ml. Cells were harvested after 5 hr and stained with anti-CD8 Pacific Blue (RPA-T8), anti-CD4 PE-Cy7 (SK3), anti-CD95 APC (DX2). Cells were then fixed in 3% paraformaldehyde in PBS and stored overnight at 4°C. The next day, the cells were permeabilized by incubating in Perm/Wash solution (BD) for 20 min followed by incubation with anti-IFN-µ FITC (4S.B3) or control mouse IgG1 FITC (MOPC-21) for 30 min at 4°C. Cells were then washed once in Perm/Wash solution and fluorescence intensities were immediately measured on a CyAn™ flow cytometer (Dako, Carpinteria, CA). All antibodies used were purchased from BD Biosciences and used according to manufacturer's instructions.

Immunohistochemistry analyses

Expression of nerve growth factor-b1 (NGF-b1) in the olfactory bulbs of rhesus macaques was used as a surrogate to determine retrograde transport of the cholera toxin mutant adjuvant CT2* according to the procedure described earlier [42]. Briefly, at the time of necropsy, the olfactory bulbs were obtained from each monkey and perfused with PBS at 25°C followed by Zamboni's fixative (4% paraformaldehyde, 15% picric acid) in 0.1 M phosphate buffer. After overnight incubation at 4°C, the tissues were placed in fresh 4% paraformaldehyde and then transferred to a 30% sucrose solution at 4°C for 48 hr. The tissues were frozen in OCT compound, cut into thin sections (6 µm), and placed on microscope slides pre-coated with 10% BSA in saline. Frozen tissue sections were subjected to NGF-b1 antibody (Chemicon International, Temecula, CA) immunohistochemistry and immunofluorescence staining by reacting with avidin-biotin conjugate followed by incubation with streptavidin-HRP or streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR), respectively. Slides were examined with a fluorescence microscope (BX50/BXFLA; Olympus, Tokyo, Japan) equipped with a digital image capture system (Olympus) and photographed.

RESULTS

Adenoviral vector lyophilization and stability assay

Ad vectors have previously been lyophilized in various buffers using a multi-stage single shelf lyophilizer showing losses of one to five logs of activity compared to pre-lyophilized Ad [35]. We chose the optimal buffer of KPBS with 1 M sucrose in the Croyle *et al.* study for all Ad vectors requiring lyophilization. The sole purpose for lyophilization in this study was to allow Ad vectors to be encapsulated and subsequently enteric coated for determining feasibility of eliciting mucosal immune responses after oral vaccination. It is important to note that HPMC capsules placed in water will become pliable in a few seconds, completely lose integrity in less than 5 min, and completely dissolve in 10 min. Thus, Ad vectors were flash-frozen within the capsules immediately prior to lyophilization.

To assess vector viability after this process, Ad-Luc-IRES-hrGFP was lyophilized within HPMC capsules using a two-stage freeze drying process. Capsules containing lyophilized vectors stored at 4°C for varying times were completely dissolved in PBS and luciferase reporter gene expression was assessed on HeLa cells. *Vector viability reduced dramatically after one week of storage and leveled off at around 5% through 4 weeks* (Fig. 1). Since vector viability was highest after 24 hr of storage, all oral immunizations with enteric-coated capsules containing vaccine vectors were timed accordingly. Viability was also determined by evaluating hrGFP expression on HeLa cells by fluorescence microscopy with similar trends observed of decreased activity over time (data not shown).

Enteric polymer dissolution and stability test

The goal of the enteric coating is to remain solid and protect the contents of the capsule in the stomach, but then release its contents in an optimum time frame within the physiological pH range and the small intestinal transit time (SITT) of rhesus macaques. Survey of the literature suggests that macaque bowel segments have similar pH profiles as humans, but can have more variable transit times (Table 2). To assess the viability of the polymer, capsules were loaded with the dye bromphenol blue, coated with polymer, and the pH-specific release of the dye at 37°C was assessed over the range of pH found in stomach (1.5) and digestive tract of non-human primates and over the pH ranges at which the polymers are reported to be soluble (6.0 – 7.5). By this assay, it was shown that the polymer remained solid at pH 1.5 and prevented dye release (Fig. 2A). As pH was increased, dye release increased with near burst release occurring at pH 7.0 and 7.5. Similar dissolution curve analysis at 4°C and 25°C gave similar kinetics (data not shown). Moreover, the dissolution curves for capsules six months old held to the same trends (data not shown).

A similar study was performed monitoring Ad release from the polymer-coated capsules. Ad transduction activity could not be used as a surrogate marker, since any virus released at low pH would be rapidly inactivated. Given this, we performed real-time PCR of viral genomes released into buffers at pH 6 and pH 7.5 (Fig. 2B). This assay demonstrated that the larger Ad virions were released more slowly than the small dye with near burst release after 80 minutes of exposure at pH 7.5. The real-time signal declined at later time points, perhaps due to DNA degradation or loss of virus or DNA to the plastic. Given data on monkey intestinal pH and transit times (Table 2), these data suggest that this particular enteric-coated capsule may begin dissolution in the jejunum and may mediate vector release prior to entry into the colon.

Immunization of Rhesus Macaques

Three rhesus macaques were immunized orally with 1.0×10^{11} v.p. of Ad-gag and 1.5×10^{10} v.p. of Ad-env-peptide lyophilized in enteric coated capsules at 0, 4, 8, and 12 weeks. Ad-gag expresses the codon-optimized HIV-1 gag gene. Ad-env-peptide expresses six conserved HIV-1 envelope peptides, 104, 111, 113, 116, 63, and 61 previously shown to elicit MHC I and II-restricted responses in multiple animal models and humans, were employed [29–34]. These peptides were expressed as a “string of beads” fusion protein fused to the c-terminus of the α -1 antitrypsin secretory leader and a linker to facilitate secretion and MHC II display. After oral adenovirus immunization, humoral and cellular immune responses were monitored against Ad, gag, and the envelope peptides through week 28. At weeks 28 and 32, the macaques received intranasal immunization 100 μ g each of six HIV-1 envelope peptides mixed with the mucosal adjuvant CT2*. This immunization would boost the env-peptide-specific responses, but not responses against Ad or gag.

Humoral immune responses against HIV antigens after oral Ad immunization

To assess if mucosal priming of the humoral immune responses was being produced by oral immunization, saliva and vaginal secretions were collected from the macaques *immediately*

before each immunization and were assayed by ELISA for antigen-specific sIgA against gag (the envelope peptides do not generate antibody responses [29]). Monkey numbers, 56 and H361, generated HIV-1 gag-specific sIgA in the saliva (Fig. 3A), and all monkeys produced vaginal anti-gag sIgA (Fig. 3B), indicating that gene delivery to the GI tract was successful. While anti-gag antibodies were observed, these were not persistent. *Parallel testing of the samples for IgA, M, and G in vaginal and salivary samples generated a similar kinetic of antibody responses and decay (data not shown).*

Humoral immune responses against adenovirus after oral Ad immunization

ELISA for anti-Ad5 antibodies in mucosal samples failed to detect anti-vector antibodies, except in the saliva of monkey H361 at week 17 (data not shown). In contrast, testing for neutralizing antibodies against Ad5 in the serum detected variable responses in the animals (Fig. 3C). Macaque 53 generated the strongest responses with geometric mean titers (GMT) of anti-Ad neutralizing antibodies above 160 at week 19 at then end of the Ad immunization sequence. These neutralizing antibodies remained at this level through week 36 or 4 months after last Ad immunization. Macaque 56 showed low, but detectable neutralizing antibody titers of 20 and 5 at weeks 19 and 28, but these declined to background by week 36. In contrast, macaque H361 showed no Ad neutralizing activity at any time point tested (Fig. 3C). The high levels of anti-vector serum humoral immune responses in macaque 53 could explain the attenuation of anti-HIV antibody responses with time. In contrast, the lack of serum neutralizing antibodies in H361 and low level response in 56 give a less obvious correlation for loss of anti-HIV antibodies in the mucosal tract.

PBMC proliferative responses after oral Ad immunization

Peripheral blood mononuclear cell (PBMC) samples were collected *before any immunization and* from the immunized macaques at varied times and were assayed for proliferation and IFN- γ production after stimulation with recombinant HIV-1 gag protein and mixtures of the cognate HIV-1 envelope peptides (Fig. 4). All three monkeys exhibited positive proliferation responses (SI values ≥ 2.0) to various combinations of the HIV-1 envelope peptides mainly during the priming phase between weeks 12 and 19 (Fig. 4A). This was true for the PBMC stimulated *in vitro* with all the six envelope peptides as a single mixture (pep-mix) as well as the gp41 and gp120 subsets (mixtures of 2 and 4 peptides respectively). With respect to Ad-gag used for priming, a low response to the recombinant HIV-1 gag protein (SI 7.7) was observed in the monkey #53 at week 12 (Fig. 4B). As a measure of success for oral delivery and release of capsule contents, monkeys #53 and #56 showed low proliferative responses to the adenoviral capsid at week 17 (SI 4.2 and 4.8, respectively), while all three monkeys exhibited strong responses at week 19 (SI 73.6, 92.5, and 25.5, respectively, Fig. 4B). This suggests that perhaps anti-vector cellular immune responses may have attenuated gene delivery to the GI tract and persistent anti-gag antibody responses (Fig. 3). Strong proliferative responses (average SI values ranging between 19.2 and 23.9) were observed with Con A in all three monkeys at all the time points tested indicating their immune competence (Fig. 4B).

PBMC ELISPOT responses after oral Ad immunization

Measurement of IFN- γ -producing cells within the PBMC samples showed no detectable IFN- γ production in response to stimulation with the mixture of all six HIV-1 envelope peptides (pep-mix) or with the gp41 and gp120 subset peptide mixtures (Fig. 4C). Similar results were obtained when the heat-inactivated HIV-1_{IIIIB} was used as the antigen for *in vitro* stimulation in this assay. Similarly, no IFN- γ -producing cells were observed when the PBMC from the animals were stimulated either with recombinant p55 HIV-1 gag protein or with adenovirus, even though strong responses were obtained with Con A stimulation. *The proliferation and*

ELISPOT data suggest that the oral mucosal immunization crossed over only moderately into the systemic compartment from the mucosal compartment.

Intranasal boosting with synthetic peptides adjuvanted with inactivated cholera toxin

After all oral Ad immunizations were completed and humoral and cellular responses due to this were assessed, the animals were boosted with protein vaccine by the intranasal route. *At weeks 28 and 32, the macaques received intranasal immunization 100 µg each of six HIV-1 envelope peptides mixed with the mucosal adjuvant CT2* [25,26]. In this case, the conserved envelope peptides were to boost the env-specific responses primed by oral capsule Ad vaccination. While env responses should be boosted, gag and Ad responses would not.*

While all three monkeys had positive proliferation responses after priming, boosting with the peptides mediated only slight increase in these responses against env when PBMCs were stimulated with the same peptides *in vitro* (Fig. 4A). In contrast, very strong proliferative responses were observed against heat-inactivated HIV-1_{III_B} preparation only after intranasal boosting whereas only negligible levels of responses were observed during the priming phase. In contrast, ELISPOT IFN-γ responses from PBMCs against the six HIV-1 envelope peptides, the gp41 and gp120 subset peptides, and against inactivated HIV were markedly increased after intranasal boost (Fig. 4C). No IFN-γ-producing cells were observed when the PBMC from the animals were stimulated either with the recombinant p55 HIV-1 gag protein or adenovirus antigens, even though strong responses were obtained with Con A stimulation (Table 3). Since recombinant gag protein was not used during the boosting phase, it is difficult to determine whether the Ad-gag priming was effective in priming any precursor populations. Similarly, given that we did not have a separate group of animals immunized with the peptide-cocktail in the presence of the CT2* adjuvant due to the limited funding of this pilot project, it is difficult to rule out that the IFN-γ responses were solely due to the intranasal immunization regimen.

Post-mortem testing of tissue samples

Animals were euthanized 36 weeks after first immunization and samples were collected from the PBMCs, spleen (SP), mesenteric lymphnodes (MLN), and intestinal tissues to determine if any memory T cells were resident in these sites. The PBMCs and splenocytes would be representative of T cells in the systemic compartment whereas the mesenteric lymphnodes and intestinal tissue should be representative of mucosal T cells, particularly relevant to oral immunization. *When T cells from these sites from macaque 53 were stimulated with the HIV-1 envelope peptides, IFN-γ-producing CD8⁺ and CD4⁺ T cells were observed only in the intestine and not in other samples (Fig. 5). Previous testing with unimmunized macaques showed no responses by the same assay (data not shown). Similar assay on H361 failed to detect any responses (data not shown). Insufficient numbers of T cells were obtained from macaque 56 for the assay. Counterstaining the PBCMs from macaque 53 for cell surface CD95 to identify memory cells, revealed that again these cells were only resident in the intestinal samples. IFN-γ⁺ CD8⁺ and CD4⁺ T cells were not detected in the peripheral blood, spleen, and lymph nodes from macaque 53. Among the CD95⁺ T cells, 24% of the CD8⁺ T cells were IFN-γ⁺ whereas 8% of the CD4⁺ T cells were IFN-γ⁺. The presence of peptide-specific CD8⁺ and CD4⁺ T cells in the intestine of macaque 53 suggest that the prime-boost immunization induced peptide-specific memory T cells in the intestine.*

Safety of CT2* as a mucosal adjuvant

We also attempted to address one important issue related to safety of using the cholera toxin mutant as mucosal adjuvant by the immunohistochemical analyses of brain tissue, specifically the olfactory bulb because antigens/adjuvants delivered by the intranasal route can be retrograde transported into the brain potentially causing complications such as Bell's palsy [43]. Based on literature reports on similar studies, we determined the expression of the nerve

growth factor beta 1 (NGF- β 1) as a surrogate for toxicity in terms of retrograde transport to olfactory bulb [42]. As a control we used the tissue similarly obtained from another monkey (H341) from a different study where the monkey was dosed with the native form of the cholera toxin (nCT) that is known to cause retrograde transport to the brain and activation of NGF- β 1 in the olfactory bulb [43]. The side-by-side immunohistochemical analyses revealed NGF- β 1 expression only in the tissues of the monkey immunized using the nCT but not those where CT2* was used as adjuvant (Fig. 6). These results are similar to those reported by McGhee's group [42] where another mutant of CT was observed to be non-toxic to rhesus macaques in terms of lack of NGF- β 1 expression, when compared with that using nCT.

DISCUSSION

The goal of this study was to generate enteric-coated capsules for oral vaccines under laboratory conditions and provide these methods to the academic vaccine community. Towards this goal, we utilized reagents and instruments that can be purchased by the basic scientist including anionic hydrophilic polymers, HPMC capsules, and standard lyophilization procedures.

A simple lyophilization procedure was adapted from [35]. Ad vectors in KPBS and 1.0 M sucrose were flash frozen in capsules on dry ice to avoid dissolution of the water-soluble capsule and were lyophilized in a standard laboratory freeze-drying system. Vectors were directly loaded into capsules to avoid having to weigh μ g masses of lyophilized virus powder to achieve relevant single doses of vaccine. By this approach, vectors lost 50% activity within a day of storage at 4°C and eventually stabilized to 95% lost activity by 3 weeks. The loss of activity for this buffer contrasts with Croyle *et al.*, in which only 10% activity was lost over 10 days at 4°C [35]. In this case, a more intricate lyophilization procedure was followed rather than our simple one, which may explain the difference in virus stability. A more recent study of lyophilized replication-competent Ad in tablets reported 33% loss in activity with the same buffer, but after extended 3-day lyophilization [44]. Alternatively, this difference in stability could be due to our flash-freezing of the virus directly into capsules versus the lyophilization in glass vials [35]. If so, one could hypothetically lyophilize in a vial and then transfer to a capsule. However, weighing the relevant number of particles into a vial would likely be problematic. We anticipate the use of more effective stabilizing agents such as 0.4% Sucrose/0.4% Mannitol/0.001% Span 20 [35] and a more rigid lyophilization procedure would likely improve vector stability.

The polymers used were Eudragit® L100 and Eudragit® S100 which are methacrylic acid esters that are insoluble below a pH of 5.0. The enteric reagents were developed as a peroral dosage form with a step-wise release of the coated ingredient within the GI tract as a function of its luminal pH. Eudragit® L100 is a 1:1 co-polymer of methacrylic acid and methylmethacrylate and Eudragit® S100 a 1:2 copolymer. The gastroresistance of polymethacrylates can be attributed to the exposed ester side chains that are very resistant to hydrolysis, and the pH dependent solubility can be attributed to the step-wise alkaline hydrolysis of the exposed esterified terminal groups [45]. However, the Eudragit® polymers are not ionized and solubilized at a specific pH, but rather a range of pH. Since the ratio of carboxyl to ester groups in the polymer chains of Eudragit® S100 is 1:2 and 1:1 in Eudragit® L100, the lower ratio of carboxyl groups in S100 causes a lesser degree of ionization in an alkaline environment and therefore a decreased rate of solubilization [46]. By varying the type of Eudragit® polymer blend and ratio, the resulting drug release and dissolution kinetics in response to changing pH can be varied accordingly [47]. A ratio of 4:1 of Eudragit® L100 to Eudragit® S100 [48,49] was used in this study (Table 1) for the purpose of allowing the increased presence of Eudragit® L100 to cause pore formation, creating channels for alkaline media to penetrate the coating, causing dissolution of the polymer coat, and releasing the capsule contents.

HPMC capsules were used in this study since they promote good polymer to polymer compatibility with anionic hydrophilic enteric polymers [21], they eliminate the need for pre-coating treatments [50], and they avoid incorporating additional adhesive polymer derivatives that may alter dissolution behavior and release kinetics [51]. In order to ensure the adhesion of the enteric coating polymer dispersion, the amount of plasticizer (TEC) was adjusted to manufacturer recommended specifications for enteric coatings (Table 1). Because pharmaceutical acrylic polymers are brittle, fragile and exhibit strong adhesiveness [52], the addition of plasticizers and glidants, such as talc (Table 1), ensures the favorable elasticity of the coatings by decreasing polymer tensile strength [52,53], lowering the film forming temperature without adverse mechanical effects [54–58], and facilitating GI transit through mucosal surfaces by avoiding polymer dispersion agglomeration.

When polymer-coated capsules were tested directly for dissolution kinetics, the polymer remained intact at low pH values and mediated burst release of small molecule dye at pH 7.0 and 7.5. Release of Ad from the capsules was more delayed, with near burst release after 80 minutes of exposure at pH 7.5. Published literature on monkey intestinal pH (Table 2) would suggest that these enteric-coated capsules should remain intact through the duodenum and begin dissolving in the jejunum. Given the 80 minute delay in Ad release at pH 7.5 and jejunum transit times from 0.5 to 6 hours, we anticipate that virus release from the capsules likely occurs prior to the colon. Whether this is indeed the case and to what degree variation in the site of Ad release affects mucosal immune responses will require additional studies.

After oral immunization with capsules bearing replication-defective Ad vectors expressing HIV-1 gag and HIV-1 envelope peptides, combined mucosal and systemic immune responses were observed. In particular, oral delivery of Ad generated detectable anti-gag IgA responses in saliva of two of the three animals and vaginal IgA in all of the animals, which declined rapidly after immunization. *Production of higher affinity IgM or IgG antibodies against gag may have reduced detection of gag-specific IgA in these ELISA assays. However, ELISA with secondary antibodies against IgA, M, and G failed to detect more persistent antibodies in the samples (data not shown). This suggests, the formation of immune complexes may not explain the loss of anti-gag antibodies in the animals.*

In contrast to the mucosal antibody responses against gag, anti-adenovirus antibodies were only detected in one sample from one animal by ELISA. This contrasted with assays for serum antibodies against Ad, where responses were detected in two animals. Macaque 53 had strong Ad neutralizing activity by the end of the Ad immunization series with GMT above 160 and these antibody levels persisted through 36 weeks. In contrast, macaque 56 had weaker serum neutralizing activity (GMT =20) and H361 had no detectable neutralizing antibodies in the serum. Given that macaque H361 had the most persistent salivary anti-gag antibodies and macaque 53 had the most persistent vaginal antibodies, a direct correlation between anti-Ad antibodies and vaccine-generated antibody persistence is unclear. In contrast, robust cellular responses were observed in all of the animals at weeks 17 and 19 and these coincided with the loss of anti-gag IgA antibodies. These data suggest anti-vector T cell responses and possibly antibody responses may have a role in attenuating anti-HIV responses after oral delivery. Even if anti-vector immunity is to blame for the eventual reduction in IgA, it is significant that responses were observed in at least some of the animals after each oral immunization, and the data suggest that the oral tract may not be as sensitive to neutralization even after four exposures to adenovirus vectors.

T cell responses generated by Ad vaccination were only be tested from the systemic compartment during immunization by assay of PBMCs. Ad immunization drove detectable proliferative responses to various HIV-1 envelope peptides in all three animals and against gag only in macaque 53. In contrast, PBMC samples showed that oral Ad drove no detectable

ELISPOT IFN- γ against envelope or gag antigens. These data suggest that the oral mucosal immunization crossed over only moderately into the systemic compartment. Previous work with the peptide vaccine showed that depletion of CD8 cells from PBMCs attenuates ELISPOT, but not proliferative responses (data not shown). Based on this, we speculate that oral Ad vaccination may drive production or crossover of helper T cells into the systemic compartment better than its ability to drive CD8 T cells into the systemic compartment from mucosal sites.

While attenuation of IgA responses was eventually observed, the ability to immunize multiple times appears effective as a priming regimen for other vaccines or for the use of other serotypes of Ad [59–62]. Proof of principle is demonstrated here where mucosal oral priming of macaques by oral Ad administration was followed by mucosal boosting with an intranasal peptide vaccine. In this case, boosting with synthetic envelope peptides adjuvanted with CT2* generated rapid T cell responses against env peptides and whole inactivated HIV virions. While we cannot formally exclude that these responses were not directly related to the priming by the peptide immunization alone, due to the limited scope of this pilot project, the rapid, strong kinetics of the responses within 2 weeks contrasts with previous work immunizing macaques with peptides alone [25]. Moreover, post-mortem T cell analysis of systemic and gut samples demonstrated robust T cell responses only in the intestinal tissues and not in PBMCs, the spleen, or in mesenteric lymph nodes. These data suggest, but do not prove, that these T cells were originally “educated” in the gut and “home” preferentially back to this site. Future work with larger groups of animals will compare different routes of systemic and mucosal vaccination in macaques to determine how this affects the establishment of memory responses at different sites.

In summary, this pilot study provides proof of principle for oral capsule delivery of replication-defective adenoviral vaccines. This simple vaccination route may provide an attractive means to deliver HIV and other vaccines across the globe if more stringent lyophilization and encapsulation approaches are perfected to maximize vector stability. Efforts to evade pre-existing and vector-induced anti-Ad immunity will likely enhance vaccine efficacy by this route. Nonetheless, the ability to deliver the same vector multiple times to the host and generate detectable responses suggests the oral route may help avoid immunity to the vector. This is consistent with previous work in mice with Ad vectors [63]. These data also suggest this route can be used for prime-boost strategies with other vaccines. Work is underway to determine the optimal combination of priming and boosting sites to afford the best barrier protection at mucosal sites for HIV-1 and other pathogens.

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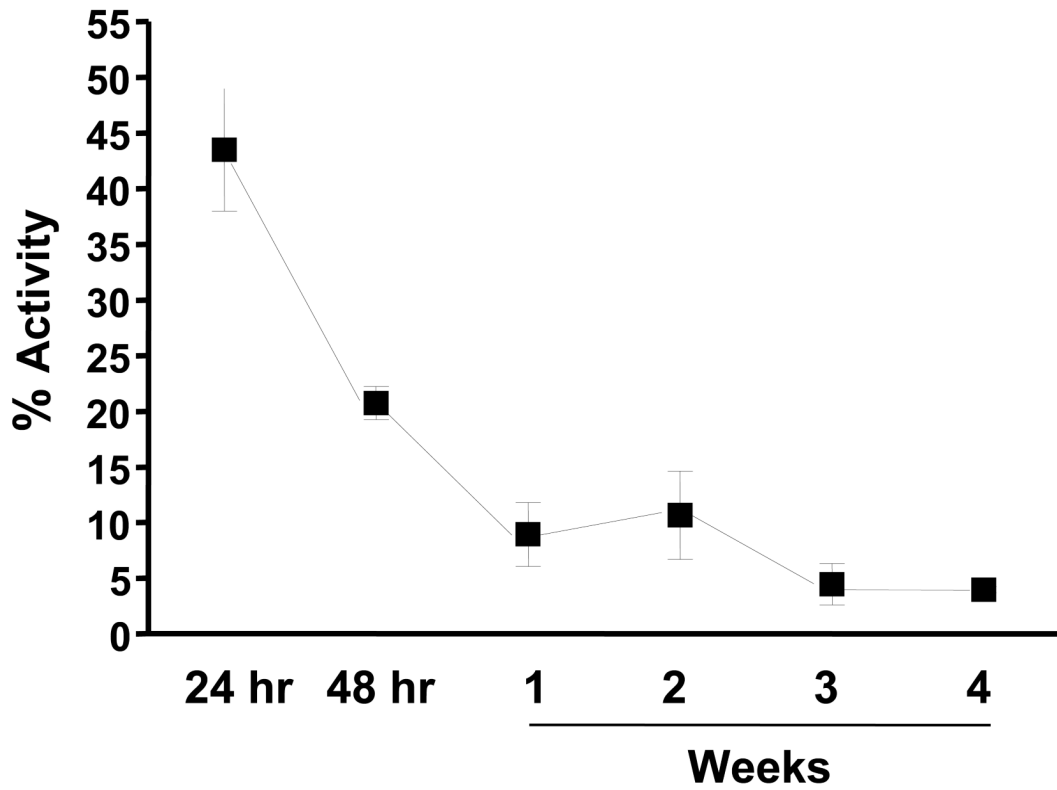


Figure 1.

Stability of Lyophilized Ad Vectors. Enteric-coated capsules containing lyophilized Ad vectors were immediately stored in a desiccator at 4°C and assayed for luciferase activity on HeLa cells upon reconstitution after storage of 24 hr., 48hr., and weekly for four weeks. Percent activity was determined by comparing to the luciferase activity of infection by the same number of particles of a freshly-diluted stability control vector as was used for lyophilization. Results are presented as the means of samples prepared in triplicate, and error bars indicate standard deviations.

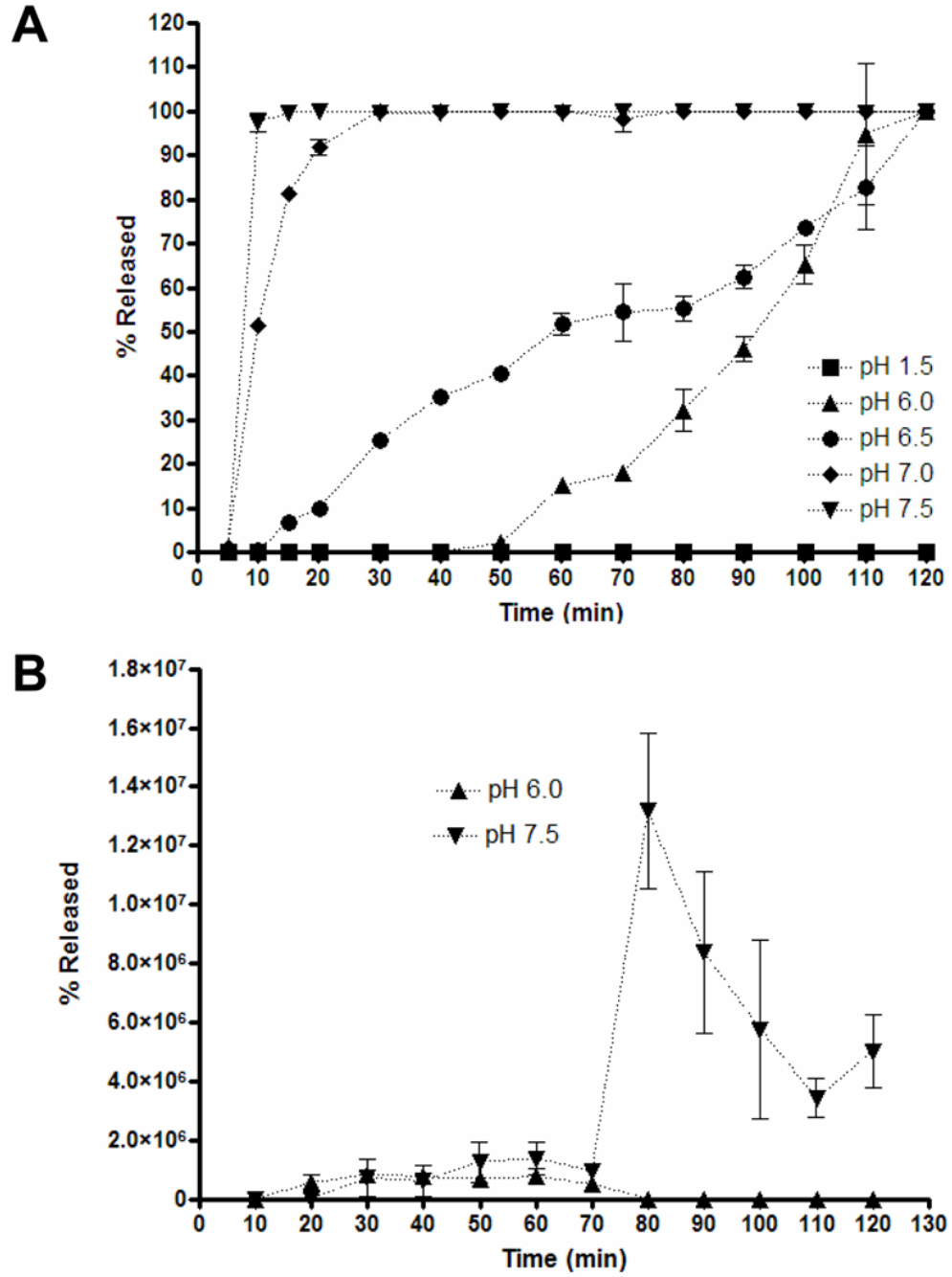
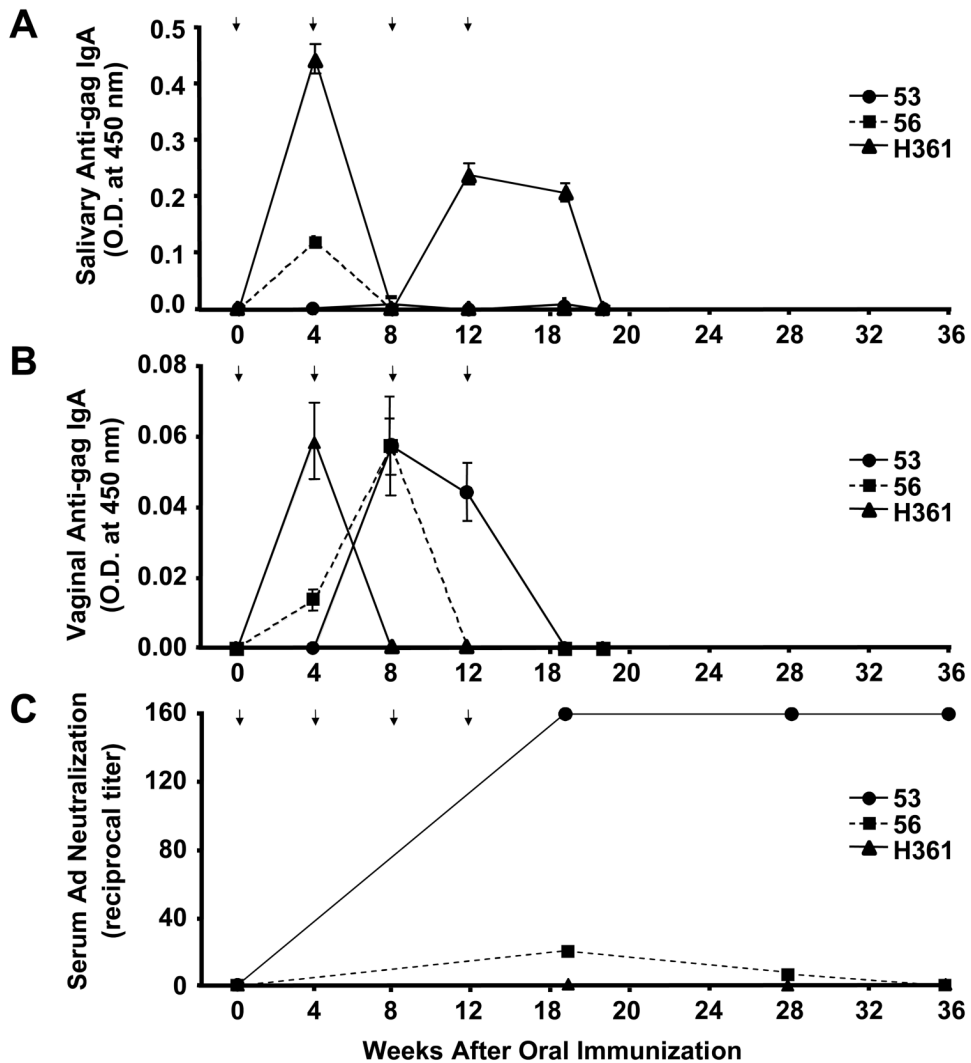
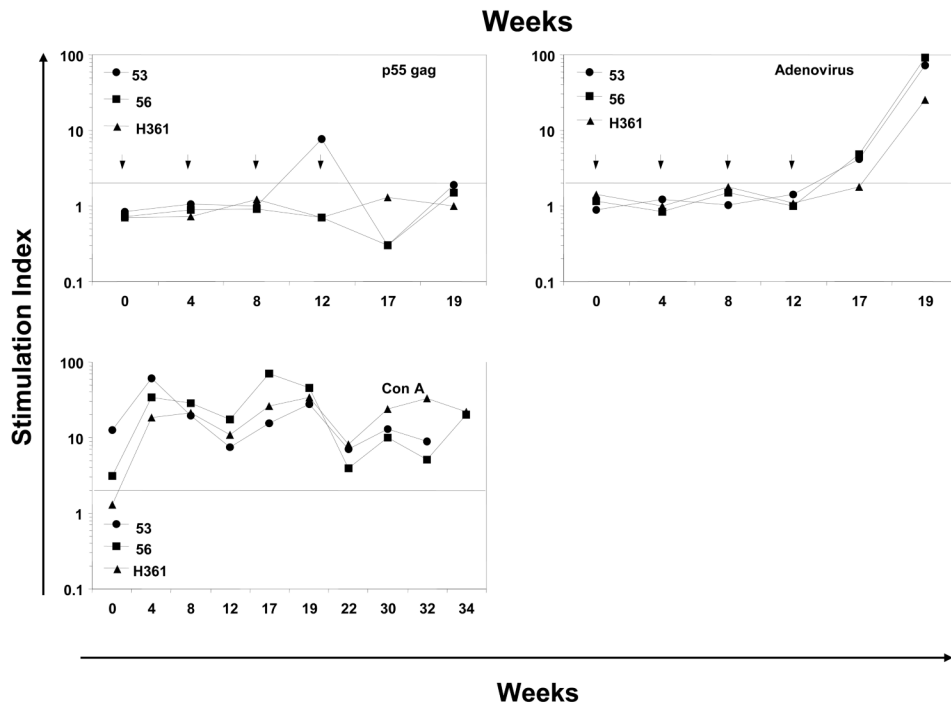
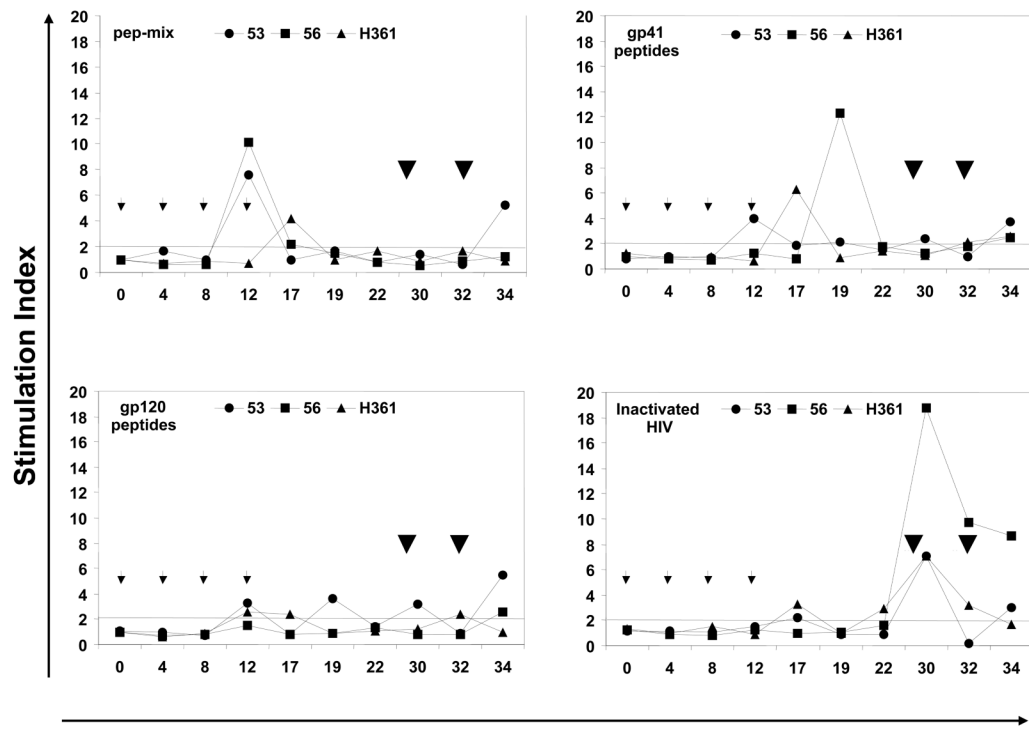


Figure 2. Enteric-coated HPMC capsule dissolution curve. Coated HPMC capsules with 13.2% w/w total solid polymer dispersion formulation of 4:1 Eudragit® L100 and Eudragit® S100 were subjected to dissolution test at 37°C in 0.05 M sodium phosphate buffers of varying pH for the indicated times and the supernatant was assayed for **A) dye release by spectrophotometry or B) virus release by real-time PCR.** Results are presented as the means of samples performed in triplicate, and error bars represent the standard deviation.

**Figure 3.**

Antigen-specific humoral immune responses. A and B, Antibody responses against HIV-1 gag in mucosal samples. C, Serum neutralizing responses against adenovirus. ELISA plates were coated with HIV-1 gag and 1:2 dilutions of saliva (A) and vaginal washes (B) were reacted. After reaction with secondary anti-IgA HRP conjugate and subsequent colorimetric substrate, data were presented as the O.D. values measured at 450 nm. Error bars indicate standard deviations of three measurements taken per time point. Statistical significance was verified for each time point against the background using a Student's paired *t* test and a *p* value < 0.05. C) Serum samples take at the indicated times were incubated with Ad5 expressing luciferase for 1 hour at 37°C prior to addition to 293 cells at 250 viral particles per cel. 24 hours later, luciferase activity was measured and gene delivery was compared to untreated Ad5 vector. Data is expressed as geometric mean titers that reduced Ad luciferase activity 50%.



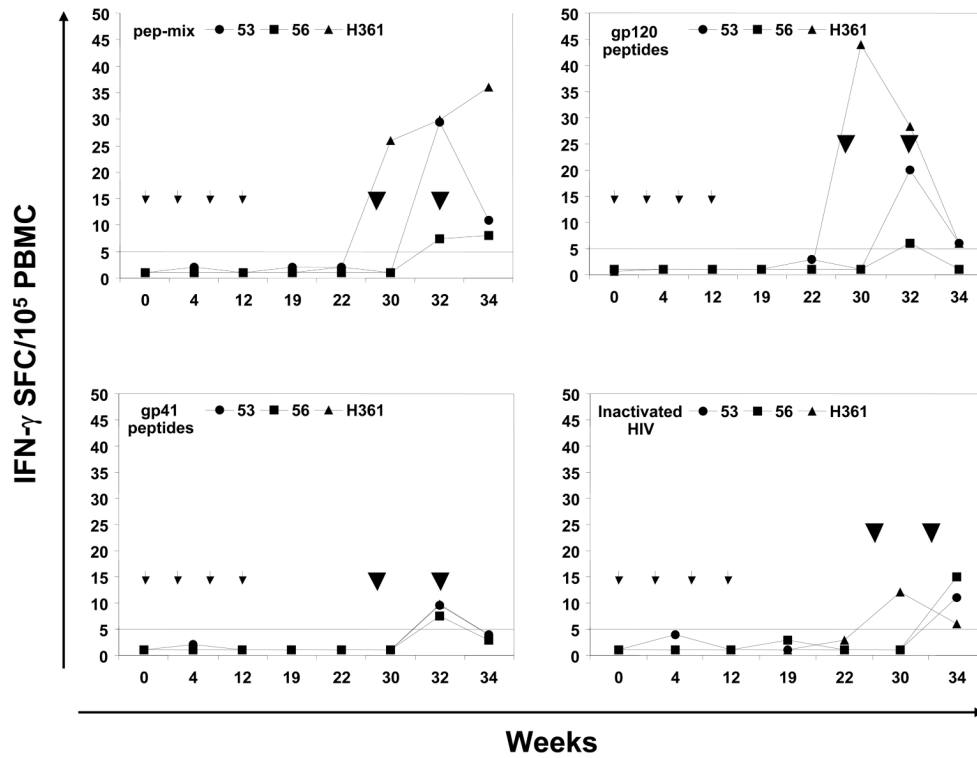


Figure 4.

Antigen-specific cellular immune responses. The proliferative responses in terms of [³H] thymidine incorporation and IFN- γ producing cells by the ELISPOT method were determined using PBMC samples from rhesus macaques immunized by oral priming with enteric-coated capsules delivering Ad-gag and Ad-env-peptide and intranasal boosting with synthetic HIV-1 envelope peptides and CT2*, the mutant cholera toxin adjuvant. (A) Systemic T cell responses using PBMC were determined in terms of proliferation in response to mixtures of all six HIV-1 envelope peptides (pep-mix), two gp41 peptides, four gp120 peptides, and heat-inactivated HIV-1_{IIIIB} preparation. The fold-increases in proliferation responses to the different antigens were calculated by comparing the values from cells in the culture medium. (B) Proliferation responses of PBMC were determined in response to recombinant HIV-1 gag protein (p55 gag), heat-inactivated Ad5, and Con A. (C) ELISPOT analyses for antigen-specific IFN- γ -producing cells for 10⁵ input PBMC in each monkey, in terms of spot-forming-cells (SFC/10⁵ PBMC) at the indicated times after oral Ad vector mediated priming and intranasal boosting with peptide-cocktail (6 peptides, 100 μ g each/dose) mixed with mutant cholera toxin, CT2* (10 μ g/dose). The horizontal line in each panel indicates the cut-off value for positive response (SI of 2.0 for the proliferation assay and SFC of 5 for the ELISPOT assay).

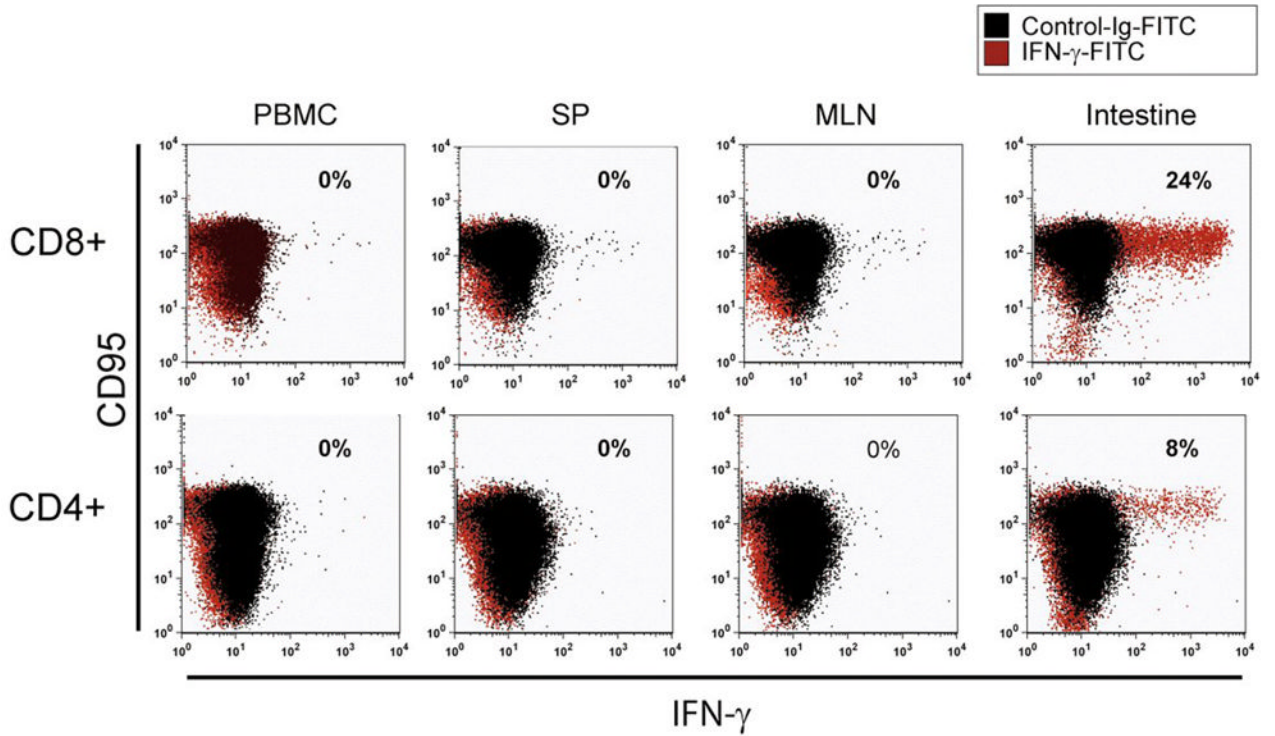


Figure 5.

Antigen-specific intracellular IFN- γ production. Cells isolated from various tissues at necropsy of macaque 53 were used for determining intracellular IFN- γ producing T cells by immunofluorescence staining after stimulation with the HIV-1 envelope peptide mixture (pepmix), cell fixation and permeabilization as described in the methods section. To define T cell subsets, lymphocytes were stained for cell surface CD4, CD8, and CD95, the later to distinguish memory-phenotype. Among the CD95⁺ T cells, IFN- γ ⁺ CD8⁺ and CD4⁺ T cells were detected in the intestinal lymphocytes, but not in peripheral blood, spleen, and lymph nodes.

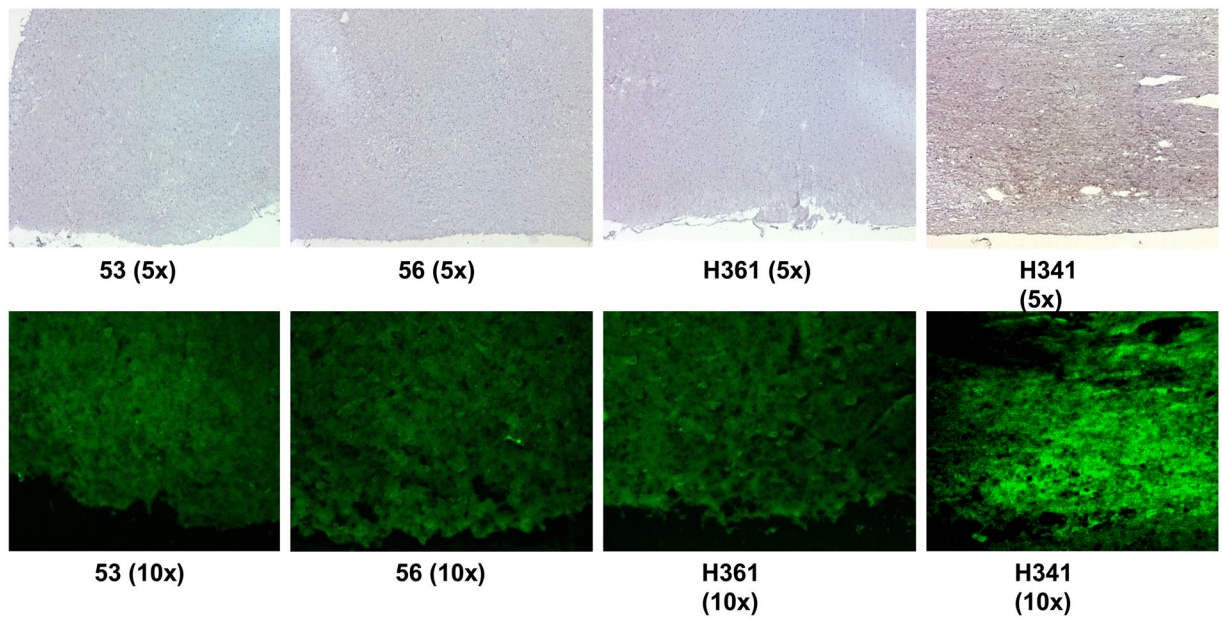


Figure 6.

Detection of NGF-b1 expression in the olfactory bulbs of rhesus macaques immunized by the intranasal route with the HIV-1 envelope peptide cocktail (pep-mix) using either the mucosal adjuvant mutant cholera toxin CT2* (the first three panels on the left-side indicated by monkey numbers, 53, 56, and H361) or native cholera toxin (nCT, right-side panel indicated by monkey number H341). Frozen tissue sections were subjected to anti-NGF-b1 antibody immunohistochemistry staining (upper panels) and immunofluorescence staining (lower panels) by reacting with avidin-biotin conjugate, followed by incubation with HRP-conjugated streptavidin or streptavidin-Alexa Fluor 488.

Table 1

Enteric Coating Formulation. Amounts reported are for making a 157-g coating dispersion for use with a Torpac Inc. Pro-Coater.

Excipients	Quantity
ddH ₂ O	6.8 ml
Isopropyl Alcohol	164.2 ml
Talc	6.93 g
Eudragit® L100	11.08 g
Eudragit® S100	2.77 g
TEC	0.809 ml

Excipients are listed in the sequence in which they are mixed.

Comparison of G.I. Physiology of Human and Non-Human Primates. The distribution of pH for the human small intestine and the gastric residence time for each segment of the small intestine take into account an average between the fed and fasted state for travel of a solid meal.

Table 2

Small Intestine Region	HUMAN		NON-HUMAN PRIMATE (Macaca Lacépède ^a ; (<i>mulatta</i> ^b , <i>fascicularis</i> ^b))	
	pH Range	Small Intestine Transit Time (hr)	pH Range	Total Small Intestine Transit Time (hr) ^c
Duodenum	6.0–6.1	1	5.6–5.8	(High Range) 4.0–6.0
Jejunum	6.1–7.2	1	5.8–6.5–7.0	(Low Range) 0.5–1.5
Ileum	7.0–7.8	2	7.0–7.4	(Avg.) 2.4
Caecum-Colon	6.5–7.0	2	6.6–7.0	5.0

The total small intestine transit time shown for non-human primates are values shown as observed averages among rhesus and cynomolgus monkeys and represent the low and high range of time required for a liquid meal to pass through the entire small intestine and through the ileo-caecal junction into the proximal colon (^agenus; ^bspecies; ^cdenotes total small bowel passage time not specific to an intestinal region) [Data compiled from 64,65–69].

Table 3
 Cumulative Summary of Antigen-specific Proliferation and IFN- γ ELISPOT Responses in Monkeys Immunized with Oral Adenoviral DNA-priming and Intranasal Peptide-boosting.

Response to	Monkey 53		Monkey 56		Monkey H361	
	Proliferation (at week)	IFN-g ELISPOT (at week)	Proliferation (at week)	IFN-g ELISPOT (at week)	Proliferation (at week)	IFN-g ELISPOT (at week)
gp41 peptide- mixture	12, 30, 34	34	19, 34	34	17, 34	34
gp120 peptide- mixture	12, 19, 30, 34	34, 38	34	34	12, 17, 32	30, 34, 38
gp41 + gp120 peptide-mixture	12, 34	34, 38	12, 17	34, 38	17	30, 34, 38
HIV-1 p55 gag protein	12					
Heat-inactivated HIV-1	17, 30, 34	38	30, 32, 34	38	17, 22, 30, 32	30, 38
Heat-inactivated Ad5	17, 19	19	17, 19		19	
Con A	All time points tested	All time points tested	All time points tested	All time points tested	All time points tested	All time points tested

Note: The weeks shown are for positive responses based on the cutoff values described in the methods section.