AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 9, Number 5, 1993
Mary Ann Liebert, Inc., Publishers

Immunogenicity of Recombinant Human Adenovirus–Human Immunodeficiency Virus Vaccines in Chimpanzees

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ABSTRACT

Recombinant human adenovirus (Ad) type 4-, 5-, and 7-vectored vaccines expressing either the HIV *env* or *gag*-protease genes were tested for immunogenicity in three chimpanzees. The first phase of the vaccination protocol consisted of a primary and two booster immunizations with Ad–HIVs by the oral route of administration, followed by a single booster immunization with Gag and/or Env subunit vaccines. The second phase of the vaccination protocol consisted of intranasal administration of Ad–HIVs previously administered by the oral route. Following the first phase adenovirus was shed into stools for only 1–7 days and modest type-specific anti-adenovirus neutralizing antibody titers were induced. Strong anti-Env binding antibody responses were detected in all three animals following the second oral booster immunization. One chimpanzee responded with a low-titered type-specific neutralizing antibody response to HIV. Cell-mediated immune responses to Env were not detected after the primary vaccination, but were detected following all booster immunizations. Administration of the Gag subunit vaccine boosted both humoral and cell-mediated immune responses to Gag antigens. In contrast, the Env subunit vaccine boosted cellular but not humoral immune responses. In the second phase of the vaccination protocol, both virus shedding and anti-adenovirus responses were enhanced. All three chimpanzees responded to the intranasal administration of Ad7–HIVs with boosted anti-HIV serum responses, including low-titered type-specific neutralizing antibodies, elicited anti-HIV antibodies at secretory sites, and stimulated cell-mediated immune responses to both Gag and Env antigens.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV) possesses a highly restricted host range. HIV infects and replicates in chimpanzees but fails to induce an AIDS-like disease in these animals. Various vaccine candidates for HIV have been tested in the chimpanzee model, most of which have stimulated the induction of humoral and cellular immunity.[1–3] A principal neutralization determinant (PND) has been identified[4,5] within the third variable domain of the external envelope glycoprotein, gp120. Neutralizing antibodies that recognize the PND tend to be strain specific, and polyclonal sera directed against the PND, when mixed *in vitro* with HIV, can prevent infection following injection into naive chimpanzees.[6] Passive immunization with a hybrid mouse–human monoclonal antibody directed at the PND was shown to protect chimpanzees *in vivo* from challenge with HIV.[7] Vaccine successes against HIV challenges have been reported utilizing the PND (in some form) both for primary and subsequent booster immunizations.[8,9] However, although these approaches have been shown to induce systemic serum neutralizing anti-HIV antibody responses, they have failed to elicit strong cellular and secretory immune responses.

Human adenoviruses possess significant advantages as vectors for recombinant vaccines, including a strong safety record

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established for the use of Ad4 and Ad7 vaccines for prevention of acute respiratory disease in military recruits, as well as the existence of multiple adenovirus serotypes that can be potentially exploited as vectors for booster immunizations. Recombinant adenovirus vectors expressing HIV antigens have been evaluated previously for immunogenicity in primate[10] and nonprimate hosts, such as the cotton rat,[11] the mouse,[10] and the laboratory beagle.[12] The latter dog model is nonpermissive for adenovirus replication but permits recombinant antigen expression.[13] Using the dog model, we have shown that Ad–HIV recombinant viruses were capable of eliciting neutralizing serum antibody responses directed at the HIV envelope. Booster immunization of dogs with heterotypic Ad–HIV recombinant viruses was necessary for the induction of high-titered neutralizing antibody responses.

Chimpanzees have been shown previously to support human adenovirus replication *in vivo* following enteric infection with either wild-type Ad7 or Ad7– and Ad4–hepatitis B surface antigen (HBsAg) recombinant viruses.[14] Low-titered neutralizing antibody responses to hepatitis B virus (HBV) were induced in two of two chimpanzees following a primary (Ad7–HBsAg) and one heterotypic (Ad4–HBsAg) booster immunization. One vaccinated chimpanzee was protected from acute hepatitis, and the other animal experienced modified disease following HBV challenge. Here we report the first immunogenicity testing of recombinant adenovirus–HIV vaccines in chimpanzees. Their abilities to induce cell-mediated, secretory, and humoral immune responses to recombinant HIV antigens following oral and intranasal immunizations and in combination with subunit HIV vaccines are described.

MATERIALS AND METHODS

Construction of recombinant viruses. The procedure for generation of recombinant adenoviruses was described previously.[15] Recombinant Ad–HIV viruses were made using Ad4, Ad5, and Ad7 vectors containing large E3 deletions. The entire gp160 or the *gag*-protease genes of HIV were inserted into these vectors, along with a cDNA copy of the HIV *rev* gene. Individual plaques that appeared 10–20 days after transfection were selected, replaqued three times, and expanded in A549 cells. To confirm the DNA structure of each recombinant, DNAs were extracted by the method of Hirt[16] and analyzed by restriction enzyme digestion.

Growth and titration of recombinant viruses. Recombinant viruses were expanded and titrated on A549 cell monolayers as described previously.[17]

Production and testing of enteric-coated capsules. Clarified and lyophilized infected A549 cell lysate supernatants were packed in gelatin capsules as described previously.[14] Capsules were coated six times with cellulose acetate phthalate (dissolved in acetone–ethanol, 1:1) by repeated dipping and air drying. The integrity of the enteric coating was tested by sequential exposure of representative enteric-coated capsules to simulated gastric fluid[18] for 1 hr at 37°C using a VanKel disintegration tester (Vankel Industries, Inc., Edison, NJ) followed by exposure to simulated intestinal fluid[18] at 37°C. All capsules tested resisted disintegration in simulated gastric fluid and dissolved within 15–30 min in simulated intestinal fluid. Infectious recombinant virus titers ($\log_{10}$/capsule) were determined by plaque assay to be as follows: Ad7–*env* (7.1), Ad7–*gag* (9.3), Ad4–*env* (10), Ad4–*gag* (10), and Ad5–*env* (10.9).

Production of Env and Gag subunit preparations. Subviral particles consisting of membrane-bound Gag antigens were harvested from Ad4–*gag*-infected A549 cells and purified as described previously.[19] Purified Env was prepared from Ad7–*env*-infected A549 cells as described previously.[12] Env (200 μg/dose) and Gag subunit (500 μg/dose) preparations were formulated in a 0.2% alum adjuvant and were administered intramuscularly.

Inoculation of recombinant adenovirus–HIV or recombinant adenovirus-derived subunit preparations to chimpanzees and collection of chimpanzee serum and fecal specimens. Three chimpanzees (approximately 1–2 years old) seronegative to human Ad4, Ad5, and Ad7 were anesthetized and immunized with enteric-coated capsules containing recombinant Ad–HIV for three successive days (Table 1) as described previously.[14] Although these animals were seronegative for Ad4, Ad5, and Ad7 and none of these strains were detected in chimpanzee stools at the time of administration, these chimpanzees were shedding either a different strain of human adenovirus or chimpanzee adenovirus in their stools at the time of inoculation. For intranasal immunizations 1-ml of suspensions of Ad–HIV recombinant virus were administered dropwise to the external nares of anesthetized chimpanzees. Chimpanzees were bled following sedation with ketamine hydrochloride (10 mg/kg). While the animals were still under anesthesia, cotton-tipped applicator sticks were used to gently swab the external nares. The sticks were placed in test tubes containing 4.5 ml of phosphate-buffered saline (PBS) supplemented with antibiotics. Fresh chimpanzee stools were collected at various times. Serum, swabs, and stool specimens were stored frozen at −70°C for further use.

Virus detection by plaque hybridization analysis. Identification of recombinant virus present in stool or swab samples was accomplished with radiolabeled DNA oligoprobes. Ten percent stool suspensions were then prepared with antibiotics [gentamicin (200 μg/ml) and amphotericin B (10 μg/ml)], clarified by centrifugation (1100 × *g*), and were plaqued on confluent A549 cell monolayers in 60-mm tissue culture dishes under a 0.5% agar overlay in Dulbecco's modified Eagle's medium (DMEM). Five to 10 days after infecting cell monolayers, plaques were visualized by neutral red staining, enumerated, and the agar overlay was gently removed, taking care not to disturb the cell monolayer. A nitrocellulose filter (type HA, 0.45 μm pore size; Millipore, Bedford, MA), presoaked in 20× SSC (3 M NaCl plus 0.3 M sodium citrate), was placed on the cell monolayer and left in contact with cells for 2 to 4 min. The filters were then removed, air dried, and baked for 2 hr at 80°C. The filters were washed twice in 3× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature and prehybridized and hybridized according to standard procedures.[20] Viral-specific ^{32}P-labeled oligoprobes to Ad4 fiber AACTCTAGCCCCTTACCAAT, Ad7 fiber GCGCGCACCGCACCAGAAAG, *env* GCATCTGTTCTACCAGG, and *gag* ATCATCTGCTCCTGTATC, were chemically synthesized, added to the hybridization buffer (1 × 10^6 cpm/ml), and incubated overnight at 42°C. The filters were washed, autoradiographed, and hybridization signals were then counted.

TABLE 1. SCHEDULE FOR IMMUNIZATION OF CHIMPANZEES WITH ADENOVIRUS–HIV AND SUBUNIT VACCINES

Immunizations (dose)[a]	*Route*	*Time (weeks)*	*Chimpanzee number*		
			312	*324*	*326*
Ad7–*env* (7.1)	Oral[b]	0	+	+	+
Ad7–*gag* (9.3)		0	−	+	+
Ad4–*env* (10)	Oral	7	+	+	+
Ad4–*gag* (10)		7	−	+	+
Ad5–*env* (10.9)	Oral	26	+	+	+
env subunit	im[c]	34	+	+	+
gag subunit		34	−	+	+
Ad7–*env* (8.0)	in[d]	46	+	+	+
Ad7–*gag* (8.0)		46	−	+	+
Ad4–*env* (9.0)	in	58	+	+	+
Ad4–*gag* (9.0)		58	−	+	+

[a] Log_{10} (pfu/dose).
[b] Oral dose per enteric-coated capsule, 1 capsule/virus/day given on three consecutive days.
[c] Intramuscular Env (200 μg/dose) or Gag (500 μg/dose) in alum adjuvant.
[d] Intranasal (1.0 ml, dropwise).

Adenovirus neutralization assays. Microtiter neutralization assays were performed to detect neutralizing antibodies as described previously.[13]

Detection of anti-HIV antibodies. Chimpanzee antibody responses to HIV antigens were measured with a commercial Western blot kit (Du Pont, Wilmington, DE) according to the instructions of the manufacturer. In certain instances biotinylated goat anti-human IgA (α-chain specific) or biotinylated goat anti-human IgG (γ-chain specific) were substituted for the biotinylated goat anti-human IgG (heavy and light chain-specific) reagent. Serum titers were taken as last serial dilution of sample to give a positive signal. Nasal secretions were tested at a 1:10 dilution. HIV neutralization assays were conducted with MT4 cells, and a 90% end point was determined as previously described.[8]

Measurement of cellular immune responses to HIV-1. Antigen proliferation assays, using chimpanzee peripheral blood mononuclear cells, were performed as previously described.[21] Baculovirus-derived recombinant gp160 (Repligen Corp., Cambridge, MA), gp120, and p24 (American BioTechnologies, Inc., Cambridge, MA) were employed as stimulating antigens. gp120 and p24 antigens were used at concentrations of 10, 5, and 1 μg/ml and gp160 was used at 2 μg/ml. Cell were grown in culture for 6 days, radioisotope was added, and cells were harvested at 7 days.

Demonstration of lymphocyte subsets in chimpanzee blood. Mononuclear cells were separated from heparinized blood by Hypaque-Ficoll gradients and stained for fluorescent-activated cell sorting with lymphoid-specific Leu-series monoclonal antibodies (Becton Dickinson, Paramus, NJ) as described previously.[22]

Immunofluorescent staining of paraffin-embedded lung sections. Lung sections were indirectly stained with an immunofluorescent kit [Adenoclone immunofluorescence assay (IFA); Cambridge Bioscience, Worcester, MA] for the presence of adenovirus antigens following treatment with xylene and ethanol to remove paraffin. Deparaffinized sections were treated as directed by the manufacturer. Cytospin preparations of infected and mock-infected A549 cells were treated identically as positive and negative controls. Independent confirmation of results was obtained from parallel studies done at the Armed Forces Institute of Pathology, Washington, DC.

RESULTS

Phase I: Virus replication following oral immunizations. Chimpanzees seronegative for Ad4, Ad5, and Ad7 were immunized orally with Ad7–HIV, Ad4–HIV, and Ad5–HIV recombinant viruses on weeks 0, 7, and 26, respectively (Table 1). One chimpanzee (312) was immunized successively with recombinants expressing only HIV gp160 (Env), whereas the two other chimpanzees (324 and 326) received primary (0 weeks) and first booster (7 weeks) immunizations with recombinants expressing *env* and *gag* genes. The second booster (26 weeks) consisted of a single Ad5–*env* recombinant. All oral immunizations with Ad–HIV recombinants were well tolerated by the chimpanzees, with no signs of clinical distress detected in any of the animals. Shedding of recombinant adenoviruses in stools was detected by plaque hybridization analysis using radiolabelled DNA oligomer probes specific for the *env*, *gag*, or type-specific adenovirus fiber antigen. Following primary oral immunization Ad7–*gag* and Ad7–*env* recombinants were detected in stools for 1–5 days postimmunization, respectively, with peak viral titers between 10^2 and 10^4 pfu/ml of stool (Table 2). Following the first oral booster immunization (7 weeks), Ad4–*env* and Ad4-*gag* were detected in stools for 1–7 days, with peak viral titers measured between 10^3 and 10^4 pfu/ml of stool (Table 2). Following administration of the second oral booster (26 weeks), recombinant Ad5–*env* was shed in stools for up to 7 days. Peak viral titers ranged from 10^2 to 10^5 pfu/ml of stool (Table 2). Viral titers of orally administered recombinant viruses consistently peaked during the 3-day inoculation period. There was no reactivation of Ad7 recombinant viruses following immunization with Ad4 and Ad5 recombi-

TABLE 2. RECOVERY OF ADENOVIRUS–HIV RECOMBINANT VIRUSES FROM FECES FOLLOWING ORAL OR INTRANASAL INOCULATION OF CHIMPANZEES

		Recombinant adenovirus titer (pfu/g stool)[a]				
			Chimpanzee 324		*Chimpanzee 326*	
Day	*Inoculation route*	*Chimpanzee 312 (Ad-env)*	*Ad*–env	*Ad*–gag	*Ad*–env	*Ad*–gag
		Ad7–*env*	Ad7–*env*	Ad7–*gag*	Ad7–*env*	Ad7–*gag*
0	Oral	0	0	0	0	0
1		0	0	19,500	300	0
2		500	5,200	3,100	0	25,000
5		0	0	0	200	0
7		0	0	0	0	0
		Ad4–*env*	Ad4–*env*	Ad4–*gag*	Ad4–*env*	Ad4–*gag*
49 (0)	Oral	0	0	0	0	0
51 (2)		1,100	20,100	2,000	250,000	16,600
54 (5)		0	8,400	200	100	800
56 (7)		0	0	0	400	100
63 (14)		0	0	0	0	0
68 (19)		0	0	0	0	0
		Ad5–*env*	Ad5–*env*		Ad5–*env*	
182 (0)	Oral					
183 (1)		0	250,000		250,000	
189 (7)		200	1,500		1,600	
196 (14)		0	0		0	
		Ad7–*env*	Ad7–*env*	Ad7–*gag*	Ad7–*env*	Ad7–*gag*
322 (0)	Intranasal	0	0	0	0	0
327 (5)		2,500	300	600	400	200
329 (7)		100[b]	0[c]	0	0	0
331 (9)		0	0	0	0	1,000
334 (12)		0	0	800	0	0
343 (21)		0	0	0	0	0

[a] Plaque hybridization titer, 0 = less than 100.
[b] Nasal swab titer, 1485 pfu/swab.
[c] Nasal swab titer, 90 pfu/swab.
Number in parentheses = number of days after last infection.

nants and no reactivation of Ad4 recombinants following immunization with Ad5 recombinants (data not shown).

Antibody response to adenovirus vectors. Primary oral immunizations generated modest neutralizing antibody responses against the Ad7 vector by chimpanzees 324 and 326, whereas a barely detectable anti-Ad7 serum antibody response was induced in chimpanzee 312 (Table 3). Following the first booster inoculation with Ad4–HIV recombinants, chimpanzees 312 and 326 developed modest anti-Ad4 neutralizing antibody responses, whereas chimpanzee 324 experienced a minimal response to Ad4 (Table 3). All three animals developed nominal serum neutralizing antibody titers to the Ad5 vector after oral administration of the second booster (Table 3). These relatively weak antibody responses are consistent with the apparently poor replication of these recombinant viruses in the chimpanzee gut.

Antibody responses to recombinant HIV antigens. Weak antibody responses against both the Gag (p24) and Env (gp160) antigens were detected by Western blot analysis following the primary oral inoculation (Table 4). Further, only weak booster responses against Gag and/or Env were detected after the first oral Ad4–HIV recombinant immunization. In contrast, the second oral recombinant virus (Ad5–*env*) booster effectively stimulated anti-Env antibodies both in terms of the magnitude of the response as well as in the specificity of the response (detection of gp120 and gp41) in all three chimpanzees (Table 4).

Oral immunization with Ad7–HIV and Ad4–HIV vaccines did not stimulate anti-HIV neutralizing antibody responses in any of the three chimpanzees (Table 5). However, a weak but significant anti-HIV neutralizing antibody response (10–20) was detected in one of the three chimpanzees following the second oral booster immunization with Ad5–*env*.

Anti-HIV immune responses following administration of subunit Env and/or Gag vaccines. A third booster immunization consisting of recombinant adenovirus-derived Gag (500 μg/dose) and/or Env (200 μg/dose) antigens was then administered 8 weeks after the second oral booster (Table 1). The two chimpanzees immunized with the subunit Gag vaccine mounted strong booster responses to p24 and p17 (Table 4). The subunit Env vaccine, however, did not elicit detectable booster responses to the HIV envelope glycoprotein in any of the three chimpanzees (Table 4). Enhanced proliferative responses to Env and Gag proteins by peripheral blood mononuclear cells (PBMCs) were demonstrated following administration of the subunit vaccines (Fig. 1).

TABLE 3. ANTI-ADENOVIRUS SERUM NEUTRALIZING ANTIBODY RESPONSES IN CHIMPANZEES FOLLOWING ADMINISTRATION OF ADENOVIRUS–HIV RECOMBINANT VIRUSES

Recombinant adenovirus serotype	*Time (weeks)*	*Anti-adenovirus antibody response*[a]		
		Chimpanzee 312	*Chimpanzee 324*	*Chimpanzee 326*
Ad7 (oral	0	<4	<4	<4
	4	4	16	16
	7	8	64	16
Ad4 (oral)	7	8	<4	<4
	11	128	<4	32
	15	128	8	128
Ad5 (oral)	26	16	8	<4
	30	512	128	64
	34	32	16	16
Ad7 (intranasal)	46	<4	8	64
	50	256	512	2048
	54	64	128	512

[a]Reciprocal neutralization titer. Chimpanzees were orally immunized with Ad7–HIV (Ad7), Ad4–HIV(Ad4), and Ad5–HIV (Ad5) recombinant viruses at weeks 0, 7, and 26, respectively. A second Ad7–HIV immunization was given intranasally at week 46.

Cellular immune responses directed at Env or Gag. Peripheral blood mononuclear cells obtained after primary oral immunization with recombinant Ad7–HIV viruses failed to proliferate on exposure to purified Env and Gag antigens in tissue culture (Fig. 1). However, a highly significant antigen proliferative response to the Env antigen was detected in cultured PBMCs obtained from chimpanzee 326 following the first booster inoculation, whereas PBMCs obtained from the other two chimpanzees were stimulated to a lesser extent. Following the second oral booster excellent cell-mediated immune responses were generated against Env antigens (gp160 and gp120) by PMBCs obtained from all three animals (Fig. 1).

Phase II: Intranasal administration of homologous adenovirus–HIV recombinant viruses. The second phase of

TABLE 4. ANTI-HIV SERORESPONSES FOLLOWING ADMINISTRATION OF HIV VACCINES AS DETERMINED BY WESTERN BLOT ANALYSIS

Weeks	*Immunogen*[a]	*Chimpanzee 312* (Env[b])	*Chimpanzee 324*		*Chimpanzee 326*	
			Env	Gag[c]	Env	Gag
0	Ad7 recombinants (oral)	–/–/–	–/–/–	–/–	–/–/–	–/–
4		1.3/–/–	–/–/–	1.3/–	–/–/–	1.3/–
7	Ad4 recombinants (oral)	1.3/–/–	–/–/–	1.3/–	–/–/–	1.3/–
9		1.3/–/–	–/–/–	1.3/–	–/–/–	1/3/–
11		1.3/–/–/	–/–/–	1.3/–	–/–/–	2.0/–
15		–/–/–	–/–/–	1.3/–	1.3/–/–	2.0/–
20		–/–/–	–/–/–	1.3/–	1.3/–/–	2.0/–
26	Ad5–*env* (oral)	–/–/–	–/–/–	–/–	–/–/–	–/–
28		2.0/1.5/2.5	1/5/–/–	–/–	4.0/3.0/3.5	–/–
32		3.5/2.5/3.0	2.5/1.5/2.5	–/–	5.0/4.0/4.5	–/–
34	Subunits (im)	2.5/2.0/2.0	2.5/2.0/–	–/–	4.0/3.5/3.5	–/–
36		2.5/2.5/2.0	2.5/2.5/–	3.0/1.5	3.5/3.5/3.0	4.0/2.5
46	Ad7 recombinants (in)	2.0/2.0/–	2.0/3.0/–	3.0/–	3.0/3.0/–	3.0/–
48		4.0/4.5/3.0	4.0/4.5/3.0	4.0/3.0	3.0/3.0/3.0	4.5/2.0
50		4.0/4.5/3.0	4.0/4.5/3.0	4.0/3.0	3.0/3.0/3.0	4.5/2.0

[a]Refer to Table 1 for immunization schedule.
[b]Log_{10} highest serum dilution showing reactivity (gp160/gp120/gp41).
[c]Log_{10} highest serum dilution showing reactivity (p24/p17).

TABLE 5. ANTI-HIV SERUM NEUTRALIZING ANTIBODY RESPONSES IN CHIMPANZEES FOLLOWING ADMINISTRATION OF ADENOVIRUS–HIV RECOMBINANT VIRUSES

Treatment	Time (weeks)	HIV-neutralizing responses[a] Chimpanzee 312	Chimpanzee 324	Chimpanzee 326
Ad7 recombinants (oral)	0	<10	<10	<10
	4	<10	<10	<10
Ad4 recombinants (oral)	7	<10	<10	<10
	9	<10	<10	<10
Ad5 recombinant (oral)	26			
	28	<10	<10	10
	32	<10	<10	20
Subunit(s) boost (im)	34	<10	<10	10
	36	<10	<10	10
	40	<10	<10	<10
Ad7 recombinants (in)	46	<10	<10	10
	48	10	10	20
	50	20	10	10
	54	40	10	10
	58	40	40	10

[a]Reciprocal titers.

the study was initiated to determine whether homologous Ad–HIV could be used as an effective boost when administered by the intranasal route to animals possessing serum antibody titers to adenovirus. Forty-six weeks after primary oral immunization with Ad7–HIV recombinant viruses, the Ad7–HIV recombinants were administered by the intranasal route (Table 1). At the time of administration, chimpanzees 312, 324, and 326 had anti-Ad7 serum neutralization titers of <4, 8, and 64, respectively. No clinical symptoms of respiratory disease were observed in these animals as a result of the intranasal administrations. Stool specimens and nasal secretions were examined for the presence of shed infectious recombinant viruses (Table 2). Ad7-*env* was detected in nasal secretions for up to 7 days postinoculation in two of the animals. In addition, recombinant Ad7-*env* and Ad7-*gag* viruses were found to be present in stool specimens for up to 12 days postinoculation. There was an inverse correlation between anti-Ad7 serum titers at the time of inoculation and subsequent virus replication as assessed by recovery of Ad7–HIV viruses in nasal secretions and stool specimens.

Antibody response to adenovirus vectors. Significant anti-Ad7 serum neutralizing antibody responses were induced in all three chimpanzees following intranasal inoculation with Ad7–HIV recombinants (Table 3).

Antibody response to recombinant HIV antigens. Intranasal immunizations with recombinant Ad–HIV resulted in significant anti-HIV booster responses in chimpanzees 312 and 324. These animals had the lowest anti-Env and anti-Ad7 serum antibody responses at the time of inoculation (Table 4). The intranasal inoculation also elicited low-titered type-specific anti-HIV neutralizing antibody titers in chimpanzees 312 and 324 (Table 5).

Induction of secretory immune responses. Anti-Env and anti-Gag antibodies were detected in nasal secretions by Western blot analysis. The anti-HIV antibody response measured in the nasal secretions contained both IgA and IgG antibodies to HIV antigens. No IgA or IgG anti-Env or anti-Gag antibodies had been detected in chimpanzee nasal secretions at the time of intranasal inoculation of Ad7-HIV recombinants. Two to 4 weeks later IgA and IgG anti-gp160 and anti-gp120 antibodies were detected in nasal secretions from chimpanzees 312 and 324. Only IgG anti-gp160 antibody was detected in chimpanzee 326. IgG anti-p24 responses were measured in both chimpanzees (324 and 326) that received the Ad7-*gag* recombinants.

Cellular immune responses directed at Env or Gag. All three chimpanzees experienced slight, but significant, cellular immune responses to HIV antigens following intranasal administration of Ad7–HIV recombinants. These responses were clearly much less then observed after the other booster immunizations (Fig. 1). The range of the chimpanzees stimulation indexes (SIs) for gp160, gp120, and p24 were 1.4–4.4, 1.6–2.0, and 1.1–2.6, respectively, at the time of intranasal inoculation. The SI for chimpanzee 312 increased for both Env antigens gp160 (2.4-fold) and gp120 (2.7-fold) by 4 weeks postinoculation. The SI for chimpanzee 324 increased for gp160 (3.6–fold), gp120 (8.8–fold), and p24 (1.5–fold) over the SI measured at the time of inoculation. The SI for chimpanzee 326 increased for gp160 (2.8-fold), gp120 (2.8-fold), and p24 (1.6-fold) by 4 weeks postintranasal inoculation.

Intranasal administration of Ad4–HIV recombinants. Three months after the first intranasal booster immunization with Ad7–HIV the chimpanzees were inoculated intranasally with Ad4–HIV recombinants (Table 1). Serum neutralization titers to Ad4 in the three chimpanzees were high (128–256) at the time of inoculation. At 3 days postinoculation, two of the animals (chimpanzees 312 and 326) were noted to have a slight cough. Two days later (day 5) the third animal, chimpanzee 324, died from acute bacterial pneumonia. The study was terminated

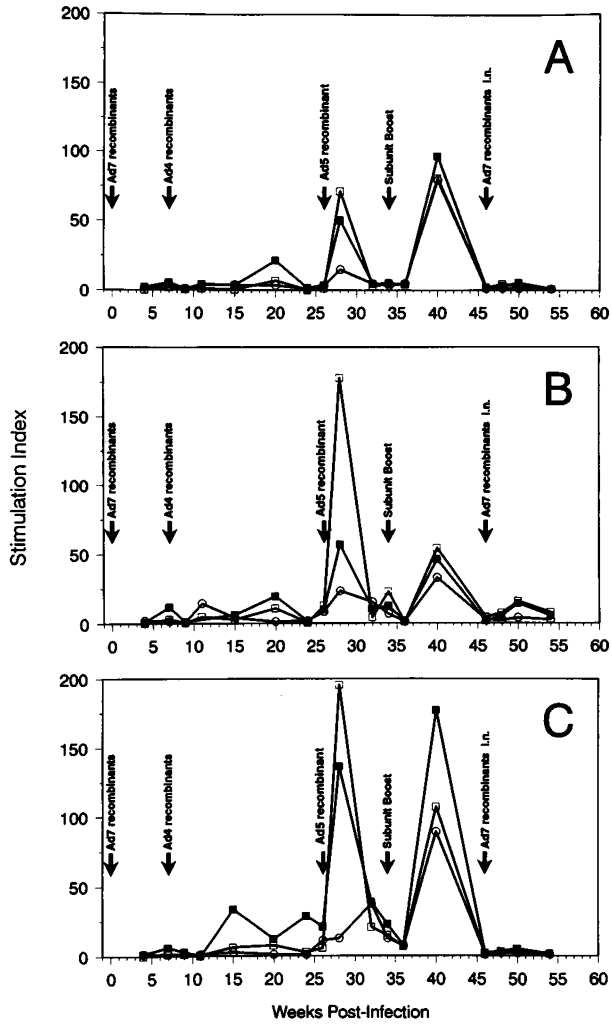

FIG. 1. Stimulation index of chimpanzee PBMCs to HIV antigens. Peripheral blood mononuclear cell cultures were prepared from chimpanzees 312 (A), 324 (B), and 326 (C) and incubated with gp160 (2 μg/ml; ■), gp120 (10 μg/ml; □), p24 (10 μg/ml; ○), or without antigen for 6 days, labeled with [^{3}H]-thymidine for 24 hr, harvested, and the amount of cell-associated radioactivity determined. Experimental and control wells were run in triplicate. Stimulation index is expressed as the (mean cpm of the antigen values)/(mean cpm of the control values).

at this time. *Streptococcus pneumoniae* was subsequently isolated from the lungs of chimpanzee 324. The other two chimpanzees presented harsh sounds by auscultation and *S. pneumoniae* was isolated from both chimpanzees. Antibiotic treatments were initiated and both animals subsequently recovered. Retrospective analysis of available clinical data indicated that chimpanzee 324 was experiencing a slight fever (38.6°C) and had an abnormal complete blood cell count (CBC) on the day of the intranasal administration of Ad4–HIV recombinants (Table 6). The CBC revealed an unusual high number of polymorphonuclear cells present at that time (64%), as well as a 5% level of band cells (immature polymorphonuclear cells), suggesting that this chimpanzee was responding to an active bacterial infection. Autopsy specimens taken from the lung, liver, spleen, and sera all tested negative for the presence of infectious adenovirus. Furthermore, lung and liver specimens (paraffin embedded) tested negative for adenovirus antigen by immunofluorescence analysis. These combined data strongly suggest that Ad4–HIV did not play a direct role in the death of chimpanzee 324. Tissue samples were examined by electron microscopy at the Armed Forces Institute of Pathology. Adenovirus virions were not detected, but intranuclear inclusions were observed in some cells. These inclusions are suggestive of the presence of an as yet unidentified virus. An indirect role for Ad4–HIV in the death of the chimpanzee cannot be ruled out.

DISCUSSION

A primary purpose of this study was to evaluate the capacity of Ad–HIV recombinant viruses to replicate and induce humoral, secretory, and cellular immune responses in the chimpanzee, the only animal known to be permissive to enteric replication by the Ad4 and Ad7 vectors used in this study. Similar Ad–*env* recombinants containing deletions in region E3 were previously shown to induce high-titered neutralizing antibody responses in dogs, which are only abortively infected by human adenovirus.[12] In this study recombinant Ad–HIV virus was shed in stools for only 1 to 7 days following oral inoculations of chimpanzees. This viral shedding pattern sharply contrasts with a previous study in which recombinant adenoviruses were shed for 5 to 6 weeks in the stools of two of two chimpanzees following primary immunization recombinant virus and for 2 to 4 weeks in three of three chimpanzees following an oral booster with a second recombinant virus.[14] Higher oral

TABLE 6. CHIMPANZEE 324 CLINICAL PARAMETERS

Time	*Mature polymorphonuclear cells with segmented nuclei* (segmented)	*Immature polymorphonuclear cells, simple elongate nucleus no constrictions (band cells)*	*Lymphocytes*	*Other*[a]	*Temperature °C*
−4.5 months	35	0	58	7	37.1
−4.0 months	22	1	63	14	36.2
−3.0 months	27	0	60	13	37.1
Day 0[b]	64	5	26	5	38.6
Day 5[c]	45	22	28	5	Not done

[a] Includes monocytes, eosinophils, and basophils.
[b] Intranasal administration of Ad4–*env* and Ad4–*gag*.
[c] Death.

doses were used in the present study and virus capsules were given on three consecutive days, whereas in the first study recombinant viruses were administered on one day only. The observation that peak stool titers fell within the 3-day inoculation period in the Ad–HIV oral study suggests that the majority of the virus shed in the stools during this time period may represent input virus rather than progeny virus produced from Ad–HIV replication *in vivo*. The reason(s) for these differences in replication pattern is unknown. Some possible explanations include the following: (1) virus structure: Ad–HIV recombinants are overpackaged from 0.25 to 2.3%, whereas Ad–HBsAg recombinants are underpackaged. The additional foreign DNA may have retarded replication of Ad–HIV recombinants *in vivo;* (2) prior experience of the animals with chimpanzee or human adenoviruses; (3) possible differences in capsule preparation: capsules were hand coated in cellulose acetate phthalate in different batches at different times; however, because representative capsules from each lot were demonstrated to be resistant to simulated gastric fluid but dissolved in simulated intestinal fluid suggests that the latter cause is unlikely.

Oral administration of recombinant Ad–HIV vaccines was efficient at inducing strong cell-mediated immune responses. Cell-mediated immune responses required priming and at least one or two oral booster immunizations with Ad–HIV recombinant viruses. Subunit vaccinations and intranasal inoculations of chimpanzees also stimulated cell-mediated immune responses.

A key issue regarding the utility of adenovirus vectors as vaccines is whether recombinant adenovirus replication will occur in individuals that possess preexisting immunity to the vector and whether the amount of recombinant antigen expression resulting from such infections is sufficient to stimulate strong immune responses to the foreign antigen. In this study, intranasal administration of Ad7–HIV recombinants to chimpanzees seropositive for Ad7 demonstrated that successful booster immunizations with recombinant adenoviruses may take place in the face of preexisting immunity to adenovirus. Related findings were made by early investigators who initially immunized human volunteers via the oral route with Ad4 vaccine and subsequently reinoculated volunteers with homologous Ad4 vaccine by the intranasal route.[23] Intranasal inoculations resulted in reinfections of individuals independent of serum antibody to Ad4. Surprisingly, enteric replication of Ad7–HIV recombinant viruses appeared to be somewhat enhanced in chimpanzees inoculated intranasally relative to direct delivery to the gut by oral immunization. The intranasal booster also appeared to stimulate anti-Env and anti-Gag responses more effectively than the previous oral boosters.

Evaluation of the safety of a live recombinant HIV vaccine is clearly of critical importance. Ad4 and Ad7 vaccines administered by the oral route to military personnel have established an enviable safety record over two decades of use. Horizontal transmission of virus among recruits following oral vaccination does not occur,[24] although transmission to family members of vaccines has been reported.[25] Transmission of adenovirus vaccine strains to family members did not result in acute disease. Whether attenuation of the Ad4 and Ad7 vaccine strains results from the route of administration alone or whether the Ad4 and Ad7 vaccine strains have been attenuated by passage in cell culture has not been clarified. Experimental respiratory infections of human volunteers with the Ad4 vaccine strain suggest that this strain is at least partially attenuated.[23] Introduction of foreign nuclei acid sequences in viruses frequently results in a compromised replication capacity of the recombinant viruses. A direct comparison of the enteric replication of the Ad7 vaccine strain and a recombinant Ad7–HBsAg virus indicated that Ad7 replicated to higher titer in the chimpanzee gut, although Ad7 was administered at a higher dose than Ad7–HBsAg. A single phase I study in humans comparing Ad7 and Ad7–HBsAg indicated that the Ad7 vaccine strain replicated to 100-fold higher titers in the human gut than Ad7–HBsAg, even though Ad7 was administered at a 10-fold lower dose. Considering the poor replication in the present study of Ad–HIV recombinant viruses in the chimpanzee gut, it seems likely that these Ad–HIV recombinants would be more attenuated than the parental vectors when administered by the oral route.

Results of the second phase of this study support the possible use of the intranasal immunization route for administration of recombinant adenovirus. This would be a particularly attractive immunization route to pursue if virus replication is consistently established in individuals possessing preexisting serum antibody to adenovirus. Clearly, intranasal administration of a recombinant adenovirus would require complete attenuation of the virus. If the present Ad–HIV vaccines are not fully attenuated, several approaches to introducing a completely attenuated phenotype could be pursued, such as the introduction of temperature-sensitive lesions (to restrict virus replication in the lower respiratory tract) or the use of replication-defective adenovirus mutants as vectors.

Intranasal inoculation of Ad7–HIV (10^8 pfu) into chimpanzees with moderate (8–64) or no (less than 4 serum neutralization titer) anti-Ad7 neutralizing antibodies did not induce overt signs of clinical illness even though all three animals were clearly infected. However, a second intranasal inoculation, using a high dose (10^9 pfu) of Ad4–HIV recombinants, was temporally associated with clinical symptoms in all three chimpanzees. One chimpanzee was determined to have had an active bacterial infection on the day of immunization and the other two chimpanzees showed signs of respiratory infections a few days later. *Streptococcus pneumoniae* was also cultured from the animals. These infections were cleared up by the use of antibiotics, indicating the infections were also bacterial in nature. Because the three chimpanzees were housed in the same cubicle, spread of a bacterial respiratory infection from chimpanzee 324 to the other animals appears to be the likely source of the infections in chimpanzees 312 and 326. It is clear that the adenovirus inoculation did not contribute directly to the death of chimpanzee 324, as virus was not isolated from the lungs of the dead chimpanzee nor was adenovirus antigen or adenovirus particles detected in lung tissue by immunostaining or electron microscopy. The high anti-Ad4 titer (128) at the time of immunization also argues against the possibility of a major role for adenovirus in the development of pneumonia. An indirect role for adenovirus in the death of this animal is not likely but cannot be excluded. Although the death of chimpanzee 324 was not due to direct involvement of Ad4 HIV in lung pathology, the safety of vaccines administered by the intranasal route remains a major source of concern.

The Ad–HIV viruses employed in this study possess extensive E3 deletions. The adenovirus E3 region has been demonstrated to contain nonessential coding regions that are not required for *in vitro* replication of virus.[26] However, E3 region gene products have been proposed to play critical roles in facilitating

adenovirus replication *in vivo*.[27,28] gp19 may prevent recognition and destruction of virus-infected cells by virus-specific class I-restricted cytotoxic T cells.[27] The 14.7K protein may confer resistance of virus-infected cells to the lytic activity mediated by tumor necrosis factor.[28] When evaluated in the cotton rat model, deletion of the E3 region of Ad5 was shown to play a critical role in enhancing the pathogenesis occurring in adenovirus-induced lung disease, raising potential concerns for enhanced virulence of E3-deleted recombinant vaccines.[29]

A limited amount of experience with E3-deleted recombinant adenovirus in humans and chimpanzees suggests that the above concerns likely do not apply to recombinant adenovirus administered by the oral route. Initial safety and immunogenicity studies involving recombinant adenoviruses expressing HBsAg that contain major E3 deletions have indicated that these recombinant viruses fail to replicate as well as wild-type viruses in human volunteers following oral enteric immunizations.[30] These recombinant viruses were well tolerated at doses of 10^7 pfu in phase I vaccine trials and did not induce any clinical symptoms of significance.[30] Virus shedding was diminished both in terms of duration and amount in stools obtained from vaccines immunized with E3-deleted recombinant viruses as compared to stools collected from vaccinees inoculated with Ad7 vaccine strain. The induction of anti-adenovirus neutralizing titers was also decreased in vaccines that received recombinant E3-deleted viruses. Likewise, oral administration to chimpanzees of recombinants containing deleted E3 regions did not result in detectable illness although significant virus replication occurred.[14] The differences in these findings and those described earlier[29] may be related to species differences, the relative dosages used, or differences in sites of virus replication.

This study indicates that oral administration of recombinant adenovirus vaccines expressing HIV antigens results in a relatively low level of virus replication in the gut. Multiple oral immunizations were necessary for induction of strong anti-HIV antibody responses and for elicitation of substantial cell-mediated immune responses. Furthermore, the results obtained from intranasal administration of Ad–HIV recombinants indicate that infection of the upper respiratory tract and gut may occur in the presence of preexisting serum antibody to adenovirus. Anti-HIV antibodies were detected in mucosal secretions following booster immunization by the intranasal route. This encouraging demonstration of secretory antibodies suggests the need to pursue further studies using this route of administration. The safety of the intranasal route of administration is of primary concern and if pursued will require careful consideration and evaluation.

ACKNOWLEDGMENTS

The authors thank Lynda Greenberg, William Magargle, Kimberly Bishop, Walter Pierzchala, Frank Wiener, Dennis Austin, and Kathy E. Cobb for their excellent technical assistance.

REFERENCES

1. Hu S, Fultz PN, McClure HM, Eichberg JW, Thomas EK, Zarling J, Singhal MC, Kosowski SG, Swensom RB, Anderson DC, and Todaro G: Effect of immunization with a vaccinia–HIV *env* recombinant on HIV infection of chimpanzees. Nature (London) 1987;328:721–723.
2. Berman PW, Groopman JE, Gregory T, Clapham PR, Weiss RA, Ferriani R, Riddle L, Shimasaki C, Lucas C, Lasky LA, and Eichberg JW: Human immunodeficiency virus type 1 challenge of chimpanzees immunized with recombinant envelope glycoprotein gp120. Proc Natl Acad Sci USA 1988;85:5200–5204.
3. Arthur LO, Bess JW Jr, Waters DJ, Pyle SW, Kelliher JC, Nara PL, Krohn K, Robey WG, Langlois AJ, Gallo RC, and Fischinger PJ: Challenge of chimpanzees (*Pan troglodytes*) immunized with human immunodeficiency virus envelope glycoprotein gp120. J Virol 1989;63:5046–5053.
4. Rusche JR, Javaherian K, McDanal C, Petro J, Lynn DL, Grimaila R, Langlois A, Gallo RC, Arthur LO, Fischinger PJ, Bolognesi DP, Putney SD, and Matthews TJ: Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc Natl Acad Sci USA 1988;85:3198–3202.
5. Javaherian KA, Langlois AJ, McDanal C, Ross KL, Ecker LI, Jellis CL, Profy AT, Rusche JR, Bolognesi DP, Putney SD, and Matthews TJ: Principal neutralizing domain of the immunodeficiency virus type 1 envelope protein. Proc Natl Acad Sci USA 1989;86:6768–6772.
6. Emini EA, Nara PL, Schelif WA, Lewis JA, Davide JP, Lee DR, Kessler J, Conley S, Matsushita S, Putney SD, Gerety RJ, and Eichberg JW: Antibody-mediated *in vitro* neutralization of human immunodeficiency virus type 1 abolishes infectivity for chimpanzees. J Virol 1990;64:3674–3678.
7. Emini EA, Schelif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE, Jett CM, Eichberg JW, and Murthy KK: Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature (London) 1992;355:728–730.
8. Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, Wurm FM, Hershberg RD, Cobb KE, and Eichberg JW: Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature (London) 1990;45:622–625.
9. Girard M, Kieny M-P, Pinter A, Barre-Sinoussi F, Nara P, Kolbe H, Kusumi K, Chaput A, Reinhart T, Muchmore E, Ronco J, Kaczorek M, Gromard E, Gluckman J-C, and Fultz PN: Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. Proc Natl Acad Sci USA 1991;88:542–546.
10. Prevec L, Christie BS, Laurie KE, Bailey MMS, Graham FL, and Rosenthal KL: Immune response to HIV-1 Gag antigens induced by recombinant adenovirus vectors in mice and rhesus macaque monkeys. J Acquir Immune Defic Syndr 1991;4:568–576.
11. Dewar RL, Natarajan V, Vasudevachari MB, and Salzman NP: Synthesis and processing of human immunodeficiency virus type 1 envelope proteins encoded by a recombinant human adenovirus. J Virol 1989;63:129–136.
12. Natuk RJ, Chanda PK, Lubeck MD, Davis AR, Wilhelm J, Hjorth R, Wade MS, Bhat BM, Mizutani S, Lee S, Eichberg JW, Gallo RC, Hung PP, and Robert-Guroff M: Adenovirus–human immunodeficiency virus (HIV) envelope recombinant vaccines elicit high-titered HIV-neutralizing antibodies in the dog model. Proc Natl Acad Sci USA 1992;89:7777–7781.
13. Chengalvala M, Lubeck MD, Davis AR, Mizutani S, Molnar-Kimber K, Morin J, and Hung PP: Evaluation of adenovirus type 4 and type 7 recombinant hepatitis B vaccines in dogs. Vaccine 1991;9:485–490.
14. Lubeck MD, Davis AR, Chengalvala M, Natuk RJ, Morin JE, Molnar-Kimber K, Mason BB, Bhat BM, Mizutani S, Hung PP, and Purcell RH: Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus. Proc Natl Acad Sci USA 1989;86:6763–6767.

15. Chanda PK, Natuk RJ, Mason BB, Bhat BM, Greenberg L, Dheer SK, Molnar-Kimber KL, Mizutani S, Lubeck MD, Davis AR, and Hung PP: High level expression of the envelope glycoproteins of the human immunodeficiency virus type 1 in presence of *rev* gene using helper-independent adenovirus type 7 recombinants. Virology 1990;175:535–547.
16. Hirt B: Selective extraction of polyoma DNA from infected mouse cell cultures. J Mol Biol 1967;26:365–369.
17. Morin JE, Lubeck MD, Barton JE, Conley AJ, Davis AR, and Hung PP: Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. Proc Natl Acad Sci USA 1987;84:4626–4630.
18. The United States pharmacopeia XXII edition. The United States Pharmacopeial Convention, Inc., Rockville, Maryland, 1990, pp. 1788–1789.
19. Vernon SK, Murthy S, Wilhelm J, Chanda PK, Kalyan N, Lee S, and Hung PP: Ultrastructural characterization of human immunodeficiency virus type 1 *gag*-containing particles assembled in a recombinant adenovirus vector system. J Gen Virol 1991;72:1243–1251.
20. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K: Screening recombinant DNA libraries section 6. In: Curr Protocols Mole Biol. John Wiley & Sons, New York, 1988. pp. 6.4.1–6.4.8.
21. Eichberg JW, Zarling JM, Alter HJ, Levy JA, Berman PW, Gregory T, Lasky LA, McClure J, Cobb KE, Moran PA, Hu S-L, Kennedy RC, Chanh TC, and Dreesman GR: T-cell responses to human immunodeficiency virus (HIV) and its recombinant antigens in HIV-infected chimpanzees. J Virol 1987;61:3804–3808.
22. Eichberg JW, Montiel MM, Morale BA, King DE, Chanh TC, Kennedy RC, and Dreesman GR: Lymphocyte subsets in chimpanzees. Lab Anim Sci 1988;38:197–198.
23. Smith TJ, Buescher EL, Top FH Jr, Altemeier WA, and McCown JM: Experimental respiratory infection with type 4 adenovirus vaccine in volunteers: clinical and immunological responses. J Infect Dis 1970;122:239–248.
24. Stanley ED and Jackson GG: Spread of enteric live adenovirus type 4 vaccine in married couples. J Infect Dis 1969;119:51–59.
25. Mueller RE, Muldoon RL, and Jackson GG: Communicability of enteric live adenovirus type 4 vaccine in families. J Infect Dis 1969;119:60–66.
26. Kelly TJ and Lewis AM: Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J Virol 1973;12:643–652.
27. Burgert HG and Kvist S: An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell 1985;41:987–997.
28. Gooding LR, Elmore LW, Tollefson AE, Brady HA and Wold WSM: A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell 1988;53:341–346.
29. Ginsberg HS, Lundholm-Beauchamp U, Horswood RL, Pernis B, Wold WSM, Chanock RM, and Prince GA: Role of early region 3 (E3) in pathogenesis of adenovirus disease. Proc Natl Acad Sci USA 1989;86:3823–3827.
30. Tacket CO, Losonsky G, Lubeck MD, Davis AR, Mizutani S, Horwith G, Hung P, Edelman R, and Levine MM: Initial safety and immunogenicity studies of an oral recombinant adenohepatitis B vaccine. Vaccine 1992;10:673–676.

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