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Human Immunodeficiency Virus Type 1-Specific Immune Responses in Primates upon Sequential Immunization with Adenoviral Vaccine Carriers of Human and Simian Serotypes

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Two triple immunization vaccine regimens with adenoviral vectors with E1 deleted expressing Gag of human immunodeficiency virus type 1 were tested for induction of T- and B-cell-mediated-immune responses in mice and in nonhuman primates. The vaccine carriers were derived from distinct serotypes of human and simian adenoviruses that fail to elicit cross-neutralizing antibodies expected to dampen the effect of booster immunizations. Both triple immunization regimens induced unprecedented frequencies of gamma interferon-producing CD8$^+$ T cells to Gag in mice and monkeys that remained remarkably stable over time. In addition, monkeys developed Gag-specific interleukin-2-secreting T cells, presumably belonging to the CD4$^+$ T-cell subset, and antibodies to both Gag and the adenoviral vaccine carriers.

More than 40 million humans are presently infected with human immunodeficiency virus type 1 (HIV-1), and most live in developing countries with no access to antiretroviral drug treatment. A vaccine to HIV-1, which would provide the only cost effective and thus viable option to stem the epidemic, remains elusive. One of the most promising vaccine candidates, now in phase 1 clinical trials, is based on an E1 deletion human serotype 5 adenoviral (Ad) recombinant (AdHu5) (5, 15). However, preexposure to this ubiquitous human pathogen resulting in the circulation of neutralizing antibodies (NAs), which are found in many adult humans (6), can strongly limit the efficacy of an AdHu5 vaccine carrier (7).

To circumvent preexisting immunity and to broaden the available repertoire of Ad vaccine carriers suitable for booster immunizations, we developed a panel of E1 deletion Ad recombinants based on chimpanzee isolates (3). We previously reported on the AdC68 vector (7) and now extend these studies to two additional chimpanzee isolates termed AdC6 and AdC7. E1 deletion vectors based on molecular clones of AdC6 and AdC7 virus were generated to express a truncated form of Gag of HIV-1. They were tested in combination with an E1 deletion AdHu5 vector encoding the same transgene product for induction of T-cell-mediated immune responses in mice and nonhuman primates (NHPs). A triple immunization protocol with sequential use of heterologous Ad vaccine carriers was shown to induce exceedingly potent CD8$^+$ and CD4$^+$ T-cell responses as well as antibodies to Gag in mice as well as in NHPs.

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MATERIALS AND METHODS

Experimental animals. Two- to three-year-old Chinese rhesus macaques purchased from Covance Research Products (Alice, Texas) were housed in the Nonhuman Primate Facility of the Division of Medical Genetics of the University of Pennsylvania. BALB/c mice were purchased from Charles River (Boston, Mass.) and housed at the Animal Facility of The Wistar Institute.

Ad recombinants. Vectors were constructed from molecular clones, propagated on HEK 293 cells, purified, and titrated as described previously (6, 11; J. M. Wilson, submitted for publication). Protein expression was confirmed by Western blot analysis (data not shown). The virus particle (vp)/PFU ratios and contents of replication-competent adenoviruses of the batches used for immunization were determined using standard methods and are shown in Table 1.

Peptides. Fifteen- and 20-mer peptides with 10-amino-acid overlaps spanning the length of the truncated Gag protein (AIDS Reference and Reagents Center, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributor, Peptide and Protein Research or AnaSpec, Incorporated) were suspended at a concentration of 10 mg/ml in 10% dimethyl sulfoxide. They were then pooled into four separate pools of equal concentrations or one pool containing all peptides.

Immunization of animals. Chinese rhesus macaques were immunized by intramuscular (i.m.) injection of the Ad vaccines diluted to 10^{12} vp in 0.5 ml of buffered saline. Groups of five adult female BALB/c mice were immunized i.m. with 5×10^9 vp of Ad vectors, and splenic frequencies of CD8$^+$ T cells were determined at 10 days and at 8 weeks after each immunization.

Preparation of sera and plasma. Sedated NHPs were bled from the cephalic vein. Plasma was heat inactivated at 56°C for 30 min.

Virus neutralization assay. NHP plasma was tested on HEK 293 cells for neutralization of adenovirus by Ad vectors expressing green fluorescent protein in a plaque reduction assay, starting with a 1:20 dilution of sample (6).

T-cell assays. ELIspot and intracellular cytokine staining (ICS) for IL-2 or IFN-γ were performed using previously described protocols for monkeys (1) and mice (7). The intracellular cytokine assays for the NHP samples included staining for CD8 (PerCy5-labeled antibody), CD3 (fluorescein isothiocyanate-labeled antibody), IFN-γ (allophycocyanin [APC]-labeled antibody), and IL-2 (phycoerythrin-labeled antibody). In all assays, most of the IFN-γ-producing CD3$^+$ T cells stained positive for CD8 whereas IL-2-producing CD3$^+$ T cells were CD8$^-$.

Enzyme-linked immunosorbent assay. Immuno plates (MaxiSorb F96; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of a solution containing 1 μg of Gag/ml in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.8). Plates were blocked overnight with phosphate-buffered saline (PBS) supplemented with 5% bovine serum albumin. Plates were washed with PBS, and serial dilutions of monkey sera were added in borate buffer (0.1 M boric acid, 47 mM sodium borate, 75 mM NaCl, 0.05% [vol/vol] Tween 20)–3%

TABLE 1. Characteristics of adenoviral vectors

Viral backbone	Insertion	Vp/PFU ratio	No. of RCA[a]
E1 and E3 deletion AdHu5	gag37	516	$<10^2$
E1 deletion AdC6	gag37	152	$<10^2$
E1 deletion AdC7	gag37	1,735	$<10^2$
E1 and E3 deletion AdHu5	rab.gp	37	$<10^2$
E1 deletion AdC6	rab.gp	110	$<10^2$
E1 deletion AdC7	rab.gp	810	$<10^2$

[a] RCA, replication-competent adenovirus.

bovine serum albumin and incubated at 37°C for 1 h. Wells were washed with PBS, and 80 μl of a 1:200 dilution of alkaline phosphatase-conjugated, goat antimonkey immunoglobulin G (Sigma Chemical Company, St. Louis, Mo.) was added and incubated at 37°C for 2 h. Wells were washed and incubated with 100 μl of *p*-nitrophenylphosphate disodium hexahydrate in diethanolamine. After 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 3 M NaOH per well. Absorbance was read at 405 nm.

RESULTS

Characteristics of the Ad vectors. The AdC7 and AdC6 viruses were sequenced and vectored as E1 deletion molecular viral clones, and a codon-optimized sequence encoding a truncated form of Gag (gag37) of HIV-1 clade B (provided by G. Pavlakis, National Cancer Institute, Frederick, Md.) was inserted basically as described previously (7). The resulting E1 deletion viruses were propagated in HEK 293 cells, which provide E1 of the AdHu5 virus in *trans*. Both viruses gave yields in HEK 293 cells that were comparable to those obtained with E1 deletion AdHu5 recombinants. Nevertheless, the vp/PFU ratio, a parameter that determines the relative infectivity level of Ad virus, was generally highest with the AdC7 vectors and lowest with AdHu5 vectors (Table 1 and unpublished data). It should be pointed out, though, that the plaque assay does not necessarily take into account the interactions between the simian vectors and the HEK 293 cells and that the ability of the simian vectors to form visible plaques may thus not accurately reflect their infectivity. Expression of the Gag protein was confirmed by Western blot analysis of lysate of CHO cells transfected to express the coxsackie adenovirus receptor that is used by AdHu5 virus and by the simian Ad viruses (not shown).

Induction of transgene-specific CD8$^+$ T-cell responses in mice. The simian Ad vectors expressing gag37, termed AdC6gag37 and AdC7gag37, together with the corresponding AdHu5 (AdHu5gag37) vector were initially tested for induction of Gag-specific CD8$^+$ T cells in BALB/c mice. Since we presume that efficacy of a vaccine to HIV-1 will only be achieved after several injections, mice were vaccinated three times in 8-week intervals with the different constructs sequentially in two different regimens. Group 7-6-5 was given a primary injection with the AdC7gag37 vector, given a booster injection with the AdC6gag37 vector, and then again given a booster injection with the AdHu5gag37 vector. Group 5-6-7 received the vectors in the reverse order, i.e., the AdHu5gag37 vector first followed by the AdC6gag37 vector and then the AdC7gag37 vector. A prime-boost regimen using heterologous vectors was chosen to avoid the dampening effect of vaccine carrier-specific NAs elicited by the first immunization on uptake of the homologous vector upon its readministration. Splenic frequencies of CD8$^+$ T cells specific for the immunodominant epitope of Gag were tested by ICS for IFN-γ 10 days after each vaccine dose to assess the acute response and then 8 weeks after immunization to assess the duration of the response. As shown in Fig. 1, frequencies of Gag-specific CD8$^+$ T cells were readily detectable after the first immunization and slightly higher upon immunization with the simian AdC7gag37 vector. Upon the booster immunization with the AdC6gag37 construct, mice given a primary injection with the AdHu5gag37 vector developed higher frequencies compared to those that received two simian Ad vectors sequentially. After the second booster immunization, relative frequencies reversed and were higher in mice that received the AdHu5gag37 vector last. Frequencies were remarkably stable when tested 8 weeks after

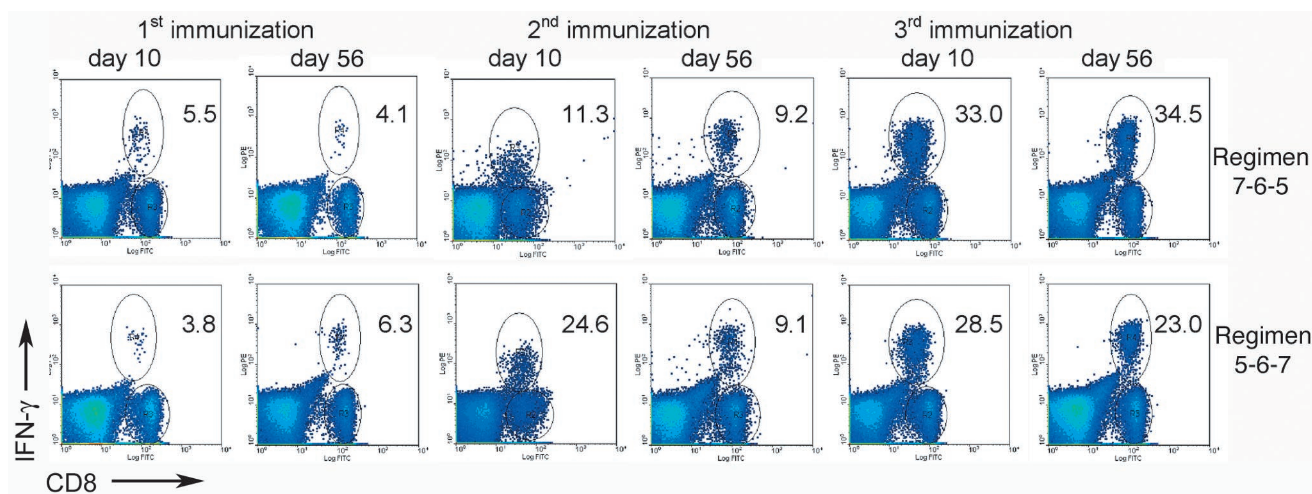

FIG. 1. Anti-Gag responses in mice. BALB/c mice were immunized at 8-week intervals with 5×10^9 vp of Ad vectors. Splenocytes were tested by ICS for IFN-γ produced by CD8$^+$ T cells in response to a peptide carrying the immunodominant epitope of Gag (AMQMLKETI). Graphs show the flow cytometry results of live cells stained for CD8 (*x* axis) and intracellular IFN-γ (*y* axis). The numbers in the upper right corners show percentages of double-positive CD8 cells over all CD8 cells − background activity in the absence of peptide.

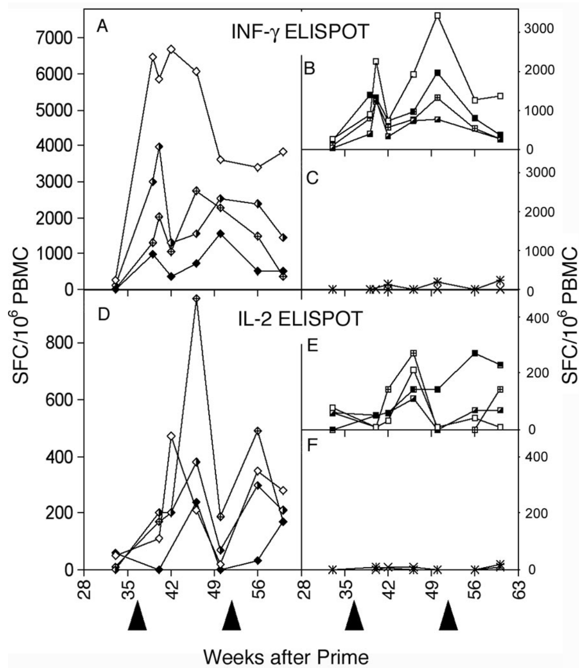

FIG. 2. Anti-Gag IFN-γ and IL-2 ELIspot responses in NHPs. PBMCs from immunized NHPs were tested at different time points after immunization by an ELIspot assay for IFN-γ (A to C) or IL-2 (D to F). (A and D) Results of 5-6-7 regimen (animals 10, 90, 97, and 115 [◇, ◆, ◈, ⊕, respectively]); (B and E) results of 7-6-5 regimen (animals 18, 48, 140, and 145 [□, ■, ◨, ⊞, respectively]); (C and F) results for control animals (animals 150 X and 164 X [*]). Heavy arrows indicate the booster immunizations. SFC, spot-forming cells.

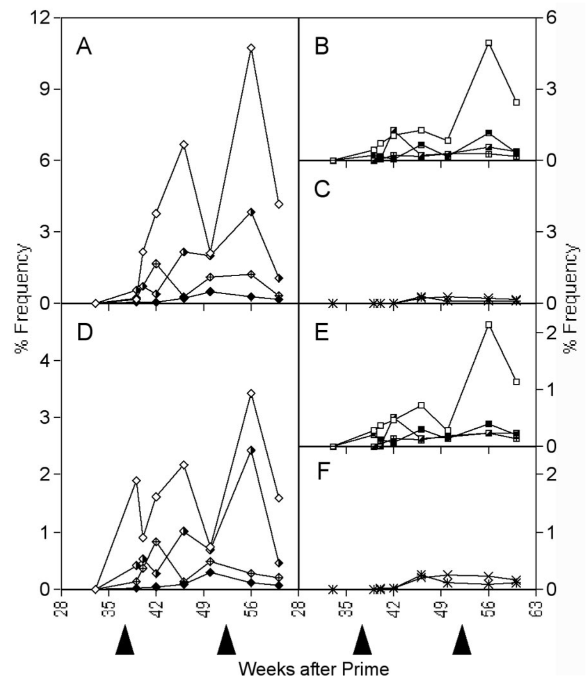

FIG. 3. Frequencies of Gag-specific IFN-γ-producing T cells in NHPs. PBMCs were tested by ICS for frequencies of $CD8^+$ (A to C) and $CD3^+$ (E to F) PBMCs in NHPs. Results of ICS testing are shown for NHP PBMCs for group 5-6-7 (A and D) and group 7-6-5 (B and E); the symbols for the animals are defined as described for Fig. 2. Cells were stimulated with four subpools of peptides that in total span the Gag protein; the resulting frequencies were added for each animal at a given time point. Frequencies were calculated as described for Fig. 1. Heavy arrows indicate the booster immunizations.

each immunization, and this stability appeared to increase upon repeated vaccinations.

Induction of transgene-specific T-cell responses in NHPs. To ensure that the vaccine carriers were suitable to induce transgene product-specific T cells in NHPs, two groups of four rhesus macaques each were immunized i.m. using 10^{12} vp of the Ad vectors and the regimens described above for the mouse study. A third group (two macaques) was immunized with the same Ad backbones carrying the rabies virus glycoprotein (rab.gp) in the same order as group 7-6-5. The animals were given a primary injection on day 0 and received the first booster injection at 8 months and the second booster injection at 12 months after the primary injection.

T-cell-mediated immune responses were assessed over time from peripheral blood mononuclear cells (PBMCs) by ICS (IFN-γ) of $CD3^+$ and $CD8^+$ cells and by ELIspot for IL-2 and IFN-γ levels to gain insight into activation of both $CD8^+$ and $CD4^+$ T cells. Animals were first tested 7 months after the first immunization. At this time point, two of four animals receiving AdHu5gag37 and four of four animals receiving AdC7gag37 had detectable responses to Gag peptides by the ELIspot assay for IFN-γ (Fig. 2A to C); none of the animal scored as positive by ICS (Fig. 3). IL-2 responses were positive in two of four animals given a primary injection with AdHu5gag37 and three of four animals given a primary injection with AdC7gag37 (Fig. 2D to F). After the first booster injection (AdC6gag37), responses of above 1,000 IFN-γ spots per 10^6 PBMCs became detectable by ELIspot for at least one of the time points in all eight animals of both groups, with the highest response in animal 10 in the 5-6-7 group (>6,000 IFN-γ spots/10^6 PBMCs; Fig. 2A). Gag-specific $CD8^+$ T-cell frequencies determined by ICS mimicked these results and were detectable in three of four animals in either group (Fig. 3A to C), with the best responder of group 5-6-7 developing frequencies of Gag-specific IFN-γ-producing $CD8^+$ T cells above 6% of all circulating $CD8^+$ cells. The other two responders in this group had peak frequencies of ~2% (Fig. 3A). The three responders of group 7-6-5 had peak $CD8^+$ frequencies of around 1% (Fig. 3B). Frequencies of IFN-γ-producing $CD3^+$ T cells paralleled those of $CD8^+$ T cells (Fig. 3D to F), indicating that most of the IFN-γ originated from $CD8^+$ T cells. Frequencies of IL-2-secreting T cells increased in all of the animals after the booster injection, and there was no correlation between frequencies of IL-2- and IFN-γ-producing T cells (Fig. 2D to F). The second booster injection augmented Gag-specific $CD8^+$ T-cell frequencies detected by ICS further in two of four animals in each group, with the highest responder (animal 10) reaching a frequency of >10% (Fig. 3A). IL-2 responses in some of the animals also increased (Fig. 2D to F), while IFN-γ responses obtained by ELIspot analysis declined in most animals after the third immunization (Fig. 2A to C). Peak frequencies seen in the animals after each immunization with the different assays are summarized in Fig. 4.

The macaques were subjected to necropsy between 7 and 17 weeks after the final immunization. PBMCs as well as lympho-

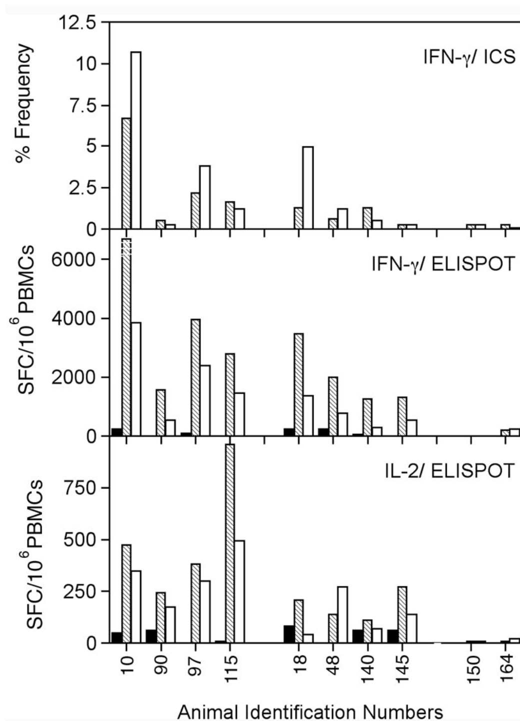

FIG. 4. Peak responses. The figure summarizes the results from Fig. 2 and 3 by showing peak responses obtained by ICS and ELIspot testing of individual animals after each immunization. SFC, spot-forming cells.

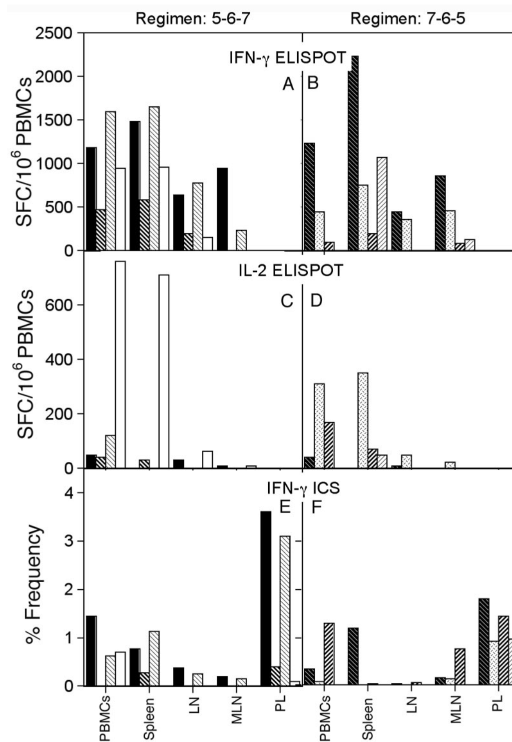

FIG. 5. Immune responses in different tissues at necropsy. Macaques were subjected to necropsy 7 to 17 weeks after the final booster immunization, and lymphocytes isolated from various tissues were analyzed for Gag-specific $CD8^+$ responses by ELIspot testing for IFN-γ (A and B) and IL-2 (C and D) and by ICS (E and F). Tissues studied included blood (PBMCs); spleen; popliteal, inguinal, and axillary lymph nodes (LN); mesenteric lymph nodes (MLN); and peritoneal lavage fluid (PL). Animals 90, 145, and 150 were subjected to necropsy at week 7; animals 48, 115, and 140 were subjected to necropsy at week 8; and animals 10, 18, 97, and 164 were subjected to necropsy at week 17 following the final immunization. Background staining for the two control animals was averaged for each tissue and subtracted from the animal results for the ICS; for the ELIspot assays, 0 spots per 10^6 cells were detected in all samples except for the spleen sample of animal 164 (50 spots per 10^6 cells). SFC, spot-forming cells.

cytes from pooled peripheral and mesenteric lymph nodes, spleens, and peritoneal lavage fluid were tested for Gag-specific T-cell responses (Fig. 5A to F). Frequencies of IFN-γ-producing cells detectable by ICS (Fig. 5E and F) and ELIspot (Fig. 5A and B) were largely comparable in spleens and PBMCs and lower in lymph nodes. Frequencies were markedly higher, especially for the 5-6-7 group, in peritoneal lavage fluid; due to lack of sufficient cell recovery, these frequencies could only be tested by ICS. IL-2-producing cells were mainly detected in PBMCs and splenocytes and only at low frequencies in lymph nodes (Fig. 5C and D). The control animals tested throughout the experiments failed to develop T-cell-mediated immune responses to Gag, as shown in Fig. 2 to 4.

Various results were obtained when the breadth of the Gag-specific $CD8^+$ T-cell responses induced by the different Ad vectors by ICS using pools of Gag peptides (Fig. 6 and Table 2) was tested. Some animals had focused responses to one out of four subpools of peptide used for the ICS, while others showed more balanced and broad responses (Fig. 6). The breadth of the responses stayed consistent in each animal throughout the experiment, indicating that Ad booster immunizations neither broadened nor narrowed the repertoire of the effector T cells.

Induction of Gag-specific B-cell responses in NHPs. To assess the simian-human Ad vector prime-boost regimen for the induction of transgene product-specific B-cell responses, sera of the two groups of rhesus macaques vaccinated with Ad vectors expressing gag37 of HIV-1 were analyzed for Gag-specific antibodies (Fig. 7A). Plasma from animals that received the rab.gp-expressing Ad vectors (Adrab.gp) controlled this experiment. Animals were bled 4 weeks after each immunization. All animals seroconverted after the first immunization. Titers in three of four animals were markedly higher upon immunization with the AdHu5gag37 compared to the results seen with the AdC7gag37 vector. After booster injections with the AdC6gag37 vector were administered, antibody titers increased significantly in all of the animals and, with one exception, titers were comparable regardless of the vaccine used for the primary injection. Upon a third immunization, all of the animals given booster injections with AdHu5gag37 displayed an increase in titers to Gag in contrast to animals given booster injections with the AdC7gag37 vector.

Induction of vector-specific antibody responses in NHPs. NA titers to the three Ad serotypes were tested before and then 4 weeks after each immunization (Fig. 7B) to assess the antibody response to the vaccine carrier. None of the animals had preexisting NAs to the adenoviruses. After the first immunization, monkeys vaccinated with the AdC7 vectors developed NA titers between 1:20 to 1:80 to AdC7 virus; titers increased

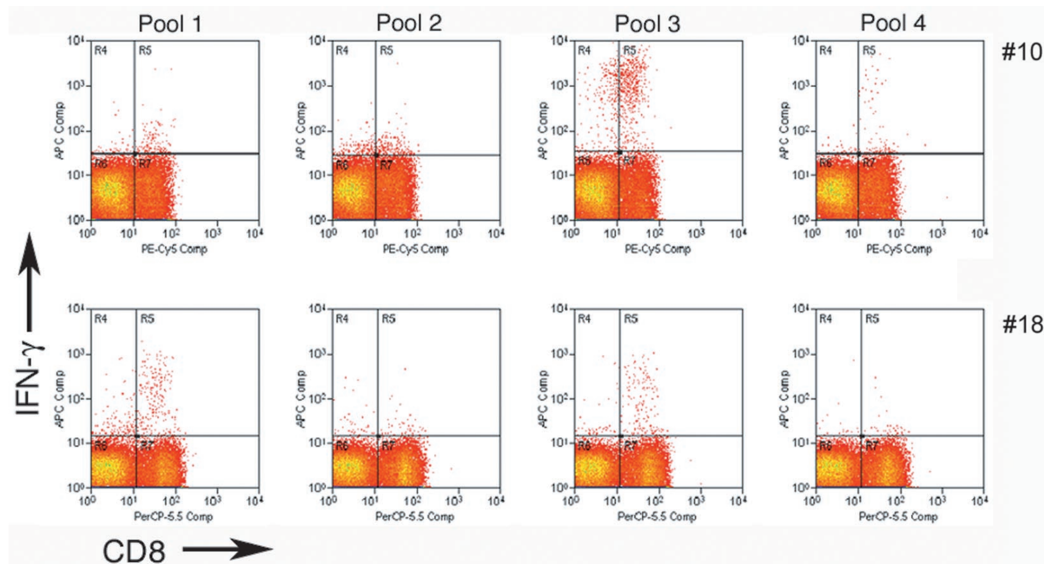

FIG. 6. Breadth of response in macaques. The panels show representative ICS data for PBMCs from animal 10 (top panels) and animal 18 (bottom panels) stimulated with four different Gag peptide subpools.

in four of six animals when tested 8 months after vaccination. Monkeys immunized with the AdHu5 vectors generated titers to AdHu5 virus ranging from 1:640 to 1:1,000 within 4 weeks; these decreased slightly by the time of booster immunization. The more-rapid kinetics for AdHu5 virus were primarily seen after the first immunization and may reflect distinct interactions of the vectors with cells of the innate immune system (19, 21). After booster injections with the AdC6 vectors were given, all of the animals developed NA titers to AdC6 virus. Interestingly, in 3 out of 10 animals the booster immunization with the AdC6rab.gp vector increased the anti-Ad antibody response generated to the initial vaccination, suggesting the presence of T-helper cells that cross-reacted between the different Ad serotypes. Upon the third immunization with either the AdHu5 or the AdC7 vectors, the animals again generated NAs to the Ad serotype used for immunization. Throughout the experiment, none of the animals developed NAs that cross-reacted between the three different vaccine carriers. Additional studies are needed to address induction of T cells to shared epitopes of the different serotypes of adenovirus and their potential effect on prime-boost regimens with heterologous Ad vaccine vectors.

TABLE 2. Breadth of the $CD8^+$ T-cell response in NHPs[a]

Vaccination schedule and animal	Frequency of $CD8^+$ IFN-γ^+ cells for peptide pool:			
	1	2	3	4
5-6-7				
10	0.11	0.11	6.19	0.26
90	0.13	0.07	0.05	0.00
97	0.13	1.88	0.10	0.08
115	0.15	0.06	0.03	0.05
7-6-5				
18	1.13	0.00	0.13	0.03
48	0.51	0.05	0.03	0.05
140	0.10	0.07	0.02	0.01
145	0.07	0.04	0.02	0.01
Control[b]				
150	0.06	0.07	0.02	0.03
164	0.14	0.08	0.01	0.03

[a] Frequencies of Gag-specific $CD8^+$ T cells for individual peptide pools are shown. NHP PBMCs were tested 2 weeks after the third immunization.
[b] Control, adenoviral vectors expressing the rab.gp.

DISCUSSION

Here we report the results of a prime-boost regimen using heterologous Ad vectors expressing a truncated form of Gag of HIV-1. E1 deletion Ad vectors have been used successfully as vaccine carriers for pathogens in experimental animals (5, 14, 15, 16, 18, 20, 21). Their ability to elicit potent cellular and humoral immune responses to the encoded transgene product is linked to some of their unique features. They achieve high levels of transgene product expression in a broad range of cells, including antigen-presenting cells (22). The E1 deletion reduces transcription of Ad antigens, which results not only in loss of cell death upon infection and thus sustained antigen presentation (20) but also in an immune response that is focused, albeit not exclusively, on the transgene product. Ad vectors initiate an inflammatory reaction, which induces maturation of dendritic cells into competent APCs (19, 22), thus negating the need for addition of adjuvants. On a practical note, Ad vectors are easy to construct and they can be propagated in vitro to high titers, thus facilitating upscaling for eventual commercial use. E1 deletion Ad vectors are replication defective and thus safer and more predictable than replication-competent vaccine carriers.

The simian AdC6 and AdC7 vectors were developed to overcome preexisting immunity to common human serotypes of adenovirus and to broaden the repertoire of Ad subunit vaccines for booster immunizations. Nearly all adults have

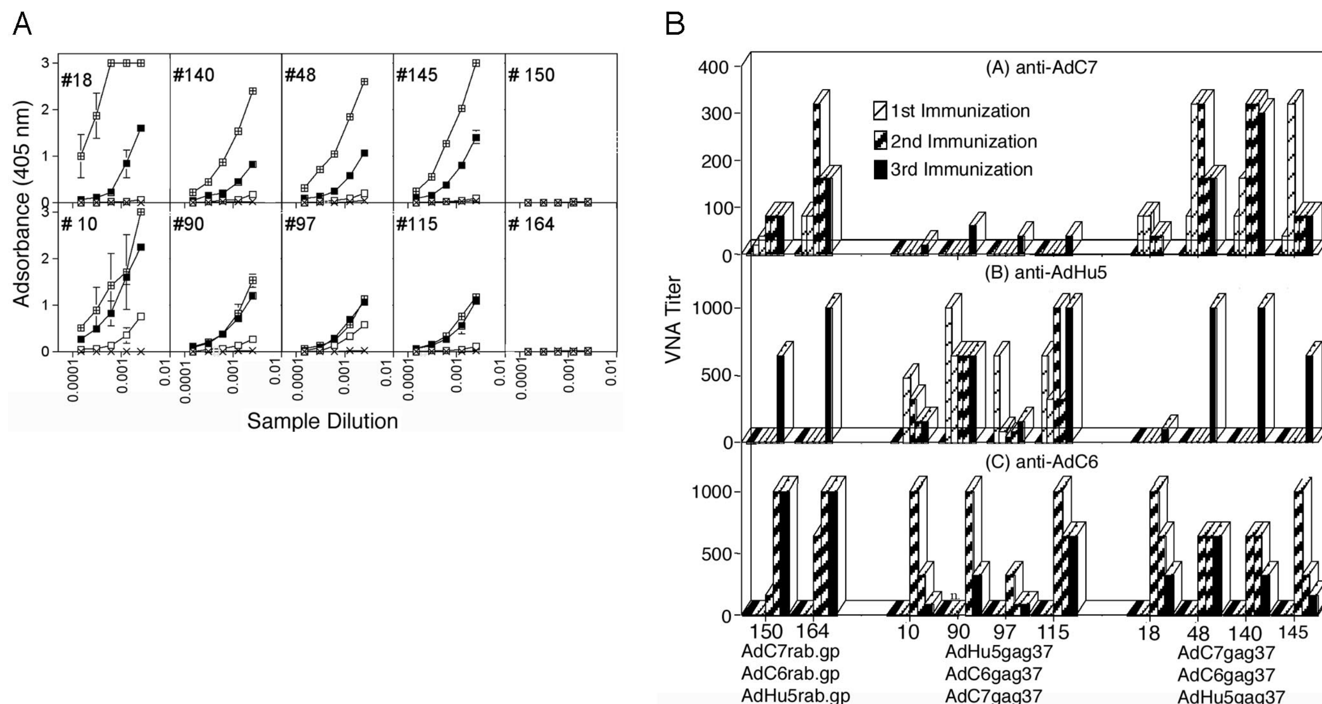

FIG. 7. Induction of antibody responses in NHP. (A) Induction of Gag-specific antibody responses in NHPs. Two groups of four NHPs each were immunized with AdC7gag37, then with AdC6gag37, and then with AdHu5gag37 vector (upper row; animals 18, 140, 48, and 145). An additional four NHPs (lower row; animals 10, 90, 97, and 115) were first immunized with AdHu5gag37, then with AdC6gag37, and then with AdC7gag37 following the same schedule and dosing as used for the other group. # 150 and # 164 represent plasma from the two animals immunized with the rab.gp-expressing vectors. Data show the results of a Gag-specific enzyme-linked immunosorbent assay expressed as means ± standard deviations of triplicate samples (×, preimmunization results; open boxes, post-first-immunization results; closed boxes, post-second-immunization results; boxes marked with crosses, post-third-immunization results). (B) The graph shows NHP serum sample titers of NAs produced in response to the different Ad serotypes. Sera were tested before immunization (black-topped columns), 4 weeks after the first immunization (first columns faced with thin stripes), and before immunization with the second vaccine (second columns faced with thin stripes). They were tested 4 weeks after the second (first columns faced with bold stripes) and before the third immunization (second columns faced with bold stripes). They were tested 4 weeks after the third immunization (black-faced columns). The *y* axis shows the titers; the *x* axis shows the animal identification numbers.

antibodies to the common serotypes of human adenovirus, and ~45% of adults in the United States have NAs to AdHu5 virus (6), with a similar prevalence of AdHu5 NAs in the human population residing in Central Africa and a markedly higher prevalence in those from Thailand (unpublished data). The chimpanzee viruses AdC6 and AdC7 are not neutralized by antibodies to AdHu5 virus, and they do not appear to circulate extensively in humans (unpublished data).

Fitzgerald et al. and Pinto et al. showed previously that the simian Ad vectors of the C68 and the C6 serotypes induce potent transgene product-specific $CD8^+$ T-cell responses in inbred mice; these responses could be boosted by the sequential use of heterologous vectors (7, 11). Here we extended these studies to two triple immunization protocols. In mice, each booster immunization increased frequencies of $CD8^+$ T cells by approximately two to threefold; although the AdHu5/simian Ad combination performed better after the double immunization than the two simian Ad vectors used sequentially, there was no clear advantage to either of the regimens after the third immunization. Surprisingly, frequencies of Gag-specific $CD8^+$ T cells were remarkably stable, especially after the third immunization, suggesting either that the repeated immunization had caused a preferential expansion of $CD8^+$ T cells destined for the memory pool or, alternatively, that antigen persisted for a prolonged time.

In NHPs, Gag-specific T-cell responses became readily detectable after the first booster immunization in all animals, although frequencies of responses, best exemplified by the ICS results for IFN-γ, differed between the animals. The highest responder in the 5-6-7 group had an unprecedented frequency of 6% of Gag-specific $CD8^+$ T cells over all $CD8^+$ T cells in PBMCs, while the lowest responder (group 7-5-6) had a frequency of only 0.4%. Although the number of NHPs was too small and the results were too variable to allow definitive conclusions, overall the 5-6-7 schedule appeared superior to the 7-6-5 schedule. We assume that the variability between animals of each experimental group, including the various results with respect to the success of the second booster immunization, is in part reflective of the use of a short transgene, which encodes too limited a number of T-cell epitopes to ensure potent and comparable responses in an outbred population. For these proof-of-principle studies we used a truncated form of Gag of HIV-1 clade B. For eventual clinical use, vectors expressing several antigens of HIV-1 will have to be developed to elicit sufficiently broad T-cell responses to prevent outgrowth of HIV-1 escape mutants (2). We would antic-

ipate that this would not only increase T-cell frequencies for HIV-1 antigens overall but also elicit more homogeneous responses in outbred NHPs.

The results with respect to the longevity of the T-cell response also differed but were remarkably stable in most NHPs over the 4-month period between the first and second booster immunizations. The second booster immunization transiently increased the frequencies of IFN-γ-producing $CD8^+$ T cells in some animals but overall did not yield the impressive results observed in mice. Booster immunizations become most effective once the effector T cells induced by primary injection have been replaced by memory T cells. It remains to be investigated whether the outcome of a triple Ad immunization protocol can be improved by a longer resting period between vaccinations. Frequencies of IFN-γ-producing T cells that increased after the second booster injection in some animals according to ICS results decreased when tested for by ELIspot assay. We infer that this relates to susceptibility of the effector T cells to activation-induced cell death upon reexposure to antigen, especially when this occurs in presence of IL-2. Cells were exposed to antigen for 6 h for ICS, while lymphocytes were stimulated for 48 h in the ELIspot assay, which is thus more affected by antigen-induced death of responding T cells (unpublished observation). The frequency of IL-2-producing T cells was substantial in some NHPs after three immunizations and thus presumably facilitated apoptosis of Gag-specific effector T cells.

NHPs were subjected to necropsy between 7 and 17 weeks after the last immunization to assess the tissue distribution of Gag-specific T cells. IL-2- or IFN-γ-producing T cells were detectable at higher frequencies in blood, spleens, and especially peritoneal lavage than in lymph nodes. In mice, so-called central memory cells home predominantly to lymph nodes whereas effector memory cells that are more active and may thus provide a first line of defense against invading pathogens migrate to nonlymphoid tissues, including the peritoneal cavity (13). Although a dichotomy of memory T cells has not yet been established formally for NHPs, our postmortem results suggest that the triple Ad immunization protocols elicit a pronounced effector memory T-cell population.

In addition to $CD8^+$ T cells implicated in providing resistance, albeit not sterilizing immunity (2), to immunodeficiency virus infections, IL-2-secreting $CD4^+$ T cells are also viewed as critical to the success of HIV-1 vaccines (8). The triple Ad immunization protocol performed well in this regard by inducing in all of the NHP lymphocytes (presumably belonging to the $CD4^+$ T-cell subset) to secreted IL-2 in response to Gag peptides.

Although antibodies to Gag may not contribute to protection against HIV-1 progression, plasma samples from NHPs immunized with the Ad vectors were tested for such antibodies to assess how the two immunization protocols performed in the induction of transgene product-specific B-cell responses. NHPs that received the AdHu5gag37 vector first developed slightly better antibody titers than animals injected with the AdC7gag37 vector. Nevertheless, titers became comparable between the groups upon booster immunization with the AdC6gag37 vector. The most remarkable difference was seen after the second booster immunization, at which time animals that received the AdHu5gag37/AdC6gag37/AdC7gag37 regimen failed to show an increase in titers after the last immunization whereas animals that received the AdHu5gag37 vaccine last again showed a robust booster effect. Numerous factors influence the magnitude of vector-induced primary or recall antibody responses. These include the effects of the vectors on cells of the innate immune system, the resulting type of the immune response, and the antigenic load and potential interference due to immune responses that cross-react between the different vaccine carriers. Additional studies are necessary to unravel the pathways that govern the booster effects of heterologous Ad vectors.

A plethora of viral recombinant vectors have undergone preclinical tests in NHPs and clinical trials in humans to assess their efficacy as vaccine carriers to simian immunodeficiency virus (SIV)–HIV-1 antigens (reviewed in references 9 and 10). Initial vaccination efforts focused on vectors encoding the viral Env antigens, the sole target for NAs. Due to the high variability of Env and its heavy glycosylation, such vaccines fared poorly in preclinical and clinical trials (4) and were subsequently replaced by vaccines designed to induce cell-mediated immune responses. Of those, E1 deletion Ad vectors (15) and poxvirus recombinants based on modified vaccinia Ankora (1, 17) or ALVAC (12) encoding either Env or Gag alone or Gag combined with other HIV-1–SIV antigens used in prime-boost regimens in conjunction with DNA vaccines resulted in the highest frequencies of Gag-specific $CD8^+$ T cells. $CD8^+$ T cells in turn provided partial protection against infections of NHPs with SIV or a pathogenic SIV–HIV chimera and prevented high viral loads and CD4 loss (1, 15). Here we used for our initial NHP studies (geared towards assessment of vector performance) Ad vaccines encoding Gag of HIV-1 for which preclinical challenge models are unavailable. Nevertheless, in spite of this limitation and the use of a single truncated HIV-1 antigen, results are promising, as frequencies obtained with the triple immunization protocol were markedly higher in the peak responders than those obtained with other SIV–HIV vaccine modalities. Additional studies are needed to confirm these results with vectors encoding antigens that permit NHP challenge experiments and to determine the effect of preexisting immunity to AdHu5 virus on the effectiveness of AdHu5 recombinant vaccine vectors as part of a prime-boost regimen with heterologous Ad vectors.

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