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Epitopes Expressed in Different Adenovirus Capsid Proteins Induce Different Levels of Epitope-Specific Immunity

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On the basis of the concept that the capsid proteins of adenovirus (Ad) gene transfer vectors can be genetically manipulated to enhance the immunogenicity of Ad-based vaccines, the present study compared the antiantigen immunogenicity of Ad vectors with a common epitope of the hemagglutinin (HA) protein of the influenza A virus incorporated into the outer Ad capsid protein hexon, penton base, fiber knob, or protein IX. Incorporation of the same epitope into the different capsid proteins provided insights into the correlation between epitope position and antiepitope immunity. Following immunization of three different strains of mice (C57BL/6, BALB/c, and CBA) with either an equal number of Ad particles (resulting in a different total HA copy number) or different Ad particle numbers (to achieve the same HA copy number), the highest primary (immunoglobulin M [IgM]) and secondary (IgG) anti-HA humoral and cellular CD4 gamma interferon and interleukin-4 responses against HA were always achieved with the Ad vector carrying the HA epitope in fiber knob. These observations suggest that the immune response against an epitope inserted into Ad capsid proteins is not necessarily dependent on the capsid protein number and imply that the choice of incorporation site in Ad capsid proteins in their use as vaccines needs to be compared in vivo.

Adenovirus (Ad)-based gene delivery systems are a promising platform for genetic vaccines because of their abilities to act as immune system adjuvants and to rapidly evoke immune responses against the transgene product and the Ad capsid proteins (1, 2, 9, 17, 29, 35, 41, 43, 49, 52, 54). One disadvantage of Ad as a vaccine carrier is that anti-Ad immunity after one immunization prevents boosting of the immune response by repeated administration (9, 10, 35, 49). A strategy to allow boosting with an identical Ad vector is to incorporate the vaccine epitopes into the Ad capsid (3, 52). The outer capsid proteins of Ad which have been targets for genetic modification include the three major capsid proteins hexon, fiber knob, and penton base, as well as protein IX, a minor capsid protein. These Ad capsid proteins can be readily engineered (5, 6, 20, 21, 31, 33, 36, 46, 48, 53).

Hexon, the largest and most abundant of the Ad capsid proteins, is the major target for neutralizing antibodies generated by the host against Ad (39, 44, 50). Hexon-modified Ad vectors included Ad vectors with antigenic peptides incorporated into one of the outer loops of the hexon protein as experimental vaccines (3, 52). In addition to hexon, antigenic epitopes have been incorporated into protein IX and penton base to optimize the use of Ad for vaccination (5, 6, 33, 36, 48). These studies focused on the analysis of exposure of the epitope, the ability to produce viable vector, or the infectivity of the modified vector in vitro, rather on a direct comparison of antiepitope immunogenicities of modified Ad vectors with epitopes incorporated into different Ad capsid proteins. To assess the latter question, the present study was directed to evaluate which of these capsid proteins can be modified without affecting the infectivity of the Ad and still induce high antiepitope immunity.

To accomplish this, we compared host immune responses following immunization with four Ad vectors with the same epitope of the hemagglutinin (HA) protein of the influenza A virus incorporated into the outer capsid protein hexon, penton base, fiber knob, or protein IX. All vectors were able to infect cells in vitro, with the highest Ad genome copy number observed for the Ad vector with the HA epitope incorporated into hexon. Interestingly, immunization of mice with either the same number of Ad particles, resulting in different total HA copy numbers, or the same number of HA copies, the highest humoral and cellular responses against HA were achieved with the Ad vector carrying the HA epitope in fiber knob, suggesting that, at least for this model system, antigenic epitopes placed in fiber knob elicit optimal immunity for an Ad-based vaccine.

MATERIALS AND METHODS

Ad vectors. The recombinant Ad vectors used in this study are E1a, partial E1b, and partial E3 vectors based on the Ad5 genome (9, 40). The expression cassettes were inserted into the E1 region and contained the human cytomegalovirus intermediate-early enhancer-promoter, the transgene, and a simian virus 40 poly(A) stop signal (12, 37). The vectors expressed either β-galactosidase (Z), luciferase (L), or no transgene (Null) (12). For the hexon-modified vector, AdZ.Hx-HA, the HA epitope YPYDVPDYA was inserted into loop 1 of hypervariable region 5 between residues 268 and 269, resulting in 720 HA epitopes per capsid, 3 for each of the 240 hexon trimers. For the protein IX-modified vector, AdLdX.pIX-HA, the HA gene was added to the C terminus of protein IX, resulting in 240 HA copies per Ad capsid. For the penton base-modified vector, AdL.L2-HA, the HA epitope was inserted into the RGD loop of penton base

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TABLE 1. Adenovirus vectors used in this study

Vector name	HA[a] incorporation site	Position (residue[s])	Reporter gene product	Structure	No. of HA copies	Reference
AdZ.Hx-HA	Hexon (HVR5)[b]	268-269	β-Galactosidase	Trimer	720	This work
AdLdX.pIX-HA	Protein IX (C terminus)	139	Luciferase	Trimer	240	This work
AdL.L2-HA	Penton base (RGD loop)	301-302	Luciferase	Pentamer	60	This work
AdL.F-HA	Fiber knob (HI loop)	543-544	Luciferase	Trimer	36	This work
AdZ	None	None	β-Galactosidase	None	None	12
AdNull	None	None	None	None	None	12

[a] The HA sequence YPYDVPDYA was incorporated.
[b] Hypervariable region 5 in loop 1 of hexon.

between residues 301 and 302, resulting in 60 copies per capsid. For the fiber knob-modified vector, AdL.F-HA, the HA sequence was placed in the HI loop of fiber knob between residues 543 and 544, resulting in 36 HA epitopes per capsid (Table 1). The vectors were propagated and purified as described previously (37, 38). Vector dosing was done on the basis of the physical particle concentration (particle units [pu]) as previously specified (25).

Western analysis. To assess the presence of the HA epitopes in the relevant capsid proteins, Ad vectors (10^{10} virus particles) were denatured by heating at 95°C for 5 min in NuPAGE sample buffer (Invitrogen, Carlsbad, CA) and separated by 4 to 15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; NuPAGE system; Invitrogen). The gel was transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), and equal loading was confirmed by GelCode silver staining (Pierce, Rockford, IL). The membrane was exposed to blocking solution (10% fat-free milk [blot grade; Bio-Rad Laboratories] in phosphate-buffered saline [PBS], pH 7.4) for 1 h and then incubated with a 1:1,000 dilution of anti-HA antibody (Sigma-Aldrich, St. Louis, MO) for 1 h. A peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich) was then added for 1 h of incubation, followed by detection with chemiluminescent peroxidase substrate (ECL+ reagent; Amersham Biosciences, Piscataway, NJ).

Mice. Female C57BL/6 (H-2^b), BALB/c (H-2^d), and CBA (H-2^k) mice were obtained from Taconic Farms (Tarrytown, NY). The animals were housed under specific-pathogen-free conditions and used at 6 to 8 weeks of age. The mice were immunized once intramuscularly with the Ad vectors diluted in 50 μl of PBS.

Accessibility of HA epitopes in Ad capsids. To evaluate if the HA epitopes are present on the capsid and are accessible for immune recognition, the four different HA vectors were immobilized on enzyme-linked immunosorbent assay (ELISA) plates by overnight incubation in 100 mM carbonate buffer (pH 9.5) at 4°C to achieve 1.8×10^{11} copies of HA for each vector/well as follows: AdZ.Hx-HA, 2.5×10^8 pu/well; AdLdX.pIX-HA, 7×10^8 pu/well; AdL.L2-HA, 3×10^9 pu/well; AdL.F-HA, 5×10^9 pu/well. The control AdNull vector was used at 5×10^9 pu/well. After extensive washes with 0.05% Tween 20 in PBS (PBST) and blocking with blocking solution (2% bovine serum albumin, 0.05% Tween 20 in PBST), the plates were incubated with a monoclonal antibody against HA (Sigma-Aldrich) for 2 h by serial $\log_2$ dilutions. Following incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma-Aldrich), detection was accomplished with a peroxidase substrate kit (Bio-Rad Laboratories) and absorbance was determined at 415 nm.

Production of recombinant HA fusion peptide. To produce a recombinant HA fusion peptide with an N-terminal His tag, the recombinant vector Smt3-HA with an N-terminal His tag was constructed by cloning the annealed oligonucleotide (sense strand, 5′-GA TCT GGT GGG TAC CCA TAC GAT GTT CCA GAT TAC GCT GGT GGA G-3′; antisense strand, 5′-GA TCC TCC ACC AGC GTA ATC TGG AAC ATC GTA TGG GTA CCC ACC A-3′) into the BamHI site of modified expression vector Smt3 (Invitrogen). The recombinant vector Smt3-His-HA was transformed into *Escherichia coli* BL21(DE3). The recombinant HA fusion peptide was purified by Ni-chelating affinity chromatography from a single transformant under native conditions. Briefly, the cultures were grown to an optical density at 60 nm of 0.8, stimulated with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C, and collected by centrifugation. The cell pellet was washed and resuspended in TBS buffer I (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, pH 7.4). Cell lysis was induced by sonification three times (each time, 10-s pulse, 1-min interval), and the lysate was cleared by centrifugation (18,000 × *g*, 4°C). Imidazole (10 mM) was added, and the crude extract was placed on Ni-nitrilotriacetic acid agarose (Prebound; QIAGEN, Valencia, CA) equilibrated with TBS buffer I. Following washout of unbound material, the protein was eluted in TBS buffer II (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 300 mM imidazole, pH 7.4) and dialyzed against PBS.

Infection with HA-modified vectors in vitro. To evaluate if incorporation of the HA epitope in the different capsid proteins interferes with the coxsackie Ad receptor (CAR)-dependent or -independent infection in vitro, infection of A549 cells (CAR-dependent infection) or dendritic cells (DC; integrin-dependent infection) was compared by analyzing Ad genome levels in the cells following infection. A549 cells (CCL185; American Type Culture Collection, Manassas, Va.), maintained in complete Dulbecco's modified essential medium (10% fetal bovine serum, 100 U of penicillin/ml, 100 mg of streptomycin/ml), were infected with 1,000 pu/cell HA-modified vectors and AdZ as a control in low-serum medium (2% fetal bovine serum) for 2 h, washed, and maintained in complete medium for 24 h. Bone marrow-derived DC were generated from bone marrow precursors as described previously (42). In brief, bone marrow cells harvested from C57BL/6 mice were grown in complete RPMI 1640 medium (10% fetal bovine serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml [GIBCO BRL, Gaithersburg, Md.]) supplemented with 10 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor and 2 ng/ml recombinant mouse interleukin-4 (IL-4) (both from R&D Systems) for 8 days. The DC were then washed and resuspended in PBS, infected with the HA-modified vectors (10,000 pu/cell) for 4 h, and washed and maintained in complete medium for 24 h. Cells were then harvested and washed, and genomic DNA was obtained with a DNeasy kit (QIAGEN).

The Ad infection level was assessed by comparing the amount of total DNA to the amount of Ad DNA. The total DNA concentration was assessed by spectrophotometry, assuming that the cellular DNA content was 6.6 pg/cell (8). Ad DNA levels were assessed by TaqMan real-time quantitative PCR with primer and probe to the Ad DNA binding protein sequence (forward, 5′-CGAGGAC CGCTCAGTACCAA-3′; reverse, 5′-CATCTAGGTAGTCGCCATGCC-3′) with an Ad plasmid as the standard and β-actin as the internal standard (forward, 5′-ACGGCCAGGTCATCACTATTG-3′; reverse, 5′-CAAGAAGGAAGCTG GAAAAGA). All data were processed by the SDS 1.6 software (PE Biosystems) to generate standard curves and to determine the target concentration in the unknowns by interpolation.

Anti-HA and anti-Ad humoral responses. To evaluate the humoral responses against the HA epitope and the Ad capsid, C57BL/6, BALB/c, or CBA mice were immunized intramuscularly with the AdZ.Hx-HA, AdLdX.pIX-HA, AdL.L2-HA, or AdL.F-HA vector at 5×10^9 pu/mouse to obtain equal particle numbers or with different numbers of particle units per mouse, ranging from 2.5×10^8 to 5×10^9, to achieve the same HA epitope number. Mice injected with AdZ at the same or the highest dose or naive mice (PBS injected) served as controls. Serum was collected from the tail vein 28 days following immunization. Anti-HA and anti-Ad specific total IgM and IgG antibodies were determined by ELISA. Microtiter plates (Nunc, Roskilde, Denmark) were coated with Smt3-HA at 0.4 μg/well or with 10^9 AdNull particles/well in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich), and incubated at 4°C for 12 h. The plates were washed three times with PBS and blocked with 5% fat-free milk (Bio-Rad Laboratories) in PBS. After three washes with PBST, the sera were added in sequential twofold dilutions starting at 1:20 and incubated for 1 h. After three washes with PBST, anti-mouse IgG-HRP or IgM-HRP (Sigma-Aldrich) was added for 1 h of incubation. Detection was accomplished with a peroxidase substrate kit (Bio-Rad Laboratories), and absorbance was determined at 415 nm. For titer determination, the absorbance values of all dilutions were extrapolated to the twofold background value with a linear fit function (34, 52).

HA-specific CD4 cellular response. To compare the HA-specific cellular immune responses induced by the HA-capsid modified vectors, CBA mice were

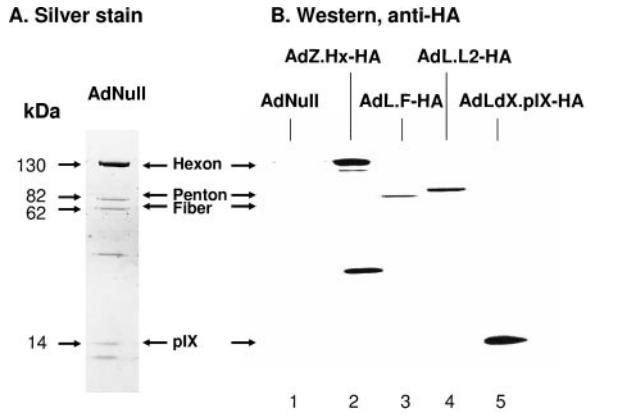

FIG. 1. Detection of HA epitopes in capsid-modified Ad vectors. Virions (10^{10}) of purified HA-modified viruses or AdNull as a control were assessed by 4 to 15% polyacrylamide gradient SDS-PAGE. Panels: A, silver staining; B, Western analysis. The proteins were transferred to polyvinylidene difluoride membrane and stained with anti-HA monoclonal antibody. Bands represent HA-modified capsid proteins as follows: lane 1, AdNull (unmodified); lane 2, hexon-HA in AdZ.Hx-HA vector; lane 3, fiber knob-HA in Ad.F-HA vector; lane 4, penton base-HA in AdL.L2-HA vector; lane 5, protein IX in AdLdX.pIX-HA vector. Hexon, penton base, fiber knob, and protein IX (pIX) and their molecular masses are identified.

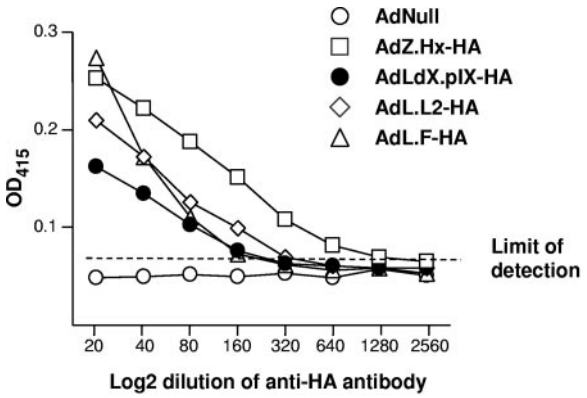

FIG. 2. HA epitope incorporated into different capsid proteins exhibits similar accessibility to anti-HA antibody in the context of an intact virion. Various amounts of purified viruses were immobilized in the wells of 96-well ELISA plates as follows: AdNull, 5×10^9 pu/well; AdZ.Hx-HA, 2.5×10^8 pu/well; AdLdX.pIX-HA, 7×10^8 pu/well; AdL.L2-HA, 3×10^9 pu/well; Ad.F-HA, 5×10^9 pu/well. All had a concentration of 1.8×10^{11} HA copies per well for each virus and were incubated with various $\log_2$ dilutions of anti-HA antibody. OD_{415}, optical density at 415 nm.

immunized intramuscularly with 5×10^9 pu of AdZ.Hx-HA, AdLdX.pIX-HA, AdL.L2-HA, AdL.F-HA, or AdZ. The frequency of HA-specific CD4 T lymphocytes was determined with a gamma interferon (IFN-γ)- and IL-4-specific enzyme-linked immunospot (ELISPOT) assay (R&D, Minneapolis, MN) 10 days following immunization. Spleen CD4 T cells were purified by negative depletion with SpinSep T-cell subset purification kits (StemCell Technologies, Vancouver, British Columbia, Canada). The purity the T cells was >95%. Splenic DC were purified from syngeneic naive animals for use as antigen-presenting cells by positive selection with CD11c MACS beads (Miltenyi Biotec, Auburn, CA) and double purification over two consecutive MACS LS+ columns (Miltenyi Biotec). The purity of the DC was >90%. DC (5×10^6/ml) were incubated for 3 h with purified Smt3-HA protein (100 μg/ml) in RPMI medium supplemented with 2% fetal bovine serum (HyClone, Logan, Utah), 10 mM HEPES (pH 7.5; BioSource International, Camarillo, CA), and 10^{-5} M β-mercaptoethanol (Sigma-Aldrich). CD4 T cells (2×10^5) were incubated with splenic DC with or without Smt3-HA protein at a ratio of 4:1 in IL-4 and IFN-γ plates (R&D) for 48 h. Following washing, biotinylated anti-IFN-γ or anti-IL-4 (both from R&D) detection antibodies were added and the plates were incubated overnight at 4°C. The plates were then washed, and the streptavidin-alkaline phosphatase conjugate (R&D) was added. For final spot detection, the 3-amino-9-ethylcarbazole substrate (R&D) was added for 1 h of incubation and rinsed with H_2O. The spots were counted by computer-assisted ELISPOT image analysis (Zellnet Consulting, New York, NY).

Statistical evaluation. The data are presented as the mean ± the standard error of the mean. Statistical analyses were performed with the nonpaired two-tailed Student *t* test, assuming equal variance. Statistical significance was defined as $P < 0.05$.

RESULTS

Expression and presentation of HA epitopes in HA-modified virions. The HA capsid-modified vectors were analyzed for the correct location of the incorporated HA epitopes. SDS-PAGE showed Ad capsid proteins of the correct size (AdNull as representative, Fig. 1A). Western analysis of the four HA-modified vectors with an anti-HA mouse monoclonal antibody confirmed the position of the HA epitopes by HA detected at the corresponding size for each capsid protein in the HA-modified vectors (Fig. 1B, lanes 2 to 5), with no HA detected in the AdNull control (lane 1). The signal intensity corresponded to the number of HA epitopes inserted into each of the capsid proteins, with AdZ.Hx-HA > AdLdX.pIX-HA > AdL.L2-HA > AdL.F-HA, corresponding to the number of the capsid proteins present in each Ad capsid. In addition to the full-length hexon size of 130 kDa, two smaller proteins were detected at around 125 and 30 kDa (Fig. 1B, lane 2), possibly reflecting some degree of degradation of the hexon protein (7).

Presentation of HA epitope on virion surface. It has been shown that the same epitope incorporated at different sites of the hexon can be recognized by a monoclonal antibody with different outcomes, suggesting that the accessibility of this epitope at each position is different (53). Therefore, to assess if the HA epitopes expressed on the surface of the modified Ad vectors were accessible for immune recognition, the four HA capsid-modified vectors, immobilized on ELISA plates at amounts adjusted to achieve the same number of HA epitopes for each vector (1.8×10^{11} copies of HA), were incubated with an anti-HA monoclonal antibody. The HA epitope was recognized by the anti-HA antibody in all four modified virions with slightly stronger binding to HA in AdZ.Hx-HA and no binding to the AdNull control (Fig. 2). This indicates that the HA epitopes in each HA-carrying vector are exposed on the outside of the virion and are accessible for binding by an anti-HA antibody.

Infection with HA-modified vectors in vitro. To evaluate if incorporation of the HA epitope into capsid proteins affected interaction with target cells, A549 cells or bone marrow-derived DC were infected with the four HA-modified vectors or AdNull and the Ad genome copy number was assessed at 48 h by viral genome measurement by real-time quantitative PCR. A549 cells were chosen as they express a high level of CAR, the native Ad receptor, and can be effectively infected by an unmodified Ad vector. In all cases, the Ad genome was detected in the cells following infection with all HA-modified vectors

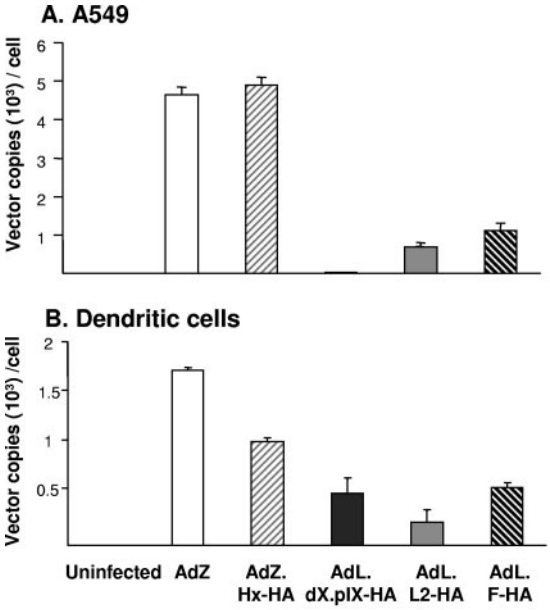

FIG. 3. Comparison of vector genomes following infection with HA-containing vectors in A549 cells and DC. (A) A549 cells were infected with each vector at 1,000 pu/cell. After 24 h, the vector genomic DNA was quantified by TaqMan real-time quantitative PCR. (B) DC were infected with each vector at 10,000 pu/cell. After 24 h, the vector genomic DNA was quantified by TaqMan real-time quantitative PCR. The vector levels from the TaqMan experiments were averaged and normalized to the genome level with the approximation that each diploid cell contains 6.6 pg of DNA. Results represent the mean ± the standard error of the mean of averaged and normalized vector levels of three independent experiments.

(Fig. 3A). The AdZ.Hx-HA vector showed an Ad genome copy number similar to that of the unmodified AdZ vector ($P > 0.3$). Lower copy numbers were observed for AdL.F-HA, AdL.L2-HA, and AdLdX.pIX-HA ($P < 0.0001$ for all three vectors compared to AdZ.Hx-HA). The infection of DC, where infection with Ad is primarily integrin dependent, showed a slightly different pattern of infection with the HA-modified vectors in vitro, with AdZ > AdZ.Hx-HA > AdL.F-HA > AdLdX.pIX-HA and AdL.L2-HA (Fig. 3B; $P < 0.01$ for all HA-modified vectors compared to AdZ), indicating that the HA motif in AdL.L2-HA could possibly interfere with binding to integrins. Overall, these data suggest that HA incorporation into hexon interferes the least with virus entry in vitro compared to unmodified Ad, whereas incorporation into fiber knob, protein IX, and penton base partially reduces the Ad genome copy numbers after infection.

Humoral immune response to HA-modified vectors in vivo. To assess the humoral response against HA following immunization with the HA-modified vectors, the serum IgG responses against a recombinant HA fusion protein was determined in BALB/c mice following intramuscular administration of the same Ad particle number of the modified HA vectors. HA-specific IgG antibodies were detected only in the mice immunized with AdZ.Hx-HA or AdL.F-HA after 28 days (Fig. 4A). Strikingly, the titers were 4.5-fold higher following immunization with AdL.F-HA compared to AdZ.Hx-HA ($P < 0.005$), although the total number of HA epitopes administered with AdL.F-HA was 20 times lower than that obtained with AdZ.Hx-HA. No significant anti-HA titers were detected in the mice immunized with AdZ, AdL.L2-HA, and AdLdX.pIX-HA. The anti-Ad IgG titers were comparable for all HA-modified vectors and unmodified AdZ ($P > 0.1$; Fig. 4B).

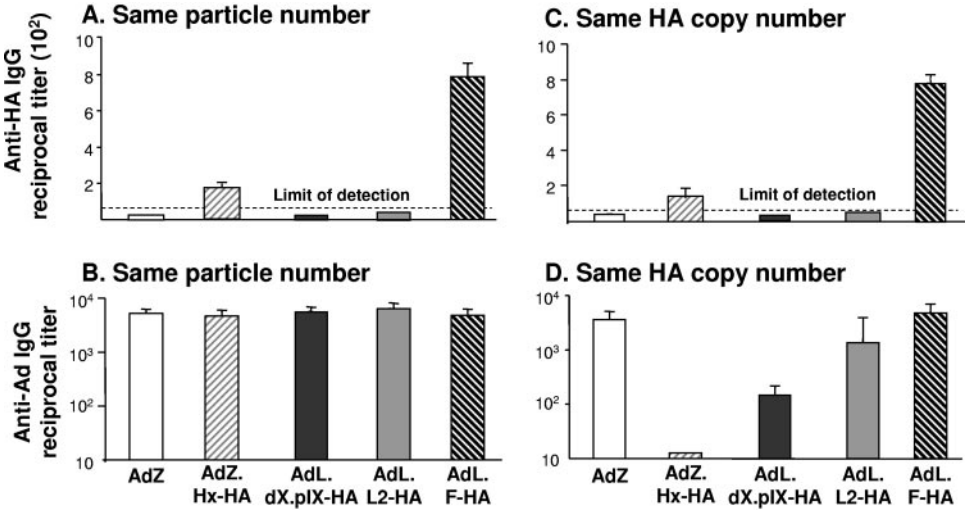

FIG. 4. Humoral response to HA following immunization with HA-modified vectors. (A and B) Vectors administered with the same number of particle units for each vector. BALB/c mice were immunized intramuscularly with AdZ, AdZ.Hx-HA, AdLdX.pIX-H, AdL.L2-HA, and AdL.F-HA at a dose of 5×10^9 pu/animal, and antibody titers were determined after 28 days. (A) Total anti-HA IgG antibody titers determined by ELISA with the HA fusion protein as the antigen. (B) Total anti-Ad IgG antibody titers determined by ELISA with AdNull virions as the antigen. (C and D) Humoral responses to HA after immunization with different doses of HA-modified vectors with the dose adjusted to achieve the same HA copy number. BALB/c mice were immunized intramuscularly with AdZ (5×10^9 pu), AdZ.Hx-HA (2.5×10^8 pu), AdLdX.pIX-H (7×10^8 pu), AdL.L2-HA (3×10^9 pu), and AdL.F-HA (5×10^9 pu). For panels A and C, the total anti-HA IgG antibody titer was determined by using the HA fusion protein as the antigen. For panels B and D, the total anti-Ad IgG antibody titers were determined by ELISA with AdNull as the antigen. Data are shown as the mean ± the standard error of the mean of five mice per group from one experiment representative of three independent experiments. The dashed lines indicate the limit of detection.

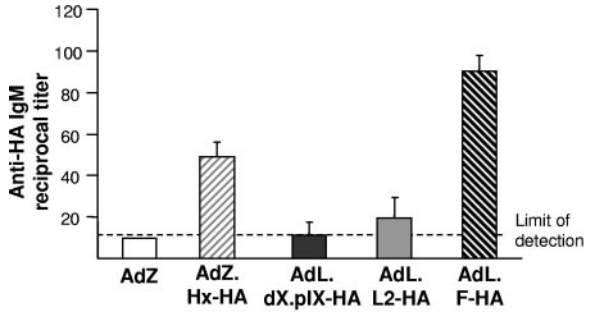

FIG. 5. Early humoral responses to HA following immunization with HA-modified Ad vectors. AdZ, AdZ.Hx-HA, AdLdX.pIX-H, AdL.L2-HA, or Ad.F-HA was administered intramuscularly to BALB/c mice at a dose of 5×10^9 pu/animal. Titers of IgM antibodies against HA were determined by ELISA at day 7 after administration with recombinant HA fusion peptide. Data are shown as the mean ± the standard error of the mean of five mice per group for one experiment representative of three independent experiments. The dashed line indicates the limit of detection.

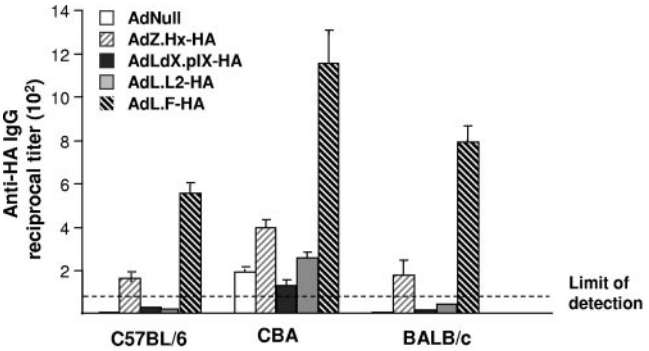

FIG. 6. Influence of strain background on the humoral responses to HA for the different HA-capsid modified vectors. C57BL/6 ($H2^b$), BALB/c ($H2^d$), or CBA ($H2^k$) mice were immunized via the intramuscular route with AdZ, AdZ.Hx-HA, AdLdX.pIX-H, AdL.L2-HA, or AdL.F-HA at a dose of 5×10^9 pu/animal. Total anti-HA IgG titers were determined at 4 weeks by ELISA with the recombinant HA fusion protein as the antigen. Data are shown as the mean ± the standard error of the mean of five mice per group for one experiment representative of three independent experiments. The dashed line indicates the limit of detection.

To analyze the humoral immune response against HA following administration of the same number of HA copies, the anti-HA IgG response was determined following immunization with different doses of Ad particles of the HA-modified vectors to achieve the same number of HA epitopes. Again, immunization with AdL.F-HA induced the strongest anti-HA IgG response compared with immunization with AdZ.Hx-HA ($P < 0.0001$; Fig. 4C). Immunization with AdL.L2-HA or AdLdX.pIX-HA at this dose did not induce HA-specific IgG titers. The anti-Ad IgG response showed decreasing titers following immunization with AdL.F-HA > AdL.L2-HA > AdL.dX.pIX-HA > Ad.Z.Hx-HA ($P < 0.04$) and equal titers with AdL.F-HA compared to AdZ ($P > 0.8$), reflecting the total particle dose administered for each of the vectors to adjust to equal HA copy numbers (Fig. 4D).

The early IgM humoral response against HA was assessed 7 days following immunization with equal doses of the Ad vectors (5×10^9 pu). IgM titers were increased at least twofold in Ad.L.F-HA-immunized mice compared to mice immunized with AdZ.Hx-HA and AdL.L2-HA ($P < 0.002$; Fig. 5), whereas for AdLdX.pIX-H and AdZ no HA-specific IgM was detected.

Strain dependency of anti-HA humoral response. To evaluate whether the preferential humoral immune response against HA after immunization with AdL.F-HA was strain dependent, three strains of mice with different major histocompatibility complex (MHC) haplotypes were used for immunization. The IgG anti-HA titers were higher in CBA ($H\text{-}2^k$) mice in all experimental vector groups compared with C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice, suggesting that the HA peptide functions in a strain-dependent manner (Fig. 6; $P < 0.02$ for all comparisons).

Immunization with AdF.L-HA induced the highest anti-HA titers in all strains compared to the other three HA-modified vectors. Therefore, the ability of the HA epitope to raise the antibody titer may be strain dependent but the preferential induction of specific anti-HA antibodies by AdL.F-HA compared to the other HA vectors seems to be independent of the genetic background of the host.

Cellular response to HA incorporated into different Ad capsid proteins. The HA epitope is well characterized as a B-cell and neutralization epitope in the human system (23, 28) but not as a T-cell epitope. The stronger anti-HA response in CBA mice could indicate that HA represents a T-helper epitope more prevalent in this strain compared to C57BL/6 ($H\text{-}2^b$) or BALB/c ($H\text{-}2^d$) mice. To assess cellular immunity to the HA-modified vectors, the frequency of T-cell responses to HA in vaccinated CBA mice was analyzed by ELISPOT assay. Seven days following immunization, purified splenic CD4 T cells of vaccinated mice were stimulated with syngeneic DC pulsed with a recombinant HA fusion peptide. The highest HA-specific IL-4 secretion (Fig. 7A) and HA-specific IFN-γ secretion (Fig. 7B) levels were detected in CD4 T cells from AdL.F-HA-immunized mice ($P < 0.01$ and $P < 0.04$, respectively [comparison of AdL.F-HA to all vectors]). Administration of AdZ.Hx-HA, AdLdX.pIX-H, and AdL.L2-HA induced only a minor increase in HA-specific IFN-γ-producing CD4 T cells compared with CD4 T cells from AdZ-immunized mice ($P < 0.02$ for all comparisons). Together, these findings indicate that AdL.F-HA induced an HA-specific cellular helper response demonstrated by the presence of IL-4- and IFN-γ-secreting CD4 T cells specific for the HA epitope.

DISCUSSION

Ad vector-based genetic vaccines are a promising means to induce antigen-specific immunity. One strategy to enhance the immunogenicity of an Ad-based vaccine is to modify Ad capsid proteins to incorporate immunogenic epitopes. To determine if the location of an epitope incorporated into an Ad capsid protein influences the antiepitope immune response, this study analyzed antiepitope immune responses induced by capsid-modified Ad vectors containing a model epitope of the influenza virus HA protein in one of the four major Ad capsid proteins, hexon, penton base, fiber knob, or protein IX. Interestingly, the Ad vector with the HA epitope incorporated into the fiber knob protein induced the strongest anti-HA humoral and CD4 cellular immunity compared to immunization with

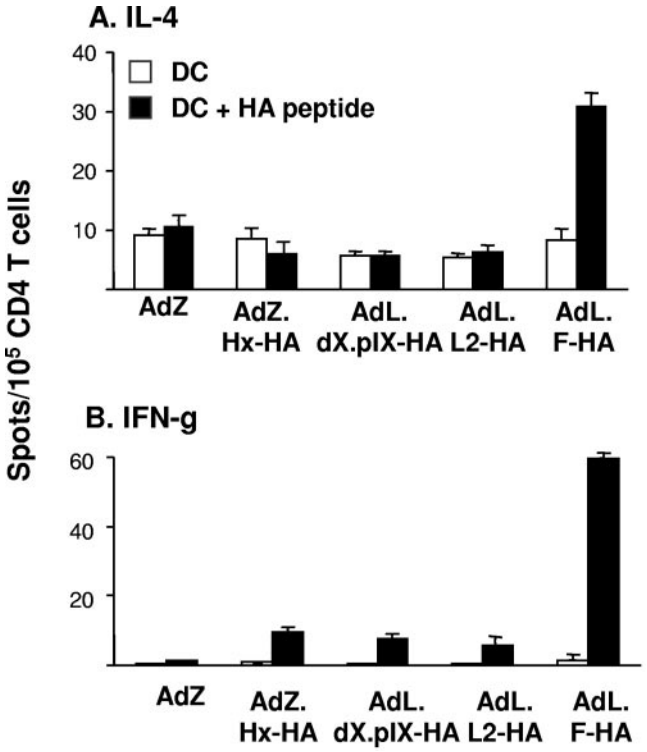

FIG. 7. HA-specific CD4 T-cell IL-4 and IFN-γ responses after immunization with the different HA-capsid modified vectors. CBA mice were immunized with AdZ, AdZ.Hx-HA, AdLdX.pIX-H, AdL.L2-HA, or AdL.F-HA at a dose of 5×10^9 pu/animal via the intramuscular route (n = 5/group). Seven days after immunization, CD4 cells were isolated from spleens and the IL-4 and IFN-γ responses were assessed following in vitro stimulation with recombinant HA fusion protein by ELISPOT assay. Panels: A, IL-4; B, IFN-γ. Shown are data for CD4 cells after immunization and in vitro stimulation with DC alone (DC) and DC plus recombinant fusion peptide (DC + HA antigen). Data represent the mean ± the standard error of the mean of pooled cells from five individual mice per group plated in triplicate from one experiment representative of two separate experiments.

Ad vectors containing the HA epitope in hexon, penton base, or protein IX. This effect was independent of administering the vectors at equal Ad particle numbers or equal HA epitope doses. Together, these data suggest that expressing an epitope in the fiber knob protein of an Ad capsid is a potent means to induce epitope-specific immunity and indicate that the antiepitope immune responses of capsid-modified Ad vectors are influenced more by the position of the antigen within the Ad capsid than the absolute number of antigens for each Ad particle.

Infectivity of HA-modified Ad vectors. Ad vaccine strategies are dependent, in part, on efficient interaction with antigen-presenting cells, particularly DC (4, 11, 19, 22, 32, 42, 45, 51). This interaction occurs through binding of the Ad capsid penton base and fiber knob proteins to cellular receptors (15, 24, 47, 51). Modifications to the Ad capsid proteins may influence infectivity and thus the immunogenicity of the Ad vaccine. For modifications made to the capsid in the present study, there appeared to be some differences in infectivity among the vectors, but this did not correlate with the in vivo responses, in which the fiber knob modifications clearly elicited optimal host responses to the HA epitope. The uptake of Ad by DC via the integrin receptors could also reflect internalization without active infection, as only the amount of intracellular vector genome was assessed. The lack of correlation between infectivity and the host responses elicited may be due to the fact that most of the humoral immune response is directed against the capsid epitopes on the intact virion and depends on the exposure of the capsid shell (3, 9, 17, 54). Alternatively, in vitro-generated DC express different patterns of surface receptors compared to DC in vivo, and thus infection of DC in vitro may not predict their responses in vivo (16).

Capsid-modified Ad vectors to enhance epitope-specific humoral immune responses. The strongest humoral immune response against the HA epitope in vivo was induced following immunization with the fiber knob-modified Ad vector, which contains the lowest number of HA epitopes in the capsid of all four vectors, 20 times lower than that of the hexon-modified Ad. Although it has been shown that the Ad hexon protein contains most of the neutralization epitopes against native Ad, several studies with humans have shown that following immunization with Ad, fiber knob- and penton base-specific total IgG responses were elicited more frequently and with a titer higher than those against hexon (7, 14). It has also been shown that shedding of fibers from Ad virions following attachment exposes fiber knob and penton base more efficiently to the host immune system (14, 30). The low anti-HA titers generated by the penton base-modified Ad may be a result of the position of the HA molecule in penton base. Integration of heterologous epitopes in foreign structures often influences the folding and exposure of the epitope (13, 53). While accessibility studies by ELISA with coated HA-modified vectors showed no difference in HA recognition by monoclonal anti-HA antibody, the recognition of HA integrated into the RGD loop of penton base in vivo could be impaired. The magnitude of the anti-HA antibody response in the mice was also dependent on the strain background. Consistent with this observation, studies with humans suggest that the humoral responses against Ad penton base and fiber knob are HLA dependent.

Anti-HA cellular immunity. Immunization with the fiber knob-modified HA Ad vector also induced a cellular CD4 helper response. The CD4 IFN-γ response was sixfold greater and the CD4 IL-4 response was threefold greater in mice immunized with the fiber knob-modified HA vector than that of those immunized with the hexon-modified HA vector. Splenocytes from Ad-infected mice demonstrated activity against hexon with the highest frequency, followed by fiber knob and penton base (18, 26), indicating that T-cell-specific epitopes are widely distributed among the proteins. The anti-HA Th2 responses only seen with the fiber knob-modified HA vector could be due to the fact that the fiber knob is involved in the maturation of DC by up-regulating MHC and costimulatory molecules (27). As the HA epitope was inserted into the HI loop of fiber knob, the HA-specific CD4 responses could be explained if the HI loop of fiber knob is one of the domains that are preferentially cleaved and loaded onto MHC class II molecules. In summary, incorporation of an epitope into the fiber knob gene is the most potent strategy to elucidate an antiepitope response when incorporating epitopes into the Ad capsid. The fact that the immune response was independent of

the dose of the epitope suggests that antigen position in the Ad capsid and its recognition by B cells are the critical components of the response to the epitope incorporated into the Ad capsid. It is conceivable that the localization of the inserted epitope within the different Ad capsid proteins could alter the immunogenicity of that epitope. Further studies are necessary to evaluate if the use of different epitopes at the same sites of the Ad proteins will yield the same immune response and confirm that better antigen exposure to B cells results in stronger immune responses. Incorporation of an epitope into Ad fiber knob may be useful in the development of Ad vectors as vaccines.

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REFERENCES

1. **Barouch, D. H., and G. J. Nabel.** 2005. Adenovirus vector-based vaccines for human immunodeficiency virus type 1. Hum. Gene Ther. **16:**149–156.
2. **Boyer, J. L., G. Kobinger, J. M. Wilson, and R. G. Crystal.** 2005. Adenovirus-based genetic vaccines for biodefense. Hum. Gene Ther. **16:**157–168.
3. **Crompton, J., C. I. Toogood, N. Wallis, and R. T. Hay.** 1994. Expression of a foreign epitope on the surface of the adenovirus hexon. J. Gen. Virol. **75**(Pt. 1)**:**133–139.
4. **Dietz, A. B., and S. Vuk-Pavlovic.** 1998. High efficiency adenovirus-mediated gene transfer to human dendritic cells. Blood **91:**392–398.
5. **Dmitriev, I. P., E. A. Kashentseva, and D. T. Curiel.** 2002. Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. J. Virol. **76:**6893–6899.
6. **Einfeld, D. A., D. E. Brough, P. W. Roelvink, I. Kovesdi, and T. J. Wickham.** 1999. Construction of a pseudoreceptor that mediates transduction by adenoviruses expressing a ligand in fiber or penton base. J. Virol. **73:**9130–9136.
7. **Gahery-Segard, H., F. Farace, D. Godfrin, J. Gaston, R. Lengagne, T. Tursz, P. Boulanger, and J. G. Guillet.** 1998. Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. J. Virol. **72:**2388–2397.
8. **Hackett, N. R., T. El Sawy, L. Y. Lee, I. Silva, J. O'Leary, T. K. Rosengart, and R. G. Crystal.** 2000. Use of quantitative TaqMan real-time PCR to track the time-dependent distribution of gene transfer vectors in vivo. Mol. Ther. **2:**649–656.
9. **Hackett, N. R., S. M. Kaminsky, D. Sondhi, and R. G. Crystal.** 2000. Antivector and antitransgene host responses in gene therapy. Curr. Opin. Mol. Ther. **2:**376–382.
10. **Hashimoto, M., J. L. Boyer, N. R. Hackett, J. M. Wilson, and R. G. Crystal.** 2005. Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. Infect. Immun. **73:**6885–6891.
11. **Herrera, O. B., S. Brett, and R. I. Lechler.** 2002. Infection of mouse bone marrow-derived dendritic cells with recombinant adenovirus vectors leads to presentation of encoded antigen by both MHC class I and class II molecules—potential benefits in vaccine design. Vaccine **21:**231–242.
12. **Hersh, J., R. G. Crystal, and B. Bewig.** 1995. Modulation of gene expression after replication-deficient, recombinant adenovirus-mediated gene transfer by the product of a second adenovirus vector. Gene Ther. **2:**124–131.
13. **Hong, S. S., M. Bardy, M. Monteil, B. Gay, C. Denesvre, J. Tournier, G. Martin, M. Eloit, and P. Boulanger.** 2000. Immunoreactive domains and integrin-binding motifs in adenovirus penton base capsomer. Viral Immunol. **13:**353–371.
14. **Hong, S. S., N. A. Habib, L. Franqueville, S. Jensen, and P. A. Boulanger.** 2003. Identification of adenovirus (Ad) penton base neutralizing epitopes by use of sera from patients who had received conditionally replicative Ad (Ad*dl*1520) for treatment of liver tumors. J. Virol. **77:**10366–10375.
15. **Hong, S. S., L. Karayan, J. Tournier, D. T. Curiel, and P. A. Boulanger.** 1997. Adenovirus type 5 fiber knob binds to MHC class I α2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J. **16:**2294–2306.
16. **Hsu, C., M. Boysen, L. D. Gritton, P. D. Frosst, G. R. Nemerow, and D. J. Von Seggern.** 2005. In vitro dendritic cell infection by pseudotyped adenoviral vectors does not correlate with their in vivo immunogenicity. Virology **332:**1–7.
17. **Jooss, K., and N. Chirmule.** 2003. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. Gene Ther. **10:**955–963.
18. **Jooss, K., H. C. Ertl, and J. M. Wilson.** 1998. Cytotoxic T-lymphocyte target proteins and their major histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. J. Virol. **72:**2945–2954.
19. **Jooss, K., Y. Yang, K. J. Fisher, and J. M. Wilson.** 1998. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. J. Virol. **72:**4212–4223.
20. **Koizumi, N., H. Mizuguchi, N. Utoguchi, Y. Watanabe, and T. Hayakawa.** 2003. Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. **5:**267–276.
21. **Krasnykh, V., I. Dmitriev, G. Mikheeva, C. R. Miller, N. Belousova, and D. T. Curiel.** 1998. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J. Virol. **72:**1844–1852.
22. **Labow, D., S. Lee, R. J. Ginsberg, R. G. Crystal, and R. J. Korst.** 2000. Adenovirus vector-mediated gene transfer to regional lymph nodes. Hum. Gene Ther. **11:**759–769.
23. **Lu, Y., J. Ding, W. Liu, and Y. H. Chen.** 2002. A candidate vaccine against influenza virus intensively improved the immunogenicity of a neutralizing epitope. Int. Arch. Allergy Immunol. **127:**245–250.
24. **Mercier, S., H. Rouard, M. H. fau-Larue, and M. Eloit.** 2004. Specific antibodies modulate the interactions of adenovirus type 5 with dendritic cells. Virology **322:**308–317.
25. **Mittereder, N., K. L. March, and B. C. Trapnell.** 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. **70:**7498–7509.
26. **Molinier-Frenkel, V., R. Lengagne, F. Gaden, S. S. Hong, J. Choppin, H. Gahery-Segard, P. Boulanger, and J. G. Guillet.** 2002. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. J. Virol. **76:**127–135.
27. **Molinier-Frenkel, V., A. Prevost-Blondel, S. S. Hong, R. Lengagne, S. Boudaly, M. K. Magnusson, P. Boulanger, and J. G. Guillet.** 2003. The maturation of murine dendritic cells induced by human adenovirus is mediated by the fiber knob domain. J. Biol. Chem. **278:**37175–37182.
28. **Muller, G. M., M. Shapira, and R. Arnon.** 1982. Anti-influenza response achieved by immunization with a synthetic conjugate. Proc. Natl. Acad. Sci. USA **79:**569–573.
29. **Nabel, G. J.** 2001. Challenges and opportunities for development of an AIDS vaccine. Nature **410:**1002–1007.
30. **Nakano, M. Y., K. Boucke, M. Suomalainen, R. P. Stidwill, and U. F. Greber.** 2000. The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. J. Virol. **74:**7085–7095.
31. **Noureddini, S. C., and D. T. Curiel.** 2005. Genetic targeting strategies for adenovirus. Mol. Pharm. **2:**341–347.
32. **Okada, N., T. Saito, Y. Masunaga, Y. Tsukada, S. Nakagawa, H. Mizuguchi, K. Mori, Y. Okada, T. Fujita, T. Hayakawa, T. Mayumi, and A. Yamamoto.** 2001. Efficient antigen gene transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. Cancer Res. **61:**7913–7919.
33. **Parks, R. J.** 2005. Adenovirus protein IX: a new look at an old protein. Mol. Ther. **11:**19–25.
34. **Plikaytis, B. D., S. H. Turner, L. L. Gheesling, and G. M. Carlone.** 1991. Comparisons of standard curve-fitting methods to quantitate *Neisseria meningitidis* group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **29:**1439–1446.
35. **Randrianarison-Jewtoukoff, V., and M. Perricaudet.** 1995. Recombinant adenoviruses as vaccines. Biologicals **23:**145–157.
36. **Rosa-Calatrava, M., L. Grave, F. Puvion-Dutilleul, B. Chatton, and C. Kedinger.** 2001. Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. J. Virol. **75:**7131–7141.
37. **Rosenfeld, M. A., W. Siegfried, K. Yoshimura, K. Yoneyama, M. Fukayama, L. E. Stier, P. K. Paakko, P. Gilardi, L. D. Stratford-Perricaudet, M. Perricaudet, S. Jallat, A. Pavirani, J.-P. Lecocq, and R. G. Crystal.** 1991. Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. Science **252:**431–434.
38. **Rosenfeld, M. A., K. Yoshimura, B. C. Trapnell, K. Yoneyama, E. R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. E. Stier, L. Stratford-Perricaudet, M. Perricaudet, W. B. Guggino, A. Pavirani, J.-P. Lecocq, and R. G. Crystal.** 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell **68:**143–155.
39. **Roy, S., D. S. Clawson, R. Calcedo, C. Lebherz, J. Sanmiguel, D. Wu, and J. M. Wilson.** 2005. Use of chimeric adenoviral vectors to assess capsid neutralization determinants. Virology **333:**207–214.
40. **Shenk, T.** 1996. Adenoviridae: the viruses and their replication, p. 2111–2148. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
41. **Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans,**

Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331–335.

42. **Song, W., H. L. Kong, H. Carpenter, H. Torii, R. Granstein, S. Rafii, M. A. Moore, and R. G. Crystal.** 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. J. Exp. Med. **186:**1247–1256.
43. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605–609.
44. **Sumida, S. M., D. M. Truitt, A. A. Lemckert, R. Vogels, J. H. Custers, M. M. Addo, S. Lockman, T. Peter, F. W. Peyerl, M. G. Kishko, S. S. Jackson, D. A. Gorgone, M. A. Lifton, M. Essex, B. D. Walker, J. Goudsmit, M. J. Havenga, and D. H. Barouch.** 2005. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J. Immunol. **174:**7179–7185.
45. **Tillman, B. W., T. D. de Gruijl, S. A. Luykx-de Bakker, R. J. Scheper, H. M. Pinedo, T. J. Curiel, W. R. Gerritsen, and D. T. Curiel.** 1999. Maturation of dendritic cells accompanies high-efficiency gene transfer by a CD40-targeted adenoviral vector. J. Immunol. **162:**6378–6383.
46. **Vellinga, J., M. J. Rabelink, S. J. Cramer, D. J. van den Wollenberg, H. Van der Meulen, K. N. Leppard, F. J. Fallaux, and R. C. Hoeben.** 2004. Spacers increase the accessibility of peptide ligands linked to the carboxyl terminus of adenovirus minor capsid protein IX. J. Virol. **78:**3470–3479.
47. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. Cell **73:**309–319.
48. **Wickham, T. J., E. Tzeng, L. L. Shears, P. W. Roelvink, Y. Li, G. M. Lee, D. E. Brough, A. Lizonova, and I. Kovesdi.** 1997. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J. Virol. **71:**8221–8229.
49. **Wilson, J. M.** 1996. Adenoviruses as gene-delivery vehicles. N. Engl. J. Med. **334:**1185–1187.
50. **Wohlfart, C.** 1988. Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. J. Virol. **62:**2321–2328.
51. **Worgall, S., A. Busch, M. Rivara, D. Bonnyay, P. L. Leopold, R. Merritt, N. R. Hackett, P. W. Rovelink, J. T. Bruder, T. J. Wickham, I. Kovesdi, and R. G. Crystal.** 2004. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. J. Virol. **78:**2572–2580.
52. **Worgall, S., A. Krause, M. Rivara, K. K. Hee, E. V. Vintayen, N. R. Hackett, P. W. Roelvink, J. T. Bruder, T. J. Wickham, I. Kovesdi, and R. G. Crystal.** 2005. Protection against P. aeruginosa with an adenovirus vector containing an OprF epitope in the capsid. J. Clin. Investig. **115:**1281–1289.
53. **Wu, H., T. Han, N. Belousova, V. Krasnykh, E. Kashentseva, I. Dmitriev, M. Kataram, P. J. Mahasreshti, and D. T. Curiel.** 2005. Identification of sites in adenovirus hexon for foreign peptide incorporation. J. Virol. **79:**3382–3390.
54. **Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.