JOURNAL OF VIROLOGY, Mar. 2009, p. 2623–2631 Vol. 83, No. 6
0022-538X/09/$08.00+0 doi:10.1128/JVI.02160-08

Host Immune Responses to Chronic Adenovirus Infections in Human and Nonhuman Primates▽†

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Received 13 October 2008/Accepted 22 December 2008

Recent studies indicate that great apes and macaques chronically shed adenoviruses in the stool. Shedding of adenovirus in the stool of humans is less prevalent, although virus genomes persist in gut-associated lymphoid tissue in the majority of individual samples. Chimpanzees have high levels of broadly reactive neutralizing antibodies to adenoviruses in serum, with very low frequencies of adenovirus-specific T cells in peripheral blood. A similar situation exists in macaques; sampling of guts from macaques demonstrated adenovirus-specific T cells in lamina propria. Humans show intermediate levels of serum neutralizing antibodies, with adenovirus-specific T cells in peripheral blood of all individuals sampled and about 20% of samples from the gut, suggesting a potential role of T cells in better controlling virus replication in the gut. The overall structure of the E3 locus, which is involved in modulating the host's response to infection, is degenerate in humans compared to that in apes, which may contribute to diminished evasion of host immunity. The impact of adenovirus persistence and immune responses should be considered when using adenoviral vectors in gene therapy and genetic vaccines.

Viruses of the *Adenoviridae* family infect a wide range of vertebrate hosts. Adenoviruses that infect mammals belong to the genus *Mastadenovirus* and encompass at least seven viral species (formerly called subtypes) that infect primates (5). The molecular biology of human-derived adenoviruses has been characterized extensively for the species C group, for which human adenovirus 2 (HAdV-2) and HAdV-5 serve as prototypes (6). Adenoviruses cause a variety of nonlethal infectious diseases in humans, and lethal disseminated adenovirus infection occurs in immunosuppressed patients (6). Attempts to evaluate the pathogenesis of human adenoviruses in animal models have been difficult since most models demonstrate a very narrow permissiveness for virus replication.

The natural history of adenovirus infections in humans was initially evaluated in prospective surveillance studies of family cohorts (7, 8). Self-limited respiratory illnesses in children were the most common syndromes associated with adenovirus infection, although virus was most easily detected in the stool. Excretion of virus in stool usually wanes rapidly following resolution of the infectious syndrome but is prolonged for a subset of individuals. Species C adenoviruses, which are the most prevalent in humans, can establish latency in adenoid and tonsil-derived lymphocytes (9).

Adenovirus vectors have been used successfully as vaccine carriers due to their ability to elicit potent cellular and humoral immune response to the encoded transgene product. Recombinant vectors based on HAdV-5 have emerged as a potent platform for activating cytotoxic T lymphocytes against tumor and viral antigens. A problematic response of the host to HAdV-5-based vectors that was not predicted based on animal testing was observed in a clinical trial, namely, there was a paradoxical increased susceptibility to infection with human immunodeficiency virus (HIV) following vaccination with an HIV-based HAdV-5 vector in subjects with evidence of prior exposure to a natural infection with HAdV-5 (2).

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 30 December 2008.

MATERIALS AND METHODS

NAb assay. Human serum samples were obtained from the University of Pennsylvania Hospital Department of Pathology and Laboratory Medicine, Philadelphia, PA. Nonhuman primate serum samples were obtained from Covance, Oregon National Primate Research Center, and Monroe Dunaway Anderson (Texas). Serum samples were heat inactivated at 56°C for 35 min. Wild-type adenovirus (10^8 particles/well) was diluted in serum-free Dulbecco's modified Eagle's medium (DMEM) and incubated with twofold serial dilutions of heat-inactivated serum samples in DMEM for 1 h at 37°C. Subsequently, a serum-adenovirus mixture was added to slides in wells with 10^5 monolayer A549 (for the human and chimpanzee adenoviruses) or BS-C-1 (for the monkey adenoviruses) cells. After 1 h, the cells in each well were supplemented with 100 μl of 20% fetal bovine serum (FBS)–DMEM and cultured for 22 h (human and chimpanzee adenoviruses) or 76 h (monkey adenoviruses) at 37°C in 5% CO_2. Next, cells were rinsed twice with phosphate-buffered saline (PBS) and stained with DAPI (4′,6-diamidino-2-phenylindole) and a fluorescein isothiocyanate (FITC)-labeled, broadly cross-reactive goat antibody (Virostat) raised against HAdV-5 following fixation in paraformaldehyde (4%, 30 min) and permeabilization in 0.2% Triton (4°C, 20 min). The level of infection was determined by counting the number of FITC-positive cells under microscopy. The neutralizing antibody (NAb) titer is reported as the highest serum dilution that inhibited adenovirus infection 50% or more compared with the naïve serum control.

Blood collection and isolation of PBMCs. Human peripheral blood mononuclear cells (PBMCs) were obtained from the Immunology Core, Center for AIDS Research, University of Pennsylvania. Nonhuman primate blood was obtained from Covance, Oregon National Primate Research Center, M. D. Anderson, and Gene Therapy Program, University of Pennsylvania. PBMCs were isolated from whole blood collected in EDTA- or heparin-containing Vacutainer tubes after Ficoll (Amersham Bioscience) or Percoll (GE Healthcare Biosciences) density gradient centrifugation at 1,000 × *g* for 25 min. Cells were collected from the interphase and washed with PBS. PBMCs were incubated with ACK lysing buffer to lyse red blood cells, washed, and resuspended in complete RPMI medium (Mediatech) containing 10% FBS, 2 mM glutamine, 10 mM HEPES, 50 μg/ml gentamicin sulfate, and penicillin-streptomycin.

Isolation of lymphocytes from intestine. Surgically extracted colon/rectum specimens from human donors provided by the Cooperative Human Tissue Network and the National Disease Research Interchange and from nonhuman primates (Gene Therapy Program, University of Pennsylvania) were cut into 0.5-cm squares, washed, and incubated with continuous agitation with Hanks' balanced salt solution containing 0.75 mM EDTA, 100 U/ml penicillin, 100 μg/ml gentamicin, 25 mM HEPES buffer, and 5% FBS. The supernatant was removed, and the settled fragments of tissue were cut into 1- to 2-mm pieces and incubated with continuous agitation for 30-min intervals with RPMI medium containing 0.5 mg/ml collagenase (type II), penicillin, gentamicin, HEPES buffer, L-glutamine, and 5% FBS. At the end of each interval, intestinal pieces were further disrupted by agitating the pieces 15 times in a 20-ml syringe. The medium, containing lymphocytes from the lamina propria (LPLs), was separated from the remaining tissue fragments by passage through stainless steel screen cups (mesh size, 40 μm). This process was repeated two or three times, until the intestinal pieces had completely dissociated into small fragments. To enrich for lymphocytes, discontinuous Percoll (GE Healthcare Biosciences) density gradient centrifugation was performed by diluting isotonic Percoll to 35% (vol/vol) and 60% (vol/vol) with RPMI and centrifuging the samples at 800 × *g* for 20 min. LPLs were isolated from the interface between the 35 and 60% gradients and resuspended in complete RPMI medium.

IFN-γ ELISPOT. Multiscreen 96-well titration plates (Millipore) were coated overnight with antibody to human (clone DK-1; Mabtech) or monkey (clone GZ-4; Mabtech) gamma interferon (IFN-γ) in PBS. Plates were washed and then blocked for 1 h with complete medium (RPMI containing 10% FBS). Plates were washed with RPMI medium, and lymphocytes were seeded in 100 μl of complete medium at 1×10^5 and 2×10^5 cells per well. Stimulant peptide pools or viruses were added to each well to a final concentration of 2 μg/ml of each peptide or at the various concentrations of viruses indicated in the text in 100 μl of complete medium. Cells were incubated at 37°C for 20 h under 5% CO_2. Plates were washed (PBS with 0.05% Tween 20) and then incubated with biotinylated antibody to human and monkey IFN-γ (clone B6-1; Mabtech) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 h and then washed. Avidin-horseradish peroxidase (Vector Laboratories) was added to each well, and plates were incubated for 1 h. Plates were washed, and spots were developed with AEC substrate (BD Biosciences). Spots were counted with an automated enzyme-linked immunospot (ELISPOT) assay reader (AID). Phytohemagglutinin (Sigma) and a CEF (immunogenic peptides from CMV, EBV, and influenza virus) peptide pool (Mabtech) were included as positive controls in each analysis. Only ELISPOT counts of >55 spot-forming units (SFU) per million lymphocytes and with values three times over the background were considered positive.

Hexon library and adenovirus stimulants. A peptide library specific for the full length of the HAdV-5 hexon was synthesized as 20-mers with 15-amino-acid overlaps (Mimotopes, Australia). The entire lyophilized library, which consisted of 188 peptides, was reconstituted with dimethyl sulfoxide and grouped into four pools (A, B, C, and D), containing 50, 50, 50, and 33 peptides, respectively. All adenovirus preparations used as stimulants were produced by Penn Vector at the University of Pennsylvania and kept at −80°C until the time of stimulation. An aliquot of each adenovirus preparation was incubated for 35 min at 56°C for heat inactivation.

Intracellular cytokine staining (ICCS). Measurement of cytokine production by isolated lymphocytes was performed by combined surface and intracellular staining with monoclonal antibodies and subsequent five-color flow cytometric analysis. A total of 10^6 lymphocytes were incubated with 10^{10} particles of heat-inactivated SAdV-48 or 2 μg of HAdV-5 hexon peptide library in the presence of purified antibodies to CD49d and CD28 (BD Biosciences) and GolgiPlug (BD Pharmingen) for 6 h. Cells were washed and stained with ECD-CD8 (Beckman Coulter), allophycocyanin-CD4 (BD Pharmingen), phycoerythrin (PE)-CD27 (BD Pharmingen), and FITC-CD45RO (BD Pharmingen) anti-human antibodies. PE-CD27 and FITC-CD45RO antibodies were used only when memory phenotype was analyzed. Cells were washed, permeabilized with 250 μl of Cytofix/Cytoperm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anticytokine antibodies, including FITC-tumor necrosis factor alpha (FITC–TNF-α; BD Pharmingen), PE–IL-2 (Beckman Coulter), and PE–Cy7–IFN-γ (BD Pharmingen) antibodies, at 4°C for 30 min. Cells were stained with only one cytokine, PE–Cy7–IFN-γ, when memory phenotype was analyzed. Cells were washed and examined by flow cytometry, and the data were analyzed using FlowJo software (Treestar, OR).

Plasmacytoid dendritic cell cytokine release assay. Plasmacytoid dendritic cells were isolated from human PBMCs obtained from the Immunology Core, Center for AIDS Research, University of Pennsylvania, using a plasmacytoid dendritic cell isolation kit (Miltenyi Biotech). Cells were infected with different adenoviruses at a multiplicity of infection (MOI) of 10^4 as indicated in the text. Forty-eight hours later, the supernatants were collected and assayed for the induction of various cytokines and chemokines, using a combination of enzyme-linked immunosorbent assay (IFN-α; Pierce) and multiplex cytokine analysis (interleukin-6 [IL-6] and macrophage inflammatory protein 1α [MIP-1α]; Millipore/Luminex). Due to the small numbers of plasmacytoid dendritic cells in peripheral blood, the data reflect cytokine levels obtained from multiple donors.

Statistical methods. Statistical significance of differences in appropriate data sets was established by a two-tailed paired Student *t* test and one-way analysis of variance for P values of <0.01.

RESULTS

We recently showed that adenoviruses are chronically shed into the stools of great apes and macaques (S. Roy, L. H. Vandenberghe, S. Kryazhimskiy, R. Grant, R. Calcedo, X. Yuan, M. Keough, A. Sandhu, Q. Wang, C. A. Medina-Jaszek, J. B. Plotkin, and J. M. Wilson, submitted for publication). These viruses were detected as infectious virions by rescue on permissive cell lines or by direct detection by PCR. Virus/virus genomes were detected in 28%/64% of chimpanzees, 38%/46% of bonobos, 21%/41% of gorillas, 0%/63% of orangutans, and 8%/56% of cynomolgus and rhesus macaques. The animals were not known to be sick at the time of sample collection. The majority of samples were from captive-bred animals, although similar findings were confirmed with a more limited number of samples recovered from chimpanzees and gorillas that live in the wild in Africa. Shedding of adenovirus is significantly diminished in humans, although the majority of subjects harbored viruses in gut tissue and gut-associated lymphocytes, as demonstrated by PCR. The adenoviruses recovered from great apes very much resembled previously identified human adenoviruses and mapped to three primary phylogenic groups, including species, B, C, and E, which are represented by the human adenoviruses HAdV-35, HAdV-5, and HAdV-4. These studies strongly suggested that the human species B, C, and E viruses were derived from cross-species transmission from great apes. The viruses recovered from macaques are quite distinct from great ape and human adenoviruses. The current study presents a comprehensive survey of host responses to natural adenovirus infections in humans, which is considered in the context of the recently discovered virology of persistent infection/latency in the gut.

Chimpanzees and macaques elicit systemic humoral but not systemic cellular immune responses to endogenous adenoviruses. A select number of captive-bred chimpanzees were evaluated for adenovirus-specific immune responses. Sera of 50 animals were evaluated for NAb. The prevalence (Fig. 1A) and the magnitude of the titer in the positive sera (Fig. 1A) demonstrated that virtually every chimp had high titers of NAb against a wide range of chimpanzee-derived adenoviruses, including representatives from species E ($n = 3$), C ($n = 1$), and B ($n = 1$). A similarly high prevalence and titer range were observed for macaque samples against macaque-derived adenoviruses from the Covance and Oregon facilities (Fig. 1A).

Chimpanzee PBMCs ($n = 11$) were screened for adenovirus-specific T cells by IFN-γ ELISPOT analysis; antigen was provided by incubating the cells with heat-inactivated viruses from species C (HAdV-5 and SAdV-40), B (SAdV-32), and E (SAdV-24 and SAdV-26) at an MOI of 10^4. Adenovirus-specific T-cell responses were in fact quite low under virtually all conditions tested (data not shown). Figure 1B presents data

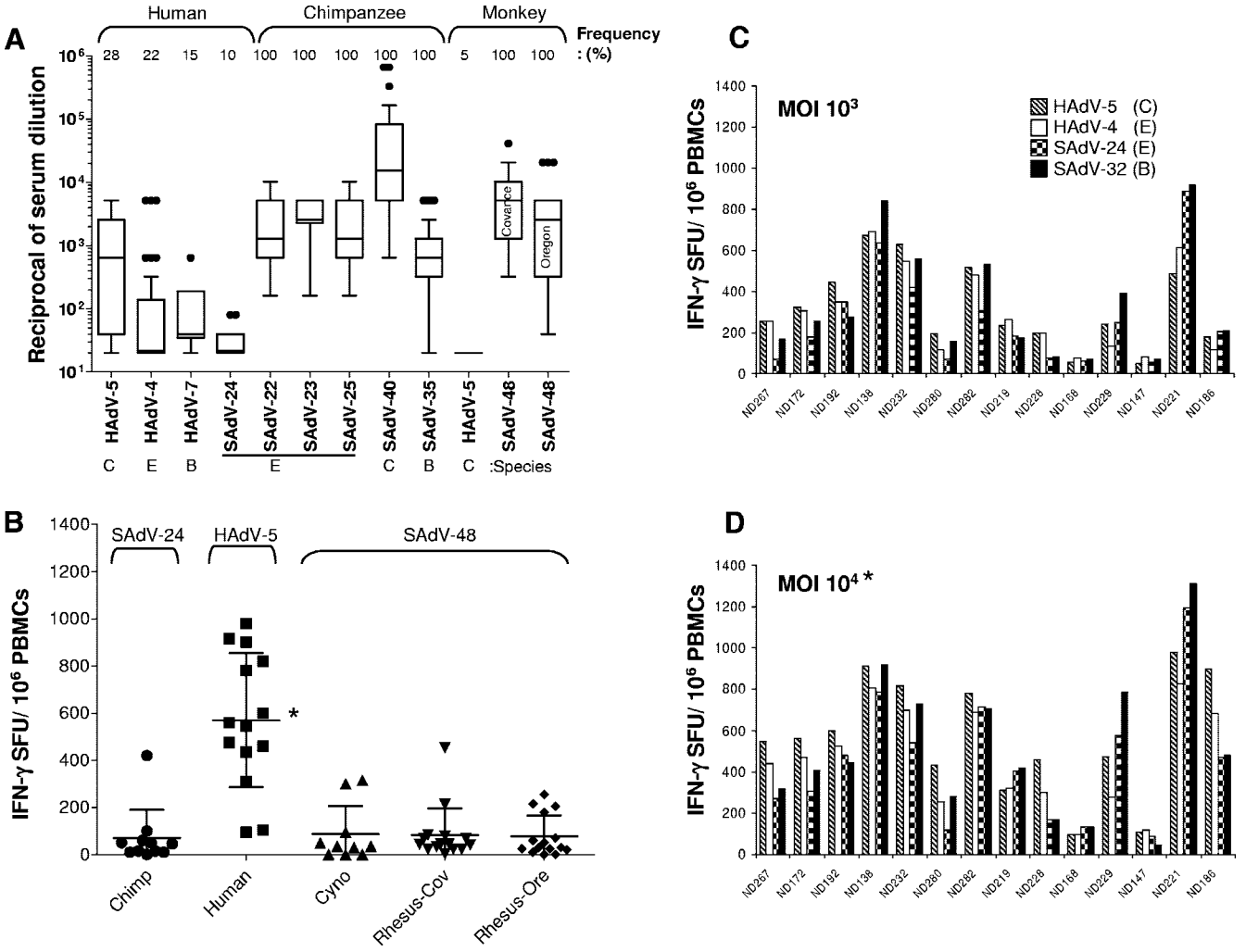
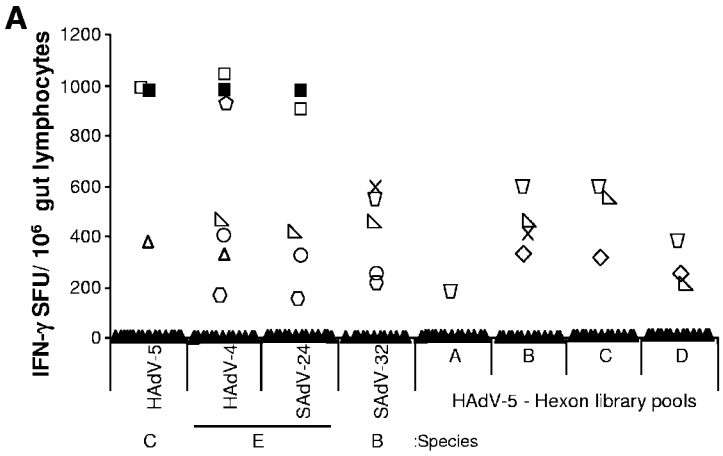


FIG. 1. T- and B-cell responses to adenoviruses in blood of human and nonhuman primates. (A) Prevalence and magnitude of NAb responses in serum in humans, chimpanzees, and monkeys. For each combination of virus and host species, the frequency of seropositivity (NAb titer of >1/20) is shown on top as a percentage. The box-and-whisker plots of NAb to different adenoviruses in sera from humans ($n = 50$), chimpanzees ($n = 50$), and monkeys ($n = 50$) describe the breadth of the response and its distribution for that subset of samples that had a titer of ≥1/20. Each box encloses 50% of the NAb titers, with the median value displayed as a line. The top (upper quartile [UQ]) and bottom (lower quartile [LQ]) of the box mark the limits of ±25% of the titer. Outliers (defined as any titer outside the range of UQ + 1.5 IQD or LQ − 1.5 IQD, where IQD is the interquartile distance) are displayed as individual points. (B) Adenovirus-specific T cells in PBMCs from human and nonhuman primates. IFN-γ ELISPOT data for PBMCs from chimpanzees ($n = 11$), humans ($n = 14$), cynomolgus macaques ($n = 10$), and rhesus macaques from Covance ($n = 15$) and from Oregon National Primate Research Center ($n = 15$) are shown, represented by the number of IFN-γ SFU per million lymphocytes stimulated by the adenovirus (represented on the *x* axis) at an MOI of 10^4. Individual values and means ± standard deviations (SD) are shown following subtraction from the no-stimulation control. An asterisk indicates a significant elevation ($P < 0.0001$) of adenovirus-specific T cells in PBMCs from humans in comparison to PBMCs from nonhuman primates by one-way analysis of variance. The mean for each group is represented by a horizontal bar. (C and D) Activation of adenovirus-specific human T cells in response to a broad range of antigens. IFN-γ ELISPOT data for 14 human PBMC samples are shown, where each bar represents the number of IFN-γ SFU per million lymphocytes stimulated with the heat-inactivated adenovirus represented on the *x* axis. Values shown are following subtraction from the no-stimulation control. All of the samples were stimulated with two different numbers of viral particles per lymphocyte, i.e., an MOI of 10^3 (C) and an MOI of 10^4 (D). An asterisk indicates a significant elevation of T-cell responses at an MOI of 10^4 ($P < 0.005$) in comparison to those at an MOI of 10^3 by a two-tailed paired Student *t* test.

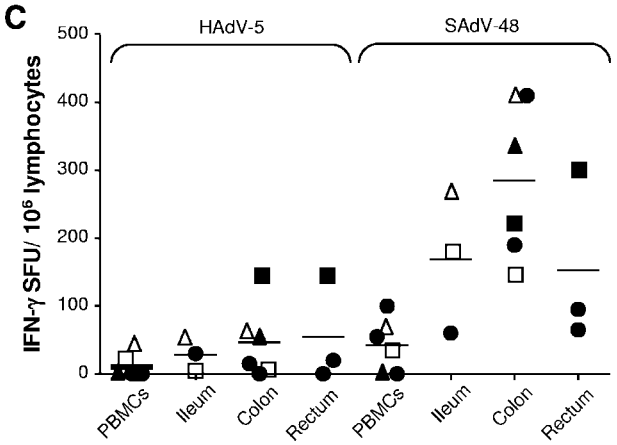
following stimulation with SAdV-24. PBMCs obtained from rhesus macaques from Covance ($n = 15$) and Oregon ($n = 15$) and from cynomolgus macaques from our facility ($n = 10$) were also evaluated for adenovirus-specific T cells against a variety of antigens (HAdV-5 [species C], SAdV-24 [species E], SAdV-32 [species B], three macaque-derived viruses [SAdV-48, SAdV-49, and SAdV-50], and HAdV-5 hexon peptides), using an IFN-γ ELISPOT assay (data not shown). ELISPOT data from cells stimulated with SAdV-48 are shown in Fig. 1B. Cells from most animals failed to respond to any adenovirus antigen evaluated, although there were some animals with low-level responses.

Adenovirus-specific CD4$^+$ T cells reside in the gut of macaques. Selected rhesus and cynomolgus macaques were necropsied, and mononuclear cells harvested from peripheral blood and various sites from the gut (ileum, colon, and rectum)



B

Sample:		Tissue (%CD4 / %CD8) Colon	Ileum	Rectum
45241	IFN-γ+	0.18 / 0.25	0.16 / 0.50	ND / ND
	TNF-α+	1.66 / 0.46	0.68 / 0.81	ND / ND
	IL-2+	1.41 / 0.14	0.43 / 0.64	ND / ND
DWIT3001-011	IFN-γ+	0.45 / <0.01	1.35 / <0.01	0.38 / <0.01
	TNF-α+	0.57 / <0.01	1.97 / <0.01	0.21 / <0.01
	IL-2+	0.32 / <0.01	0.98 / <0.01	0.12 / <0.01
45291	IFN-γ+	0.33 / <0.01	ND / ND	ND / ND
	TNF-α+	0.29 / <0.01	ND / ND	ND / ND
	IL-2+	0.12 / <0.01	ND / ND	ND / ND



D

Sample:		Tissue (%CD4 / %CD8) Colon	Ileum	Rectum
△ R-366	IFN-γ+	0.12/ <0.01	0.09/ <0.01	0.35/ 0.07
	TNF-α+	0.38/ 0.16	0.21/ 0.13	0.46/ 0.14
	IL-2+	0.21/ <0.01	0.14/ 0.16	0.34/ 0.08
□ 90-201	IFN-γ+	0.05/ <0.01	0.07/ <0.01	ND
	TNF-α+	0.07/ <0.01	0.14/ <0.01	ND
	IL-2+	<0.01/ <0.01	0.11/ <0.01	ND
▲ C24980	IFN-γ+	0.15/ <0.01	<0.01/ <0.01	ND
	TNF-α+	0.28/ <0.01	<0.01/ <0.01	ND
	IL-2+	0.13/< 0.01	<0.01/ <0.01	ND
■ C24948	IFN-γ+	ND	0.05/ <0.01	ND
	TNF-α+	ND	0.06/ <0.01	ND
	IL-2+	ND	0.07/< 0.01	ND

FIG. 2. Analysis of adenovirus-specific T cells in macaque and human guts. (A) Adenovirus-specific T-cell responses in human colon. Numbers of IFN-γ SFU per million lymphocytes from 16 human LPL specimens from 15 individuals stimulated with an HAdV-5 hexon library and heat-inactivated adenoviruses of human or chimpanzee species B, C, and E at an MOI of 10^4 are shown. Values shown are following subtraction from the no-stimulation control. Samples with ELISPOT counts of <55 SFU per million lymphocytes and with values less than three times above the background were plotted as zero. (B) Assessment of functionality of adenovirus-specific human LPLs isolated from colon, ileum, and rectum. Levels of $CD4^+$ and $CD8^+$ T cells that produce IFN-γ, TNF-α, and IL-2 cytokines in human gut LPLs are shown. Three human surgical samples were stimulated with the HAdV-5 hexon peptide library, and frequencies of cytokine-secreting cells within the CD4 or CD8 population (minus those with the no stimulation background; <0.01) are shown. (C) Adenovirus-specific T-cell responses of PBMCs and gut-associated lymphocytes in macaques. IFN-γ ELISPOT data for rhesus macaque ($n = 2$) (open symbols) and cynomolgus macaque ($n = 4$) (full symbols) PBMCs and LPLs (colon, ileum, and rectum) following stimulation with an adenovirus (MOI, 10^4), minus the stimulation background, are represented as numbers of SFU per million lymphocytes. The mean for each group of samples is illustrated by a horizontal bar. (D) Assessment of functionality of adenovirus-specific nonhuman primate LPLs. Cytokine production of IFN-γ, TNF-α, and IL-2 by $CD4^+$ and $CD8^+$ T cells in gut LPLs from two rhesus and two cynomolgus macaques following stimulation with heat-inactivated SAdV-48 (MOI, 10^4), minus the stimulation background, is shown. ND, not determined.

were evaluated for the presence of adenovirus-specific T cells, using HAdV-5 and SAdV-48 as antigens (Fig. 2C and D). Animal welfare considerations precluded similar studies with great apes. Adenovirus-specific T cells were detected throughout the gut when SAdV-48 was used to stimulate the cells; they demonstrated a mixture of cells with central memory and effector memory phenotypes (data not shown). Interestingly, the presence of adenovirus-specific T cells in the gut was not predicted based on analysis of the corresponding PBMCs, which were consistently negative or had very low responses. T cells generated in response to endogenous adenovirus infections in macaques were not effectively stimulated with HAdV-5, using cells from blood or the mucosa (Fig. 2C). ICCS of gut samples from several macaques for adenovirus, using SAdV-48 as an antigen, revealed a predominance of $CD4^+$ T cells, although the frequencies were low but positive for expression of IFN-γ, TNF-α, and IL-2 (Fig. 2D). Stool extracts were also assayed for the presence of NAb to SAdV-35 (species B) and SAdV-40 (species C). Both the prevalence and titer were found to be consistently lower than those in serum (data not shown).

Systemic NAb and T cells to adenoviruses are highly prevalent in humans, although gut-associated T cells are more variable. Several studies have screened human sera for the presence of NAb to adenoviruses. We screened a population from the United States for NAb against a variety of human- and chimpanzee-derived adenoviruses, including those from species C (HAdV-5), E (HAdV-4 and SAdV-24), and B (HAdV-7) (Fig. 1A). The prevalence and magnitude of NAb to

human-derived adenoviruses in humans were substantially lower than those observed in chimpanzees against chimpanzee viruses. The highest levels were observed against HAdV-5, which is consistent with previous reports (4). Analysis of samples from subjects in Africa confirmed these findings, except for an increased prevalence of NAb against HAdV-5 (data not shown).

PBMCs from 14 healthy human subjects were evaluated for evidence of adenovirus-specific T cells to heat-inactivated HAdV-5, using the ELISPOT assay to detect secretion of IFN-γ following exposure to antigen. Cells were initially stimulated with HAdV-5 at various MOIs (10, 10^2, 10^3, and 10^4). Figure 1C and D present the data for MOIs of 10^3 and 10^4; comparisons to data from nonhuman primates are presented in Fig. 1B. For humans, the number of spots produced after stimulation with heat-inactivated HAdV-5 was significantly above background in each sample at both MOIs, although the response was significantly higher when the MOI chosen was 10^4 ($P < 0.005$) (Fig. 1D; see Fig. S1 in the supplemental material). T-cell responses to both human and simian adenoviruses were very similar in magnitude, with no significant difference among them ($P > 0.05$), suggesting that epitopes from the adenovirus capsid protein responsible for stimulating human T cells appear to be conserved across a broad array of human- and chimpanzee-derived viruses, including HAdV-5, HAdV-4, SAdV-24, and SAdV-32. Cells stimulated with a library of peptides spanning the HAdV-5 hexon resulted in frequencies equal to or greater than (1,215 ± 862 [mean ± standard deviation] SFU/10^6) that seen following stimulation with heat-inactivated HAdV-5, indicating that hexon is a source of dominant epitopes.

The same human PBMC samples were assessed for reactive T cells to HAdV-5, using ICCS (Fig. 3). Following 6 h of stimulation with the HAdV-5 hexon library, cells were stained with fluorochrome-labeled antibodies to CD4, CD8, IFN-γ, TNF-α, and IL-2 and analyzed by five-color flow cytometry. All 14 samples showed positive cytokine responses to HAdV-5, and the responses were almost exclusively mediated by $CD4^+$ T cells and dominated by TNF-α and IFN-γ production ($P < 0.005$), followed by IL-2 (Fig. 3A for $CD4^+$ T cells and Fig. 3B for $CD8^+$ T cells; also see Fig. S1 in the supplemental material). The frequencies of multiple cytokine-producing cells were further examined using a Boolean gating algorithm, yielding seven unique response patterns comprising every combination of the three individual measurements (Fig. 3C). Among the cytokine-producing $CD4^+$ T cells, almost 25% secreted both IFN-γ and TNF-α and about 10% secreted all three cytokines, while very few cells secreted only IL-2 or IL-2 and IFN-γ. PBMCs were also stained with antibodies to CD45RO and CD27 to characterize memory phenotypes, which showed that they were primarily central memory T cells (Fig. 4A). Low levels of adenovirus-specific $CD8^+$ T cells were noted in some individuals (Fig. 3B).

The potential of the presence of persistent adenovirus antigens in the gut due to chronic shedding, latency, or sequestered viral proteins compelled us to evaluate gut-associated lymphoid tissues for the presence of adenovirus-specific T cells. Our studies focused on healthy human tissue from surgical specimens. Sixteen samples from 15 subjects were obtained from the ileum, colon, and/or rectum for extraction of LPLs. Cells from 14 of the patients were incubated with heat-inactivated adenoviruses representing species C (HAdV-5), E (HAdV-4 and SAdV-24), and B (SAdV-32) and analyzed for activation through the use of an IFN-γ ELISPOT assay (Fig. 2A); 12 of the samples were stimulated with the HAdV-5 hexon library. Samples from eight of the subjects showed clear T-cell responses against at least one of the antigens, and seven were negative. Samples that responded to stimulation with HAdV-5 were also reactive against virtually all other human and simian adenovirus antigens tested. An increased sensitivity was observed when small peptide pools were used as stimuli in the assay versus the intact virus. This may be due to immunodominance of particular hexon peptides but more likely is explained by increased efficiency of major histocompatibility complex (MHC) I presentation of the peptides in the assay. Cells from three other patients were analyzed by ICCS (Fig. 2B); these studies included colon-derived cells as well as cells from the ileum and rectum, when available. Adenovirus-specific responses were due mainly to $CD4^+$ responses (6/6 specimens), yet in some humans there was also a $CD8^+$-mediated response observed (2/6 specimens) (Fig. 2B). All samples contained a significant number of adenovirus-specific $CD4^+$ T cells that expressed IL-2, IFN-γ, and TNF-α; expression of TNF-α was higher than that of IFN-γ. Figure 4B and C present the coexpression of cytokines in antigen-specific $CD4^+$ T cells. Staining with CD45RO and CD27 revealed that the $CD4^+$ T cells from the rectum and ileum had effector and effector memory phenotypes (Fig. 4A; see Fig. S2 in the supplemental material).

Innate immune responses differ significantly between species B, C, and E adenoviruses. In order to probe the question of whether the differences in adaptive immune responses between monkeys, apes, and humans and their respective viruses were due to distinct interactions and responses at an innate level, selected members of the species B, C, and E adenoviruses were evaluated for activation of innate immunity after exposure (MOI, 10^4) to cultured human plasmacytoid dendritic cells. Supernatants were sampled for the presence of a number of cytokines and chemokines, and Fig. 5 shows the data for IL-6, IFN-α, and MIP-1α. There were remarkable similarities in the profiles of cytokine/chemokine expression with adenoviruses belonging to the same species group. The species C viruses failed to elaborate significant quantities of any cytokine/chemokine, while high levels of several cytokines/chemokines were produced after exposure to species E viruses. Detectable but more variable levels of cytokine were found after exposure to the species B viruses. Within each species group, there were no consistent differences between human and nonhuman primate adenoviruses.

The E3 loci of human adenoviruses contain fewer and smaller CR1 ORFs than those of the corresponding simian adenoviruses. Previous studies of human adenoviruses have shown that the E3 locus encodes proteins important in modulating host responses but not essential for replication (3, 10, 12). Variations in the structure and function of E3 open reading frames (ORFs) may contribute to the marked differences in host responses that were observed between great apes and humans.

The E3 region of adenovirus can carry up to nine ORFs. Many of these ORFs were highly conserved across human and

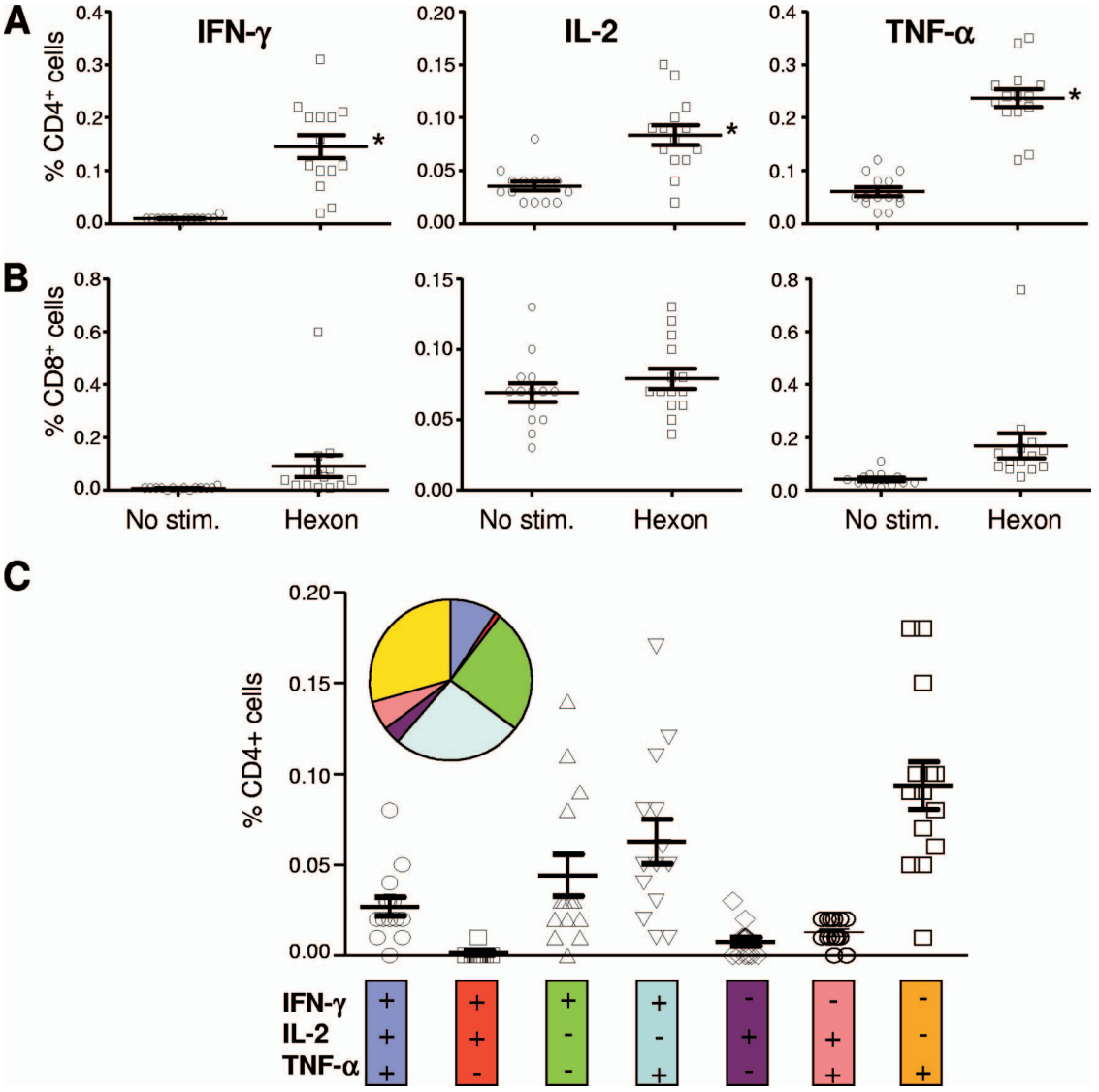


FIG. 3. Functional analysis of adenovirus-specific T cells in human PBMCs. ICCS analysis of the HAdV-5 hexon-specific T-cell response was performed with 14 human PBMC samples. Cytokine production of IFN-γ, TNF-α, and IL-2 by $CD4^+$ (A) or $CD8^+$ (B) T cells was measured following stimulation with medium only (no stim.) or with the hexon peptide library of HAdV-5 resuspended in medium. Individual sample data and means ± SD are shown. An asterisk indicates a significant elevation ($P < 0.0001$) in comparison to the nonstimulated control by a two-tailed paired Student *t* test. (C) Functional profile of the $CD4^+$ T-cell response to HAdV-5 hexon. The contributions of multiple subpopulations were determined for each individual sample, with the mean ± SD represented by horizontal bars. The colored pie chart represents the relative contribution of each functional subpopulation within the total adenovirus-specific $CD4^+$ T-cell response.

great ape adenoviruses, including the last three ORFs, encoding the antiapoptotic proteins RID-α and RID-β and the 14,700-molecular-weight (14.7K) protein (12), the first E3 ORF, encoding a 12.5K protein of unknown function, and the ORF encoding the gp19k protein, which is known to downregulate MHC class I expression (1).

The remaining E3 ORFs, numbering from two to four in different isolates, encode transmembrane proteins of unknown function that contain a conserved domain—CR1 (pfam PF02440)—and are designated CR1-α, CR1-β, CR1-γ, and CR1-δ. Each species group of viruses demonstrated a characteristic structure of the CR1 ORFs that varied between different species groups (Table 1). However, within a species group, there were consistent differences between human and great ape isolates, as described below (Table 1).

Species C great ape viruses all contained three CR1 ORFs, those for CR1-α, CR1-β, and CR1-γ; human representatives of the species C family consistently were missing the CR1-δ ORF. Species B great ape viruses contained the full spectrum of the CR1s; human representatives of the species B family were either missing CR1-δ (species B2) or contained a substantially truncated version of CR1-δ (species B1). Species E great ape viruses also contained all four CR1 ORFs; the sole human representative of this family, HAdV-4, contained a substantially truncated version of CR1-γ.

It may be speculated that the apparent degeneration of CR1

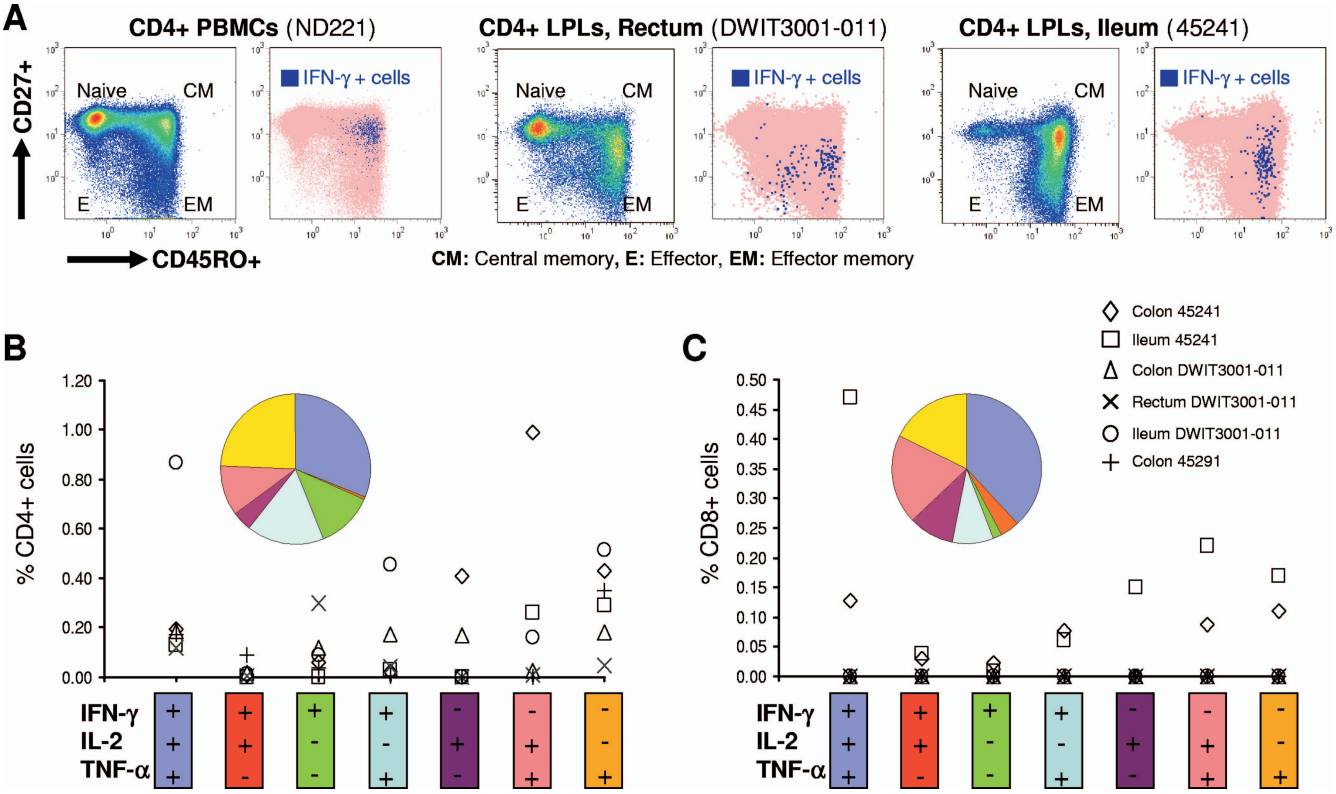


FIG. 4. Memory phenotype characterization of HAdV-5 hexon-specific human $CD4^+$ T cells and functional analysis of adenovirus-specific T cells in human gut. (A) Intracellular cytokine analysis, represented by dot plots of lymphocytes isolated from different sources, as denoted above. Cells were stimulated with an HAdV-5 hexon peptide library and stained for memory markers CD27 (*y* axis) and CD45RO (*x* axis). Naïve cells were defined as $CD27^+$ $CD45RO^-$, central memory cells were $CD27^+$ $CD45RO^+$, effector memory cells were $CD27^-$ $CD45RO^+$, and effector cells were $CD27^-$ $CD45RO^-$. (Left) Density plots represent the memory phenotype of the overall population of lymphocytes from the particular source. (Right) The graphs overlay blue dots on top of the overall lymphocyte population to highlight IFN-γ-secreting cells upon stimulation with the HAdV-5 hexon peptide library. (B and C) Intracellular cytokine secretion profiles of human LPLs isolated from six specimens from three healthy donors in response to the HAdV-5 hexon peptide library. The cytokine production of IFN-γ, TNF-α, and IL-2 by $CD4^+$ (B) or $CD8^+$ (C) T cells following stimulation with the hexon peptide library of HAdV-5 was determined. The pie charts represent the relative contribution of each functional subpopulation within the overall HAdV-5 response.

structure in human viruses compared to that in great ape viruses may impact the ability of the viruses to evade host immune detection and elimination.

DISCUSSION

Analysis of the host's response to natural adenovirus infections was studied in the setting of persistence/latency in clinically asymptomatic individuals. This was considered in the context of virology data that showed high shedding in great apes and macaques and low shedding in humans. It is interesting that chimpanzees and macaques continue to shed virus despite high levels of broadly cross-reactive NAbs in serum, which might be a function of the increased risk of reexposure due to the high incidence of shedding in these populations. Clearly, these antibody titers in the circulation are not translated to effective humoral immune responses at the gut mucosa. In fact, we were unable to detect adenovirus NAb in stools of chimpanzees (data not shown). Adenovirus-specific T cells were not found in PBMCs of chimpanzees or macaques, although they were found in the gut mucosa of most macaques. The gut is host to a large number of commensal organisms that exist without inciting inflammatory responses of the host. It appears that the population of adenovirus-specific T cells detected in the gut mucosa of macaques is ineffective in eliminating the persistent adenovirus infection.

Humans present a much different response to antecedent adenovirus infections than do monkeys. The scope and extent of systemic humoral immunity are diminished in magnitude and breadth compared to what is observed in nonhuman primates, possibly representing lower rates of infection/reinfection. The most dramatic difference was the presence of an adenovirus-specific T-cell response in PBMCs of all individuals surveyed, which were largely absent in chimpanzees and macaques, as well as high levels in the gut mucosa of at least 50% of those surveyed. More detailed characterization of these cells in humans indicated that they were primarily $CD4^+$ T cells in blood, with a central memory phenotype, and a mix of $CD4^+$ and $CD8^+$ T cells in the gut, with effector and effector memory phenotypes. T cells in each compartment expressed multiple cytokines. Production of IL-2 in combination with IFN-γ and TNF-α has been demonstrated with several experimental systems to be a more accurate predictor of the protective capacity of responder T-cell populations than IFN-γ secretion alone

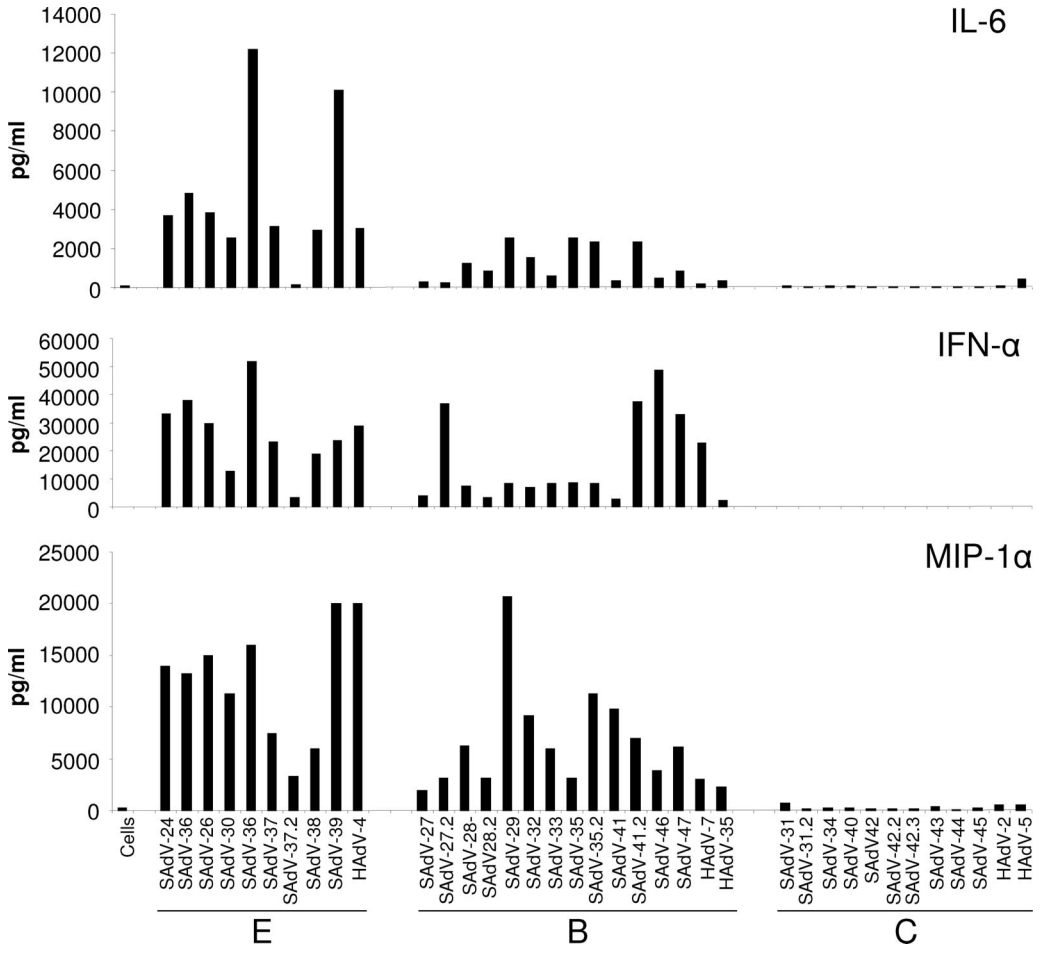


FIG. 5. Activation of human plasmacytoid dendritic cells by human and nonhuman primate adenoviruses. Levels of several cytokines and chemokines were determined by assays to measure stimulation and release of human plasmacytoid dendritic cells by multiplex analysis. The levels of IL-6 (top), IFN-α (middle), and MIP-1α (bottom) in the culture supernatant following exposure of cells to adenoviruses (MOI, 10^4) belonging to subgroups E, B, and C are shown.

(11). The cellular immune response to adenovirus in humans appears to play an important role in better controlling ongoing replication and persistence.

The mechanisms responsible for differences in virology and immunology of adenovirus infections in nonhuman primates versus humans are largely unknown, although our studies provide some insight. Activation of innate immunity, as measured in human dendritic cell cultures, appears to be a function of the phylogenetic species of adenovirus, not the source of the virus (i.e., humans versus great apes). Analysis of the E3 regions of great ape- and human-derived adenoviruses, which are involved in modulating host responses to virus infection, did demonstrate structural differences that may further explain the apparent differences in host responses. A family of related ORFs within the E3 locus, called CR1-α, -β, -γ, and -δ, was either truncated or missing in human adenoviruses compared to that in related great ape adenoviruses within species B, C, and E. The function(s) of the CR1 ORFs in adenoviruses has not been defined, although they share homology with the human cytomegalovirus RL11 gene family (5). It is possible that CR1 proteins play a role in maintaining fitness of the virus in great apes that is not critical in humans. A secondary consequence of the loss of CR1 function may be a less effective evasion of host responses and a different immunologic niche in humans than in great apes. We are currently investigating whether the expression of the E3 CR1 protein(s) may impact immune evasion in vivo.

The persistence of adenovirus antigens and adenovirus-specific T cells in the gut may have implications for the use of

TABLE 1. Comparative structures of E3 loci in adenoviruses from higher-order primates[a]

Virus species	Length (aa) of E3 structure			
	CR1-α	CR1-β	CR1-γ	CR1-δ
Species C				
Human	**62–66**	**101–138**	**0**	0
Simian	**148–180**	**291–303**	**272–283**	0
Species B				
Human B1	146	174–181	187–195	**41–94**
Human B2	131	174–181	187–195	**0**
Simian	142–150	177–246	146–209	**101–119**
Species E				
Human	210	220	**54**	274
Simian	207–209	199–246	**203–206**	285–295

[a] The lengths (numbers of amino acid residues) of CR1 proteins present in simian adenoviruses isolated from chimpanzees, bonobos, and gorillas are shown compared to the cognate ORFs in sequenced human adenoviruses. Bold numbers indicate the predicted lengths of the CR1 proteins for which the human counterparts are clearly shorter.

adenovirus-based vectors for gene therapy and genetic vaccines. One such example is the STEP trial, which measured the efficacy of vectors based on HAdV-5 expressing HIV type 1 (HIV-1) antigens in a population of subjects at high risk of contracting HIV-1 infection (2). There was no demonstrated efficacy of the vaccine for the cohort that was seronegative for HAdV-5 prior to receiving the vaccine. The real surprise was that the vaccine increased the acquisition of HIV-1 infection in the cohort that had preexisting circulating NAb to the adenoviral vector. A number of hypotheses are being considered to explain this finding. However, most agree that it is prudent to consider it a valid finding that deserves further study. Our present study provides insight into the natural biology of adenoviral infection in humans with potential relevance to the STEP trial and future HIV-1 vaccine trials using alternative adenovirus vaccines. Although any direct connection is speculative, one hypothesis is that the adenovirus vaccine led to an increased number of activated adenovirus-specific T cells in the epithelia of the intestine, thereby increasing the number of target cells for HIV infection. We show the existence of adenovirus-specific T cells in the periphery of many humans, which could be expanded subsequent to vaccination. The presence of persistent adenovirus antigen in the gut may facilitate migration to and expansion in the gut mucosa. The finding that these T cells can be activated further with adenovirus capsids of various species suggests that alternative adenovirus vectors may suffer the same fate.

Our work points to another important issue to consider in assessing the utility of the macaque model for gene therapy and genetic vaccine studies. Important differences in the virology and immunology of the viruses used to generate the viral vector might impact their safety and efficacy profile, depending on the host species. The profile of adenovirus infection in humans is very different from that in macaques. Humans have low viral loads in stools and robust central and mucosal T-cell responses, while macaques have high viral loads and T-cell responses that are low in blood and moderate in the gut. Furthermore, endogenous macaque adenoviruses are sufficiently different from human viruses in that memory T cells in macaques are not responsive to human adenoviruses of the type used as vectors.

In summary, these studies illustrate the complex biology of adenovirus infections in great apes, macaques, and humans, which may confound the outcome and interpretation of human clinical trials with vectors based on these viruses.

ACKNOWLEDGMENTS

We thank the Cooperative Human Tissue Network (CHTN) of the University of Pennsylvania Medical Center and the National Disease Research Interchange (NDRI) for providing human gut specimens. We also thank the Immunology Core personnel (University of Pennsylvania, Gene Therapy Program), i.e., Qiuye Qin, Surina Boyd, Jennifer Miliaresis, and Lynne Gallery, for their help in isolating lymphocytes from blood and tissues.

This work was sponsored by grants from GlaxoSmithKline, NIDDK (5P30DK047757-15), NHLBI (5P01HL059407-10), and the Cystic Fibrosis Foundation (R881-CR07).

L.H.V., S.R., S.S., and J.M.W. are inventors on patents licensed to various biopharmaceutical companies. R.C. and L.W. have no conflicts of interest.

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