RESEARCH ARTICLE

Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV

K-Q Xin¹, N Jounai¹, K Someya², K Honma³, H Mizuguchi⁴, S Naganawa⁵, K Kitamura⁵, T Hayakawa⁴, S Saha¹, F Takeshita¹, K Okuda³, M Honda², DM Klinman⁶ and K Okuda¹

¹Department of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, Yokohama, Japan; ²AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; ³Department of Microbiology, Tokyo Dental College, Chiba, Japan; ⁴Laboratory of Gene Transfer and Regulation National Institute of Biomedical Innovation, Osaka, Japan; ⁵Department of Public Health, Yokohama City University, Graduate School of Medicine, Yokohama, Japan; and ⁶Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, MD, USA

Immunization involving a DNA vaccine prime followed by an adenovirus type 5 (Ad5) boost elicited a protective immune response against SHIV challenge in monkeys. However, the hepatocellular tropism of Ad5 limits the safety of this viral vector. This study examines the safety and immunogenicity of a replication-defective chimeric Ad5 vector with the Ad35 fiber (Ad5/35) in BALB/c mice and rhesus monkeys. This novel Ad5/35 vector showed minimal hepatotoxicity after intramuscular administration with the novel Ad5/35 vector. In addition, an Ad5/35 vector expressing HIV Env gp160 protein

(Ad5/35-HIV) generated strong HIV-specific immune responses in both animal models. Priming with a DNA vaccine followed by Ad5/35-HIV boosting yielded protection against a gp160-expressing vaccinia virus challenge in BALB/c mice. The Ad5/35-HIV vector was significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector. These findings indicate that an Ad5/35 vector-based HIV vaccine may be of considerable value for clinical use. Gene Therapy (2005) 12, 1769–1777. doi:10.1038/sj.gt.3302590; published online 4 August 2005

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Introduction

A vaccine capable of preventing HIV infection is needed to control the global AIDS pandemic. In the past decade, multiple strategies to produce an immunogenic HIV vaccine have been explored. This included production of HIV subunit peptide vaccines,¹ DNA vaccines,² recombinant virus-vector vaccines (including modified vaccinia virus,³ adenovirus (Ad),^{4,5} rabies virus,⁶ flavivirus,⁷ sendai virus,⁸ Venezuelan equine encephalitis virus,⁹ and adeno-associated virus^{10,11}), and bacterial vector-vaccines (bacille Calmette–Guerin,^{12,13} and *Lactococcus lactis*¹⁴). Each of these strategies showed some promising results in animal models, either alone or in combination.

Among these vectors, the replication-defective human Ad type 5 (Ad5) recombinants (with the deletion of a replication-essential gene, E1) and the replication-defective modified vaccinia Ankara (MVA) elicited the most potent CD8⁺ T-cell responses and provided the highest degree of protection in non-human primates.^{3,4,15,16} A major limitation for the clinical application of the Ad5 and MVA vectors is the pre-existing immunity against

these viruses in humans, since most of the human population has been infected with Ad5¹⁷ and vaccinia virus on being administered the smallpox vaccine. The pre-existing antiviral immunity may strongly influence the efficacy of the HIV vaccine using Ad5 and MVA vectors.

Human Ads are classified into six subgroups from A–F.¹⁸ Most of Ad serotypes belonging to subgroups A, C, D, E, and F use the coxsackievirus and adenovirus receptor (CAR) as a cellular receptor.¹⁹ The Ad5 (subgroup C) has well-defined biological properties and has been widely used as a vector for gene therapy and vaccine. The replication-defective Ad5 vector can easily be produced in high titers and is highly effective in boosting HIV-specific immunity.^{4,15} However, this virus uses CAR as its primary attachment receptor, which confers tropism for liver parenchymal cells.^{19–22} This raises important safety concerns,²² particularly because the administration of an Ad5-based vector for gene therapy resulted in the death of a patient.²³ In response to these shortcomings, our laboratory has examined the immunogenicity and safety of a replication-defective chimeric Ad5 vector with Ad type 35 fiber (Ad5/35) (Ad35 virus was classified as subgroup B). The Ad35 fiber showed 25% amino-acid homology with the Ad5 fiber.²⁴ Cell entry of Ad35 is CAR independent and may involve CD46 receptor, which expresses on most human cells.²⁵ Ad35 can be transduced to liver nonparenchymal cells on a level 4–5 log orders lower than Ad5, but not to

Correspondence: Dr K Okuda, Department of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

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liver parenchymal cells.²⁰ In the present study, we found that the Ad5/35 recombinants not only induced strong antigen-specific humoral and cellular immune responses and exhibited minimal hepatotoxicity in both mice and non-human primates, but were also significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector.

Results

Biodistribution of Ad in mice

In the initial experiments, mice were injected intramuscularly (i.m.) with 10^{11} viral particles (vp) of a luciferase-expressing Ad5 (Ad5-Luc) or Ad5/35 vector (Ad5/35-Luc). Luciferase expression was monitored using an *in vivo* imaging system (IVIS) on days 3 and 10 after administration. As shown in Figure 1a, all of the Ad5/35-Luc vector remained at the injection site. In contrast, substantial amounts of the Ad5 vector migrated to the liver. This difference in vector distribution was confirmed by studies involving LacZ-expressing Ad5 and Ad5/35 vectors (data not shown). Studies on serum glutamic-oxaloacetic transaminase (GOT) and serum glutamic-pyruvic transaminase (GPT) levels revealed that mice injected with the Ad5-Luc vector had changes indicative of liver damage (Figure 1b). We also analyzed serum levels of key proinflammatory cytokines (IFN- γ and IL-6) on days 0, 3, and 10 after administration of virus vectors. The levels of IFN- γ and IL-6 were significantly elevated following administration of Ad5-Luc vector, but not of Ad5/35-Luc vector (Figure 1c). Thus, the hepatotoxicity caused by the Ad5 vector was circumvented by the use of an Ad5/35 vector.

Time-course study of HIV-specific immune responses in mice. Ad5/35 vector can efficiently transfect antigen-presenting cells^{18,21,26,27} and muscular cells (Figure 1a). In order to explore whether the virus vector can be used as a vaccine vector, we constructed an HIV Env gp160-expressing Ad5/35 vector (Ad5/35-HIV). The expression of HIV gp160 was confirmed by Western blotting (Figure 2a). The HIV Env gp160-expressing DNA vaccine (DNA-HIV) used in this study was reported previously.²⁸ The mice were immunized with 10^{10} vp of Ad5/35-HIV vector, and the HIV-specific cellular immune response was periodically monitored by the intracellular cytokine staining (ICS) assay. The assay has been widely utilized to distinguish the relative contributions of CD8⁺ cells to the overall T-cell responses.²⁹ On day 3, HIV-specific IFN- γ -secreting CD8⁺ T cells can be detected (Figure 2b) and peaked 2 weeks after immunization. On day 50 and month 7 after final immunization, 2.5 and 1.2% of HIV-specific IFN- γ -secreting CD8⁺ T cells still persisted, respectively.

Mice were vaccinated with Ad5/35-HIV vector to explore the humoral immune response 7 weeks after the final immunization. The animals immunized with 10^{10} vp of Ad5/35-HIV vector developed a high-titered anti-gp160 antibody (Ab) response (Figure 2c). The specificity of the Ab response was confirmed by Western blotting (Figure 2c, upper panel). The magnitude of this response was not significantly altered by preimmunization with the DNA-HIV vaccine (Figure 2c). DNA-HIV

Figure 1 Biodistribution and safety of Ad vectors. BALB/c mice were injected i.m. with 10^{11} vp of the Ad5-Luc or Ad5/35-Luc vector. (a) Using an IVIS CCD camera, vector distribution was detected after the addition of luciferin (3 mice/group) (expressed in relative light units (RLU)). One of the mice is represented and other mice used show the same pattern. (b) Serum GOT and GPT levels were measured on days 0, 3, and 10 after injection (5 mice/group). IU: international unit. (c) Serum IFN- γ and IL-6 levels were measured on days 0, 3, and 10 after injection (5 mice/group). *Mean values are significantly different between Ad5-Luc-administered mice and Ad5/35-Luc-administered mice or naïve mice at the same time point.

vaccination alone generated a low level of HIV-specific serum Ab (Figure 2c, bottom panel). HIV-specific neutralizing Ab was only detectable in the Ad5/35-HIV

Figure 2 Time course of HIV-specific immune responses in mice. (a) HIV Env protein expression of DNA-HIV vaccine and Ad5/35-HIV on HEK293 cells was confirmed by Western blotting using an HIV Env-specific mAb. (b) Time-course study of cellular immune responses after a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector (3 mice/time point). D: day; M: month. (c) HIV-specific Ab was detected by Western blotting using 100-fold diluted antisera (serum pool of 10 mice/group) (upper panel) and ELISA (10 mice/group) (bottom left panel).

vaccinated mice (1:186) and DNA prime/Ad5/35-HIV boosted mice (1:206).

Immune responses and challenge in mice 2 weeks after vaccination. There is growing evidence that cellular immunity contributes to protecting the host against HIV infection.^{3,4,30,31} The ability of the Ad5/35 vector to trigger the activation and proliferation of antigen-specific T cells was monitored. Vaccination with the DNA-HIV vaccine induced the number of HIV-

specific IFN- γ -secreting CD8⁺ T cells to increase from background levels (<0.2 – 0.7%) ($P < 0.05$) (Table 1). This was significantly less than the effect of vaccination with the Ad5/35-HIV vector (10^{10} vp/mouse) that increased the IFN- γ -secreting CD8⁺ T cells to 5.5% ($P < 0.05$). Priming with the DNA-HIV vaccine followed by an Ad5/35-HIV vector boost led to a further three-fold increase in the number of IFN- γ -secreting CD8⁺ T cells ($P < 0.05$).

A tetramer-binding assay was used to verify that the IFN- γ -secreting cells were MHC class I-restricted HIV-specific CD8⁺ T cells.³² A single immunization with Ad5/35-HIV vector elicited a significant increase in the number of tetramer-binding CD8⁺ T cells (Table 1). When compared with DNA-HIV vaccination alone, immunization with the Ad5/35-HIV vector yielded five-fold more HIV-specific CD8⁺ T cells ($P < 0.05$). Priming with the DNA-HIV vaccine, followed by Ad5/35-HIV boosting, further increased the tetramer binding ($P < 0.05$).

To examine the protective activity of the Ad5/35-HIV vector, immunized mice were challenged with 10^8 plaque forming units (PFU) of vPE16 2 weeks after final immunization. The animals that were vaccinated with the Ad5/35 vector alone or in combination with the DNA-HIV vaccine were completely protected from infection (Table 1); however, the DNA-HIV vaccination alone had little impact on the susceptibility to infection by vPE16.

Long-term cell-mediated immune responses and challenge in mice. The durability of these vaccine regimens was explored. HIV-specific cellular immune responses persisted through 7 months after final immunization (Table 1 and Figure 2b). To determine whether this immune response was protective, vaccinated mice were challenged with vPE16 (10^8 PFU/mouse) 7 weeks after final immunization. The viral load of Ad5/35-HIV-immunized mice was reduced by 10^5 as compared with that of the control mice ($P < 0.05$). DNA-HIV vaccination by itself was not protective, but the combination of DNA-HIV priming and Ad5/35-HIV boosting yielded a prolonged and complete protection (Table 1).

Biodistribution of Ad in rhesus macaques

To study the biodistribution of Ad in monkeys, 10^{11} vp of Ad5-Luc and Ad5/35-Luc vectors was injected i.m. into two rhesus monkeys for each vector. The luciferase activity in the tissues was detected 3 days after administration, because high luciferase activity in the mouse liver was observed at that time point. Liver infection with Ad5 vector was 20- to 40-fold stronger than that with Ad5/35 vector (Figure 3a). It is important to note that the luciferase activity of the cerebellum and the posterior cerebrum in the monkeys that received the Ad5-Luc vector was two- and four-fold higher, respectively, than that of the monkeys that received the Ad5/35-Luc vector.

Immune response in rhesus monkeys after vaccination

To explore the immunogenicity of the Ad5/35-HIV vector in monkeys, two rhesus macaques were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector. A detectable HIV-specific serum Ab response developed

Table 1 HIV-specific cell-mediated immune responses and virus challenge after vaccination

	Week 2			Week 7			Month 4	Month 7
	ICS (%)	Tetramer (%)	Ovary viral titer	ICS (%)	Tetramer (%)	Ovary viral titer	Tetramer (%)	Tetramer (%)
Nonimmune	0.1±0.1	0.1±0.1	8 × 10 ⁸ ±35	0.1±0.1	0.1±0.1	1 × 10 ⁹ ±65	0.0±0.0	0.0±0.0
DNA-Empty	0.1±0.1	0.1±0.1	2 × 10 ⁹ ±45	0.0±0.0	0.0±0.0	8 × 10 ⁸ ±32	0.0±0.0	0.0±0.0
Ad5/35-Luc	0.2±0.1	0.2±0.2	2 × 10 ⁹ ±25	0.0±0.0	0.0±0.0	4 × 10 ⁸ ±46	0.0±0.0	0.0±0.0
DNA-HIV	0.7±0.1	1.0±0.3	6 × 10 ⁶ ±42	0.4±0.2	0.6±0.1	5 × 10 ⁷ ±51	0.3±0.1	0.1±0.1
Ad5/35-HIV	5.5±0.3	5.2±0.3	ND	2.5±0.8	3.1±0.2	2 × 10 ⁸ ±34	2.5±0.5	1.2±0.4
DNA-HIV+Ad5/35-HIV	17.2±0.8	19.4±2.1	ND	8.2±1.2	8.9±0.8	ND	7.1±0.6	4.1±0.3

Mice were immunized with DNA plasmid or Ad5/35 vector, either alone or in combination. At 2 weeks, 7 weeks, 4 months, and 7 months after final immunization, HIV-specific cellular immune responses were detected by ICS assay and tetramer assay. The data represent the percentage of IFN- γ - or tetramer-positive CD8⁺ T cells (5–10 mice/group). The backgrounds were less than 0.1% IFN- γ -secreting CD8⁺ T cells when cells were stimulated with control peptide (influenza NP peptide, TYQRTRALV). The vaccinated mice (10 mice/group) were challenged with vaccinia virus vPE16 2 or 7 weeks after final immunization. At 6 days after the challenge, the vPE16 titer in mouse ovaries was measured. ND, not detectable.

Figure 3 Biodistribution and HIV-specific immune responses in rhesus monkeys. Rhesus monkeys (2 monkeys/group) were administered i.m. 10¹¹ vp of Ad5-Luc or Ad5/35-Luc vector. The luciferase activity in the organs of the monkey (expressed in RLU) was examined 3 days after administration (a). Rhesus monkeys were immunized i.m. with 10¹¹ vp of Ad5/35-HIV vector at 0 and 8 weeks. PBMCs were isolated at weeks 0, 2, 8, and 12. HIV-specific Ab titers were measured in triplicate by ELISA (E) (●, ■) and neutralizing assay (N) (○, □) (b). The detecting limitation of the neutralizing assay was 100 ND₅₀/ml. PBMCs were stimulated with HIV Env gp120 protein, and the number of cells activated to secrete IFN- γ was determined in triple wells by ELISPOT (c). SFC: spot-forming cells.

within 2 weeks of immunization (Figure 3b). The animals were boosted at 8 weeks. After 4 weeks, titers in excess of 1:50 000 were achieved. Similar results were observed in neutralizing Ab. A increase in the number of HIV-specific IFN- γ -secreting T cells was also detected in the peripheral blood mononuclear cells (PBMCs) (Figure 3c). Boosting with Ad5/35-HIV vector further increased this T-cell response.

Effect of pre-existing immunity on vaccination

To evaluate the effect of the anti-Ad5 neutralization Ab (found in 60% of the adult human population)¹⁷ on the Ad5/35 vector, the infectivity of the vector was examined after incubation with serially diluted serum from subjects with high titers of anti-Ad5 Abs (anti-Ad5 neutralizing titer=1:64). As shown in Figure 4, the human antisera had 1:8 anti-Ad5/35 neutralizing titer and normal human sera against either Ad5 or Ad5/35 vector was less than 1:4. The sera derived from Ad5/35-HIV-immunized monkeys showed two-fold higher neutralizing Ab titer against Ad5/35 vector than Ad5 vector.

To examine the effect of pre-existing anti-Ad5 immunity on the activity of the Ad5/35 vector *in vivo*, mice were injected i.m. with 10¹⁰ or 10¹¹ vp of Ad5-Luc. After 8 weeks, these animals were immunized with 10¹⁰ vp of Ad5-HIV or Ad5/35-HIV. The HIV-specific responses were detected by the tetramer assay 2 weeks after immunization. Although pre-existing immunity to Ad5 reduced the immune response elicited by both vectors, Ad5/35-HIV was significantly more immunogenic than Ad5-HIV ($P < 0.05$; Figure 4).

Discussion

This study demonstrates that an Ad5/35-HIV vector vaccine induces strong cellular and humoral immune responses with minimal toxicity in mice and rhesus macaques. A prime-boost strategy involving the DNA-HIV vaccine and the Ad5/35-HIV vector generated protective immunity against viral infection in mice.

A widely used HIV vaccine should have high immunogenicity, low cost of production, and low or no pathogenicity. Replication-defective Ad5 is one of the best vectors for HIV vaccine development. Vaccination

with recombinant Ad5 has achieved great success in inducing protection against virus infection in several animal models.^{4,15,33} Ad5 is well characterized, and its subclinical disease association in humans is well known.^{34,35} However, a majority of the human population (more than 60%) is infected with the Ad5 virus.^{17,36,37} The neutralizing Ab and the cellular immune responses against the Ad5 fiber and capsid may reduce the efficacy of the Ad5 vector when it is used in a clinical trial.^{37,38} The switching of the Ad serotypes^{37,39} and the use of animal Ads^{40–44} enables the partial bypass of the pre-existing immune responses to Ad5 viruses. However, there are a few drawbacks: lack of knowledge regarding the biology of these viruses, including tropism on human cells; potential difficulties in manufacturing; and the possibility of *in vivo* recombination with other human viruses leading to unknown diseases. Animal Ad vectors may induce the antigen-specific responses as strongly as Ad5 in animal models.⁴³ However, their immunogenicity in humans is still unknown. This study used a chimera Ad5 vector with Ad35 fiber, which relates with cell tropism. The Ad5/35, similar to Ad5, has a high productive titer in tissue culture cells, because it is commonly known that human subgroup B Ads, such as Ad5, have a considerably higher titer as compared with

other subgroup viruses, including Ad35. Nevertheless, the virus displayed the cell tropism of Ad35. We explored the immunogenicity of the Ad5/35 vector encoding HIV Env gene in both mice and non-human primates. The results indicate that the Ad5/35-HIV vector elicited strong HIV-specific humoral and cellular immune responses that conferred protective immunity (Table 1 and Figure 3b and c). Coupled with the evidence that an Ad5/35 vector transduces human dendritic cells more efficiently as compared with an Ad5 vector,^{18,21,26,27} these findings suggest that the Ad5/35-HIV vector is a promising candidate for human trials.

Another concern regarding the use of the Ad5 vector in clinical trials is its strong tropism to hepatocytes that is caused by the high expression of CAR in the hepatocytes. Our experiments showed a high expression of the Ad5 vector in the liver in both mice and non-human primates after i.m. administration, but not of the Ad5/35 vector (Figure 1). In contrast to Ad5 vector, Ad/35 vector did not elevate the levels of serum markers (GOT/GPT) of hepatotoxicity and key proinflammatory cytokines (IFN- γ and IL-6) in mice (Figure 1b and c). These results demonstrate that, as a vaccine vector, Ad5/35 vector is safer than Ad5 vector. However, low expression of Ad5/35 vector in monkey liver was still detected after i.m. administration of Ad5/35-Luc vector to monkeys (Figure 3a). It may have resulted from low capacity of Ad5/35 to infect liver nonparenchymal cells, but not liver parenchymal cells.²⁰ Interestingly, we found a certain magnitude of Ad5 vector expression in the posterior cerebrum and cerebellum of monkeys; however, the Ad5/35 vector was not expressed (Figure 4a). Nevertheless, in the present experiment, we could not precisely define the location of the Ad5-infected cells or determine whether the infection potentially causes local inflammation or toxicity. However, potential brain infection after Ad5 vector administration is a safety concern because intranasal administration of the Ad5 vector has been reported to result in the infection of the central nervous system.⁴⁵

In this study, the effect of pre-existing anti-Ad5 immunity on the Ad5/35 vector was explored along with several immunization protocols as follows. (1) Both *in vitro* and *in vivo* studies demonstrate that the Ad5/35 vector is significantly less susceptible to neutralization by anti-Ad5 Abs as compared with a conventional Ad5 vector (Table 2 and Figure 4). The administration or infection of Ad can induce immune responses against the Ad hexon, penton, and fiber antigens. The exchange of fiber can partially reduce the inhibition of the

Figure 4 Effect of pre-existing antiviral immunity. Ad5-Luc and Ad5/35-Luc vectors were incubated with an equal volume of serially diluted normal human sera (No. 2, anti-Ad5 neutralization titer <1:4), human antisera (No. 2, anti-Ad5 neutralization titer = 64), or monkey antisera from Figure 3b and c (No. 2) in triplicate and were subsequently added to infected Vero cells in a 96-well plate at 10^7 vp/well. The luciferase activity was measured 48 h after infection. The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background. Average and standard deviations for three independent experiments are shown. *Mean values are significantly different between groups.

Table 2 Effect of pre-existing antiviral immunity

	Prime	Anti-Ad5 neutralizing Ab titer	Boost	Tetramer assay (%)
Control	Non	<1:4	Ad5-HIV (10^{10} vp/mouse)	4.8 ± 0.2
Low dose	Ad5-Luc (10^{10} vp/mouse)	1:102	Ad5/35-HIV (10^{10} vp/mouse)	5.1 ± 0.2
			Ad5-HIV (10^{10} vp/mouse)	2.3 ± 0.4 }
High dose	Ad5-Luc (10^{11} vp/mouse)	1:248	Ad5/35-HIV (10^{10} vp/mouse)	4.6 ± 0.6 }
			Ad5-HIV (10^{10} vp/mouse)	0.5 ± 0.1 }
			Ad5/35-HIV (10^{10} vp/mouse)	2.6 ± 0.4 }

After 8 weeks, naive mice or mice pretreated with 10^{10} or 10^{11} vp of Ad5-Luc vector (6 mice/group) were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV vector. At the time of vaccination, anti-Ad5 neutralizing titers were measured in Ad5-Luc-treated mice. At 2 weeks after vaccination, the HIV-specific responses were detected by an HIV-specific tetramer assay. *Mean values are significantly different between the groups.

pre-existing immunity against the parent Ads (Table 2 and Figure 4). The exchange of other genes, including hexon and penton, may further reduce the inhibition of pre-existing anti-Ad5 immunity. (2) We also explored the immune responses by using the same vector for prime/boost. When the mice were immunized i.m. with 10^{10} vp of Ad5/35-HIV 1–3 times at 4-week intervals, the HIV-specific cell-mediated immune responses were detected by the tetramer assay. An increased response was observed after the second immunization but not after the third immunization (data not shown). High anti-Ad5/35 neutralizing Ab after second immunization may block the Ad5/35-HIV infection (Figure 4). These results are in agreement with the data from our study on monkeys (Figure 3b and c) and with that from studies by other groups,^{33,43} and other virus such as MVA vector may be applicable for further boost if high immune responses are required. The DNA-HIV vaccine prime/Ad5/35-HIV vector boost regimen greatly increased HIV-specific cell-mediated immune responses (Table 1 and Figure 3b and c), but not the humoral immune response (Figure 2c). This suggests that DNA vaccination enhances adenoviral recombinant-induced cell-mediated immunity rather than humoral immunity, as described by other groups.^{4,38} Furthermore, DNA vaccine priming can reduce the humoral response to the adenoviral antigens and can counterbalance the impaired B-cell response to the antigen expressed by the adenoviral recombinant in mice that are preimmune to Ad.⁴⁶ This DNA prime/Ad5/35 boost regimen might be highly suitable for use in humans with previous exposure to the Ad5 virus. (3) To examine the immunogenicity and protective immunity of the Ad5/35 vector, we used HIV-1 IIIB in this study, because the strain has been well studied and we can compare our data from the new vector with that from other studies. For the clinical trial, we have constructed DNA vaccine and Ad5/5 vector expressing ENV and GAG of a Clade C HIV-1 isolate and similar results with HIV-1 IIIB strain were obtained in a mouse model (manuscript in preparation).

Considered together, using Ad5/35 vector, we developed an HIV vaccine with a higher immunogenicity and low pathogenicity. The Ad5/35-HIV vaccine induced strong HIV-specific immune responses in both BALB/c mice and rhesus monkeys. Priming with a DNA-HIV vaccine followed by Ad5/35-HIV boosting yielded protection against viral infection in mice. The Ad5/35 vector may be a promising vaccine for human clinical trial.

Materials and methods

Recombinant vectors

E1,E3-deletion, replication-defective recombinant viruses were constructed with an Ad generation kit (Avior Therapeutics Inc., Seattle, WA, USA).²¹ Briefly, a 5.2k bp *Sall/PstI* fragment containing CAG promoter-HIV_{IIIB} rev/env gp160-polyA was isolated from pCAGrev/env.²⁸ A shuttle plasmid (pLHSP) containing Ad5 positions 22–342, Ad5 3523–5790, *Escherichia coli ori*, and ampicillin-resistant gene was obtained from Avior Therapeutics Inc. (Seattle, WA, USA). The 5.2k bp blunted fragment was subcloned into blunted *EcoRI* site

of pLHSP plasmid vector to generate pLHSP-HIV shuttle plasmid. The pLHSP-HIV shuttle plasmid was linearized with *PacI* and transfected with E1,E3-deletion, chimeric Ad5 or 5/35 genome to human embryonic kidney (HEK293) cells using calcium precipitation method to generate recombinant virus, Ad5-HIV and Ad5/35-HIV, respectively. The recombinant virus (Ad5/35-HIV, Ad5-HIV) was propagated in HEK293 cells and purified by two repetitions of the CsCl methods described elsewhere.⁴⁷ The total concentration of virions in each preparation was calculated from the optical density at 260 nm (OD_{260}), using the formula $1 OD_{260} = 1 \times 10^{12}$ vp/ml. The HIV_{BH8} gp160-expressing replication-competent vaccinia virus (WR strain, vPE16; HIV_{BH8} gp160 has 97.32% amino-acid homology with HIV_{IIIB} gp160) was obtained from the AIDS Research and Reagent Program, National Institutes of Health, Rockville, MD, USA (Cat. No. 362). The vPE16 vectors were propagated in CV1 cells. The Ad5-Luc and Ad5/35-Luc vectors expressing luciferase coding gene were described previously.²⁶ The DNA-HIV vaccine (pCAGrev/env) containing HIV_{IIIB} rev and env genes has been previously reported.²⁸

Biodistribution of Ad5 and Ad5/35 vectors in vivo

The experiment was performed as previously described.^{48,49} In brief, the Ad5-Luc or Ad5/35-Luc vectors (10^{11} vp/mouse) were injected i.m. into BALB/c mice. On days 3 and 10, the mice were anesthetized with a 2% isoflurane/air mixture, and a single dose of 150 mg/kg luciferin in normal saline was administered intraperitoneally. The CCD images were obtained using a cooled *in vivo* imaging system (IVIS) CCD camera (Xenogen, Alameda, CA, USA) and analyzed. To study the viral biodistribution in primates, two rhesus monkeys (2 years old, male) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc. At 3 days after administration, the luciferase activity was detected in the monkey organs (brain, liver, kidney, spleen, heart, and lung) using the Luciferase Assay Systems (Promega, Madison, WI, USA). Serum GOT and serum GPT were measured in mouse or monkey sera at the Kitayama-Rabesu Institute (Ina, Nagano, Japan). The concentration of serum IFN- γ and IL-6 was measured using the IFN- γ and IL-6 ELISA kits (Biosource, Camarillo, CA, USA), respectively, according to the manufacturer's protocol.

Animal immunization

Female BALB/c mice (8-week-old; H-2D^d) were purchased from Japan SLC Inc., Shizuoka, Shizuoka-ken, Japan. The mice were immunized with an i.m. injection of 100 μ g of pCAGrev/env or pCAGempty plasmid DNA in phosphate-buffered saline (PBS) at 0, 1, and 2 weeks and were boosted with 10^{10} vp of Ad5/35-HIV or Ad5/35-Luc vector at week 3. For the time-course study, the mice were administered a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector per mouse. To study the effect of pre-existing antiviral immunity on vaccination, the mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc vector and then immunized 8 weeks later with 10^{10} vp of Ad5-HIV vector or Ad5/35-HIV vector. For rhesus monkey immunization, 10^{11} vp of Ad5/35-HIV vector was injected i.m. into two rhesus monkeys (2 years old, male) at weeks 0 and 8.

Intracellular cytokine staining assay

IFN- γ -secreting CD8⁺ T cells were detected by the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus kit, PharMingen, San Diego, CA, USA). In brief, lymphocytes were isolated from the mouse spleen. A single cell suspension was incubated with 10 μ g/ml of the HIV V3 peptide (NNTRKRIQRGP GRAFVTIGKIGN) for 24 h at 37°C. At 2 h before the end of incubation, 1 μ g/ml of GolgiPlug was added. The cells were washed with staining buffer (3% fetal calf serum (FCS), 0.1% sodium azide (NaN₃) in PBS), blocked with 4% normal mouse sera, and stained with phycoerythrin (PE)-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen). The cells were then suspended in 250 μ l of Cytofix/CytoPerm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anti-mouse IFN- γ Ab conjugated with fluorescein isothiocyanate (FITC) (PharMingen) at 4°C for 30 min, followed by flow cytometric analysis.

Tetramer assay

The tetramer assay used a PE-conjugated H-2D^d/p18 tetramer (RGPGRFVTI), as previously described.²⁸ In brief, splenocytes were isolated from mice and incubated for 30 min at 4°C with 4% normal mouse serum in PBS. Cells were stained with FITC-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen) for 30 min at 4°C. After washing twice with the staining buffer (3% FCS, 0.1% NaN₃ in PBS), the cells were incubated with the tetramer reagent for 15 min at 37°C, followed by flow cytometric analysis (Becton Dickinson).

Recombinant vaccinia virus used for the challenge study

Using vPE16 vaccinia virus, the virus challenge experiment was performed as described previously.²⁸ Vaccinated female mice were intraperitoneally challenged with 10⁸ PFU of vaccinia virus vPE16 at 2 or 7 weeks after the final immunization. At 6 days after challenge, the mice were killed, their ovaries were sonicated, and the vPE16 titer was determined by serial 10-fold dilution on a plate of CV1 cells. Infected cells were detected by staining with crystal violet and plaques were counted at each dilution.

Detection of HIV-1-specific Ab

The HIV-1-specific Ab was detected by the Western blotting method and the enzyme-linked immunosorbent assay (ELISA). By Western blotting method, the HIV envelope glycoprotein gp160-coated membrane from the New Lav Blot 1 kit (Bio-Rad, Marnes-la-Coquette, France) was incubated with a 100-fold dilution of mouse serum followed by an affinity-purified horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (ICN Pharmaceuticals Inc., OH, USA). HIV gp160 protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech).

ELISA was performed as described elsewhere.¹⁰ To summarize, 96-well microtiter plates were coated with 10 μ g/ml of HIV_{III} gp120 protein (donated by AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They

were then treated with 100 μ l of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified HRP-labeled anti-mouse Ab or anti-monkey Ab (both from Sigma). The mean Ab titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 s.d.

The HIV-specific neutralizing titer of immune mice or monkeys was also measured. The serially diluted antisera were incubated with 200–300 blue spot-forming units (BFU) of HIV-1_{LAI} at 37°C for 1 h. The mixture was incubated with confluent MAGIC5 cells (from Dr Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan),^{50,51} Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 0.2 mg/ml of G418 in a 96-well plate at 37°C for 2 days. The cells were fixed with fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min and stained with staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, 0.4 mg/ml X-gal in PBS) at 37°C for 18–24 h. The staining was stopped by removing the staining solution and the cells were washed twice with PBS. The blue spot in each well was counted after the staining, and the neutralizing titer was calculated as (1 – (% infection/% infection of control wells)) $\times$ 100. The 50% neutralization dose (ND₅₀) is defined as the concentration of the Ab that reduced the number of infected cells by 50%. The detecting limitation of the assay was 100 ND₅₀/ml.

ELISPOT assay

The frequency of HIV-specific IFN- γ -secreting cells in monkeys was determined using an ELISPOT assay kit (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's manual. In brief, 2 $\times$ 10⁵ monkey PBMCs were stimulated in triplicate wells with 1 μ g/ml of the HIV_{III} gp120 protein for 16 h at 37°C. Nonstimulated cells were used to assess the background. The cells were transferred to an anti-IFN- γ Ab-coated 96-well plate and incubated for 5 h at 37°C. The cells were removed and 200 μ l/well of ice-cold deionized water was added to lyse the remaining PBMCs. Subsequently, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with biotinylated anti-IFN- γ Ab for 1 h at 37°C. After 10 washings with PBS-T, 50 μ l of gold-labeled anti-biotin Ab was added and incubated for 1 h at 37°C. The plate was washed 10 times with PBS-T, and 30 ml of activator solutions was added. The plate was incubated in the dark for 30 min at room temperature to develop spot formations. After 30 min incubation, the plate was washed with deionized water and air-dried; spots were counted by a computer-assisted video image analysis. The results were expressed as spot-forming cells (SFC) per million cells.

Ad-specific neutralizing assay

Ad5-Luc or Ad5/35-Luc vector (10⁷ vp) was incubated with an equal volume of serially diluted normal human sera (anti-Ad5 neutralizing titer <1:4), human antisera (anti-Ad5 neutralizing titer = 1:64), or monkey sera (at weeks 0, 2, 8, and 12 after immunization with Ad5/35-HIV vector) at 37°C for 2 h. The mixture was incubated with confluent Vero cells in a 96-well plate at 37°C for an additional 48 h. The luciferase activity was detected by Luciferase Assay Systems (Promega, Madison, WI, USA).

The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background.

Data analysis

All values were expressed as means $\pm$ standard error (s.e.). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at $P < 0.05$ in the statistical analysis.

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