compromised patients with massive ascites are excellent candidates for neoadjuvant chemotherapy, as it avoids postoperative fluid shifts, which can stress the cardiovascular integrity of these patients.

Some patients who are receiving long-term maintenance or even palliative chemotherapy continue to have stable disease beyond the time that the tumour cells would have been expected to develop drug resistance. A closer approximation to antiangiogenic scheduling may explain the improved outcome of empiric treatment of 'slower growing' human cancer using continuous infusion 5-fluorouracil in breast cancer and colorectal cancer [116-118], weekly paclitaxel in recurrent ovarian cancer and pretreated solid tumours [119,120], and daily oral etoposide in non-small cell lung cancer and in supratentorial malignant glioma in children [121-123]. If this hypothesis proves generalisable, it may suggest which agents and on which schedules chemotherapy may be best combined with more specific angiogenesis inhibitors for improved antiangiogenic and anticancer efficacy.

Molecular-targeted therapy could be considered, using novel agents capable of homing in on a single molecular target that is overexpressed in cancer cells, but lacking in normal cells. These gene- and target-based therapies are able to become new treatment strategies with less toxicity than conventional treatment modalities. The application of these new treatment strategies to ovarian cancer is still in its infancy. Recently, it has been reported that in a stringent preclinical model, standard chemotherapy followed by a novel maintenance regimen resulted in disruption of pericyte support by plasmid-derived growth factor receptor and subsequent metronomic chemotherapy and/or VEGF receptor inhibitors target consequently sensitised endothelial cells, collectively destabilising pre-existing tumour vasculature and inhibiting ongoing angiogenesis [124]. This exciting translational work requires many disciplines and organisations to work together internationally to accelerate patient benefit.

8. Expert opinion

Poor prognosis of ovarian cancer compared with uterine cervical cancer and endometrial cancer is due to incapability of early diagnosis. Ovarian cancer presents at a late clinical stage in > 75% of patients, and is associated with a 5-year survival of 35% in this population. By contrast, the 5-year survival for patients with Stage I ovarian cancer is > 90%, and most patients are cured of their disease by surgery alone. Therefore, increasing the number of women diagnosed with Stage I disease should have a direct effect on the mortality and economics of this cancer without the need to change surgical or chemotherapeutic approaches. A global view of the proteome would enhance the possibility of identifying protein signatures for ovarian cancer. Surface-enhanced laser desorption and ionisation with time of flight detection (SELDI-TOF) spectral analysis was linked with a high-order analytical approach using samples from women with a known diagnosis to define an optimum discriminatory proteomic pattern. This pattern was used to predict the identity of masked samples from unaffected women, women with early and late-stage ovarian cancer, and women with benign disorders. Following proper validation, serum proteomic pattern analysis might be ultimately applied in medical screening clinics, as a supplement to the diagnostic workup and evaluation. A negative value, if the sensitivity remains at 100% on further trials, could be used for reassurance, whereas a positive value may be sufficient to warrant further evaluation. An important future goal is confirmation of sensitivity and specificity for the prospective detection of Stage I ovarian cancer in trials of high- and low-risk women, respectively. It will be important to design the trial to evaluate the efficacy of the approach as a standalone approach or one to be combined with current screening options. Such trials should benefit patients, particularly ovarian cancer patients.

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Application of Expression Genomics for Predicting Treatment Response in Cancer

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ABSTRACT: **During tumor progression, multiple genetic changes in the genome vastly alter the transcriptomes of cancers. Some of these changes, including the mutations of various growth regulatory genes as well as alterations in the transcription of a large number of genes, may lead to resistance to treatment. Therefore, capturing such genomic information of the tumors would enable a physician to decide on the course of treatment options clinically available. Currently, it is still not feasible to identify all the genetic mutations that have occurred in a patient's cancer genome. However, the advent of DNA microarray coupled with the completion of the human genome sequence and the identification of all its genes, have made possible genome-wide gene expression profiling of the cancer genome. In this review, we will focus on the application of expression genomics for identifying signature gene expression profiles in primary cancers to predict response to either radio- or chemotherapy. We envision that transcription profiling of the cancer genomes ultimately will not only reveal how altered gene expression results in resistance to treatment, but also be exploited for predicting and personalizing cancer therapy.**

KEYWORDS: **microarray; expression genomics; transcription profiling; drug resistance; genomic medicine; expression profiling; radiation therapy; cancer; chemotherapy; predictive; personalized**

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RESISTANCE TO CANCER TREATMENT

Besides surgery, cancer treatments are largely limited to radio- and chemotherapy. The ability of tumor cells to evade killing by either radio- or chemotherapy leads to treatment failure, and the resulting failure to respond to these treatment modalities suggest that tumor cells are either intrinsically resistant to therapy or have acquired the resistance during treatment.[1] Resistance to radiotherapy may have resulted from altered modulation of the complex DNA repair pathways that normally protect cells from the damage inflicted by ionizing radiation as well as from DNA-damaging agents.[2,3] More remarkably, tumor cells seem to harbor resistance or be capable of developing resistance to virtually every drug used in cancer chemotherapy in the clinic, thus further compounding the limited success of these treatment modalities.

ABCs OF TUMOR RESISTANCE

One of the most important mechanisms of resistance to cancer chemotherapy is the overexpression of P-glycoprotein, encoded by the *ABCB1* gene in human, in which cancer cells exhibit broad spectrum resistance to a variety of anticancer drugs with different chemical structures and properties, and mechanisms of action.[4] The discovery of P-glycoprotein almost 30 years ago[5,6] and the subsequent identification of its superfamily of ATP-binding cassette (ABC) transporters that confer multidrug resistance to tumor cells in various cell culture as well as tumor xenograft models,[4,7] raised the possibility that overexpression of this class of transmembrane proteins is sufficient and may account for the observed clinical multidrug resistance in cancers.

Since then, noncytotoxic small molecules termed chemosensitizers that compete with anticancer drugs as substrate for binding to the ABC-transporters were developed as a rational approach to circumvent multidrug resistance, so that a net increase in intracellular accumulation of chemotherapeutic agents can be achieved in cancer cells.[8–10] Consequently, the use of these chemosensitizers including verapamil and cyclosporine A in conjunction with chemotherapy, demonstrated the ability to reverse multidrug resistance in cell culture and tumor xenograft models.[11] In limited clinical study, use of some chemosensitizers or P-glycoprotein modulators seemed to enhance drug accumulation in P-glycoprotein–expressing tumors and normal tissues in patients using Sestamibi retention imaging.[12] However, results from clinical studies to reverse multidrug resistance were less encouraging, as only a few patients with solid tumors benefited from the concomitant use of chemosensitizers with anticancer agents during chemotherapy.[13] In contrast, given the combination of chemosensitizers and the respective treatment regimens specific for each malignancy, patients with hematological cancers such as leukemia, lymphoma, and multiple myeloma showed a mix of response, though not spectacular, but generally with better overall outcome compared to patients with solid tumors.[13,14]

These results further raise questions whether the ABC-superfamily of transporters were the culprits of treatment resistance and also suggest that additional tumor-specific cellular factors might contribute to drug resistance in cancer. Alternatively, the modestly positive outcome observed in some hematological cancers also lends support to the notion that the ABC-transporters may conceivably have a more significant role in multidrug resistance in this group of cancers than in solid tumors.[15]

TUMOR TRANSCRIPTOME AND TREATMENT RESISTANCE

As is the multifactorial nature of the development of cancer, it is clear that multidrug resistance in cancer is not attributable to overexpression of the ABC-superfamily of transporters alone.[16,17] It is well documented that during tumorigenesis, a large number of growth regulatory genes including oncogenes and tumor suppressor genes are genetically altered.[18–20] Through yet unknown mechanisms, altered expressions of some of these genes including MYC, ERBB2, TP53, BRCA1 and 2, and others, are known to be associated with drug resistance.[15,21–23] Some of these affected oncogenes and tumor suppressor genes are transcription factors, which regulate the expression of a large number of downstream target genes. Hence, the development of drug resistance during tumorigenesis may be due to the vastly altered transcriptomes of cancers that are accompanied by changes in the expressions of a large number of genes,[24] and some of whose expression may contribute to drug resistance by virtue of their extended normal cellular functions in transport,[7] metabolism,[25] signaling,[26] DNA repair, and death and survival.[27,28] It is also evident that the development of resistance even to a single anticancer agent can be attributed to multiple cellular factors associated with multiple genetic changes which result in the expression of their corresponding genes that confer multidrug resistance.[24]

These and other mechanisms of drug resistance, as well as the ABC-transporter–mediated drug efflux mechanism, are all derived from studies in cell culture systems and some *in vivo* mouse models. Though correlative studies have been examined for some of these markers in cancer samples from patients, [12,29] however, their roles in conferring drug resistance in human cancers have not been fully validated.

Cancer treatment whether by radio- or chemotherapy is often empirical owing to the inability to predict the individual's response to these treatment modalities. Clearly, this is further confounded by the multiple cellular factors, whose aberrant expression contributes to drug resistance. Therefore, monitoring the expression of these genes that have role in drug resistance in cancer will not only provide insights into the mechanisms of resistance, but also ultimately help guide and improve cancer treatment.[16]

EXPRESSION GENOMICS AND TREATMENT RESISTANCE

Completion of the human genome sequence is an important advancement in biomedical research that has led to the identification of all the genes in the genome.[30,31] Efforts in functional genomics are currently ongoing to annotate all these genes. These developments coupled with the discovery of DNA microarray,[32,33] which enables genome-wide gene expression profiling in a single setting, may help to identify all the genes involved in drug resistance.

Since treatment failure in cancer therapy is multifactorial, for as long as cancer treatment continues to rely on intervention using either small molecules or radiation, therefore, the application of expression genomics in cancers for the identification of signature gene expression profiles that contribute to treatment resistance may be exploited for predicting *a priori* the susceptibility of tumors to various treatment algorithms.[16] Such an approach will enable the personalization of treatment that may yield the best response.

Gene expression profiling is increasingly applied in human clinical specimens.[34,35] It is anticipated that expression genomic data from human tissue specimens in combination with *in vitro* laboratory data will be a robust resource for *in silico* systems biology modelin,[36–38] and for querying and predicting response to treatment, as well as outcome and susceptibility to toxicity. It is also envisioned that, the combination of expression genomic data and other genomic information including single nucleotide polymorphisms (SNPs), genomic sequence, and proteomic profile will be the cornerstone for the practice of genomic medicine, which would enable a physician to perform molecular diagnosis, and to prescribe the right drug, at the right dose, for the right patient, in the not too distant future.

PREDICTIVE CANCER TREATMENT

Cancer treatment is currently a one-size-fits-all approach, which does not take into account whether patients may or may not respond to the treatment modality. Currently, there are no clinical markers for physicians to predict, *a priori*, whether patients would respond to treatment. The advent of genomics promises to revolutionize the practice of medicine, as we know it. We show here two examples of proof-of-principle pilot studies on the application of transcription profiling of primary cancer samples from patients and the feasibility of predicting treatment response based on their signature gene expression information.[39,40] This new approach in predictive medicine promises to offer a much-needed avenue in cancer treatment that will obviate the unpredictable trial-and-error and one-size-fits-all approaches of clinical medicine today. As a result, patients will be spared from unnecessary treatment and exposure to their associated toxic side effects.

Predicting Response to Radiotherapy

In this example, we show the combined approaches of expression genomics and pattern recognition algorithm for the analysis of human cancer specimens that led to the prediction of treatment response. We asked whether a voice/speech pattern recognition algorithm used in the telecommunication industry can be adapted for the analysis of expression genomic data and for the molecular classification of cervical cancer and the prediction of patients' response to radiotherapy.[40]

We applied a statistical approach to pattern recognition in this study,[41,42] A combination of linear discriminant analysis for training set, feature extraction by Bayesian parameter estimation, decision by nearest neighbor classification, and classifier performance evaluation were performed with the gene expression data. Patients were selected from each category, "sensitive" and "resistant," for training and feature selection. The process was iterated until a signature gene expression pattern was obtained and used for further testing with samples set aside from each category that were not used for training.

It is also noteworthy that we used patients' primary cervical cancer samples, taken at the time of diagnosis and before radiotherapy, to obtain their transcription profiles. Gene expression profiling was conducted with cDNA microarrays containing 10,692 elements corresponding to all human transcripts, of which approximately 7,000 of elements correspond to known genes and the remainders to unknown tran-

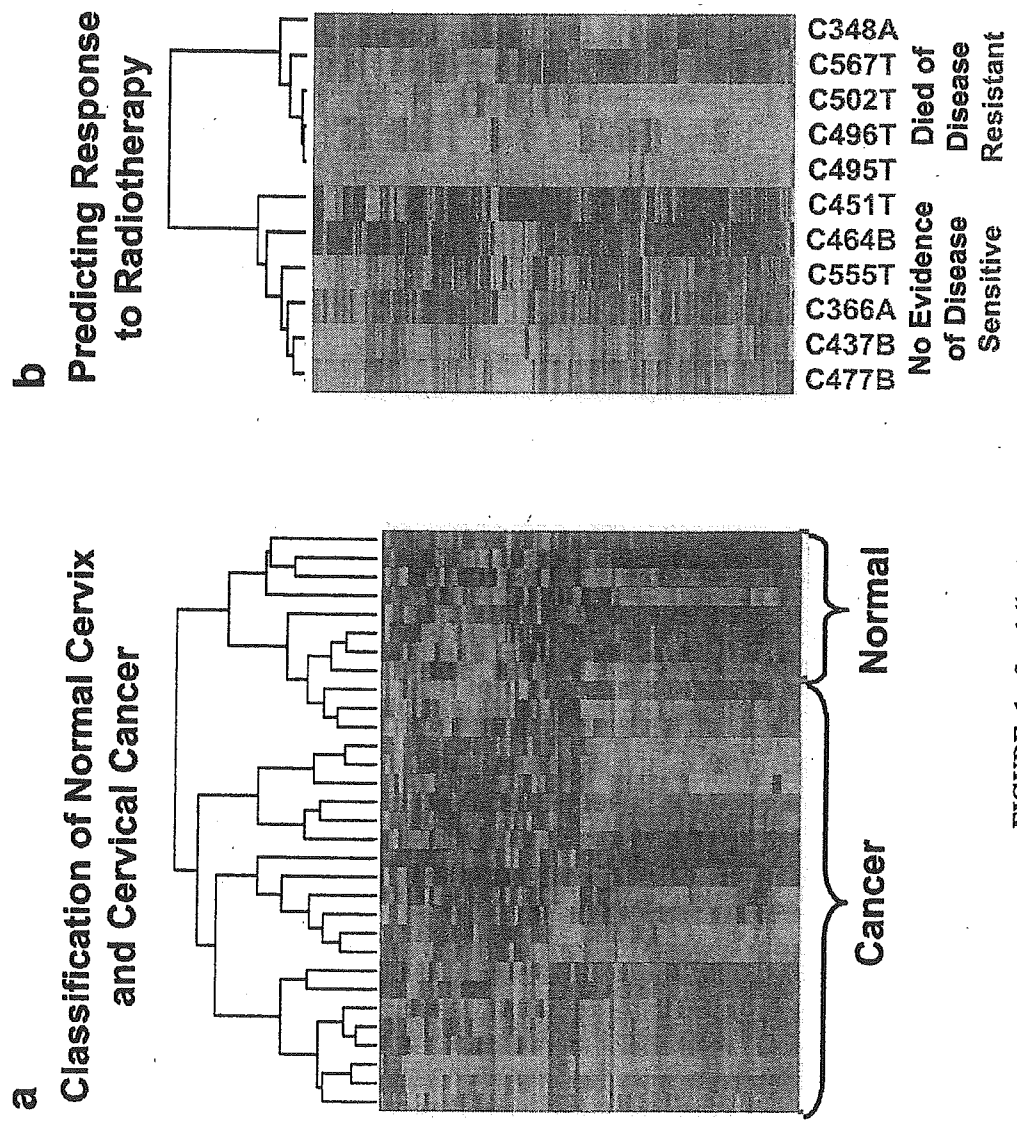

FIGURE 1. *See following page for legend.*

scripts or genes. We first assessed the suitability of voice/speech pattern recognition algorithm for gene expression data analysis by testing whether the process can distinguish the expression profiles of normal cervices derived from hysterectomy from cervical cancer samples. A pattern recognition algorithm classified all the samples correctly as either normal or cancer based on their gene expression data (FIG. 1a).

Cervical cancer is pathologically staged into four stages according to the FIGO system. We next showed by pattern recognition that a subset of the cancer samples in our study can be correctly classified into either Stage IB or Stage IIB. Together, these and the above results suggest that the voice/speech pattern recognition approach can be used for gene expression data analysis.

The patients in our study were given radiotherapy as primary treatment and were further stratified into two groups: radiotherapeutic sensitive (responder) or resistance (nonresponder). The clinical data indicated that patients who did not respond to radiotherapy had a mean survival time of 22.2 months, while the group who responded had a mean survival time of 66.5 months. Voice/speech pattern recognition analysis correctly classified the samples as either radiotherapy-sensitive or radiotherapy-resistant based on their gene expression profiles (FIG. 1b).

By this analysis, the signature expression profile correctly predicted the patients' response to radiotherapy. These signature predictors were made up of a set of genes with diverse physiologic functions, which include transcription factors, proteins with cytoskeletal, membrane and cell structural functions. Bioinformatics searches of these predictors did not reveal insights into their roles in modulating cellular sensitivity or resistance to radiation therapy. In addition, further analysis of a larger cohort of cervical cancer patients treated by radiotherapy will be required to confirm the signature predictors and to determine the role of these genes in modulating response to radiotherapy.

Predicting Response to Chemotherapy

In ovarian cancer, emergence of drug resistance during chemotherapy results in death for more than 90% of patients with metastatic disease. The poor prognosis has prompted major efforts to identify prognostic factors, improve surgical staging, and develop adjuvant therapies that could improve patient outcome.[43] The above analysis examined cervical cancer patients given only radiation treatment. To test whether treatment response can be predicted in a more complex treatment model, we examined the expression profiles of primary ovarian cancer samples from patients given a platinum-based combination chemotherapeutic regimen of either Cisplatin/ cyclophosphamide, or Carboplatin/taxol, or Cisplatin/taxol. Patients were stratified into two groups based on their chemotherapeutic response.[39]

Pattern recognition analysis of the transcription profiles of the ovarian cancer samples generated a signature predictor set that correctly predicted the response of

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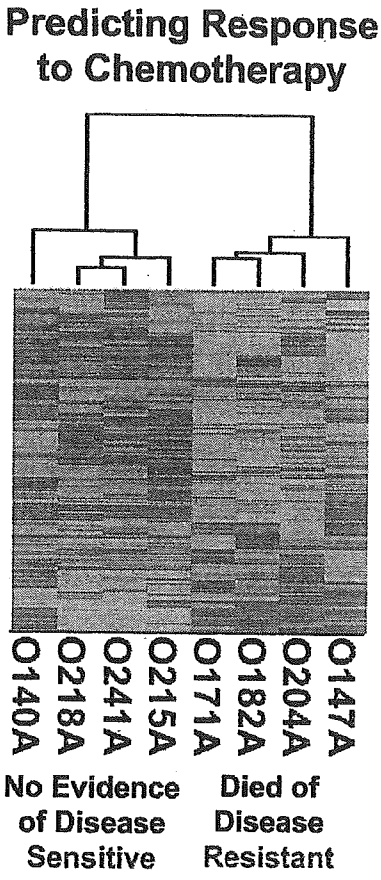
FIGURE 1. Transcription profiling of cervical cancer. **a**, Molecular classification of cervical cancer by DNA microarray. Normal cervix and cervical cancer samples were correctly classified by voice/speech/pattern recognition. **b**, Prediction of radiotherapy response in cervical cancer based on transcription profiles of primary cervical cancer samples obtained at time of diagnosis before therapy was given.



	Treatment
O147A	Cisplatin/Cyclophosphamide
O204A	Cisplatin/Cyclophosphamide
O182A	Cisplatin/Cyclophosphamide
O171A	Cisplatin/Taxol
O215A	Carboplatin/Taxol
O241A	Carboplatin/Taxol
O218A	Cisplatin/Cyclophosphamide
O140A	Cisplatin/Cyclophosphamide

FIGURE 2. Predicting response to chemotherapy in ovarian cancer. Pattern matching algorithm correctly predicted treatment response and the displayed dendrogram showed patients who either responded to or failed the given treatment regimens.

the two groups of patients as either chemotherapy sensitive or chemotherapy resistant (FIG. 2).

In this signature predictor set, we observed an increase in glutathione S-transferase expression, which is well known to confer increased resistance to Cisplatin.[29] Further examination of some of these predictor genes reveals a cluster of transcription or protein factors that bind DNA, whose expression are increased in the cancer samples of patients who did not respond to treatment. This signature expression pattern is reminiscent of the expression profiles found in ovarian cancer cell lines exposed to Cisplatin in temporal gene expression studies (unpublished data), thus suggesting that these nucleic acid binding proteins may have an important biologic role in conferring either intrinsic or acquired drug resistance in ovarian cancer. Whether the transcriptional changes in the expression of these predictor genes are associated with the etiologic causes of drug resistance or treatment failure in ovarian cancer given the above combination platinum-based therapies remains to be determined in future studies with a larger cohort of patients. Nevertheless, these proof-of-principle studies provided support that gene-expression profiling analysis is suited for the molecular classification of cancer and prediction of treatment response.

EPILOGUE

Monitoring gene expression profiles by DNA microarray in human cancer as well as other diseases will be the gateway genomic approach to diagnosis, which will provide pharmacogenomics information for molecular classification of diseases and predict susceptibility to drug toxicity and treatment outcome. Transcription profiling has already been increasingly used in studying human cancer samples for predicting treatment response and disease outcome.[40,44–53] This approach will undoubtedly bring an end to the trial and error and one-size-fits-all medical practice in the treatment of cancer and other human diseases. Patients whose expression profiles exhibit pattern associated with resistance to treatment will be given alternative or supplementary modality of treatment that may result in improved responsiveness or cure, thus personalizing treatment for individuals based on their gene expression patterns.

[*Competing interests statement:* The authors state that they have no competing financial interests.]

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RESEARCH ARTICLE

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

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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.
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Keywords: *Ad5/35 vector; HIV; animal model; vaccine; immune response*

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 Ad5[17] 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 transducted to liver nonparenchymal cells on a level 4–5 log orders lower than Ad5, but not to

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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-tittered 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 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

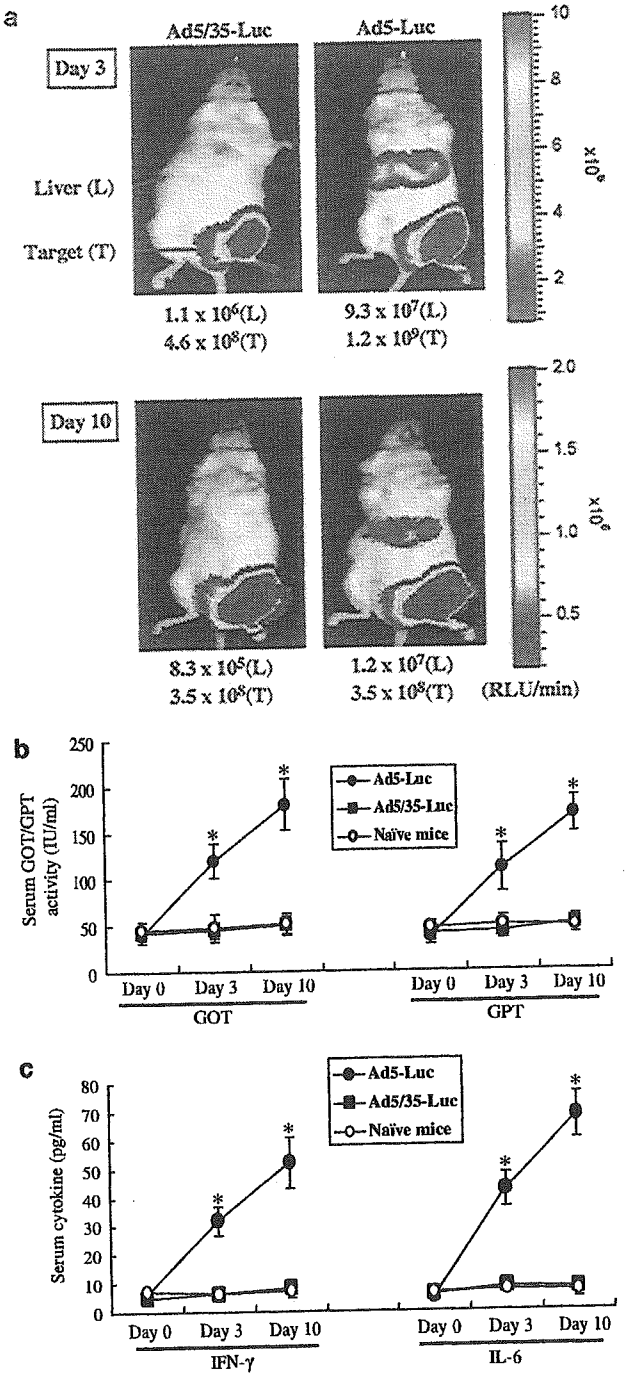

*Figure 1 Biodistribution and safety of Ad vectors. BALB/c mice were injected i.m. with 10^{11} vp of the Ad5-Luc or Ad5/35-Luc vector. (a) Using an IVIS CCD camera, vector distribution was detected after the addition of luciferin (3 mice/group) (expressed in relative light units (RLU)). One of the mice is represented and other mice used show the same pattern. (b) Serum GOP 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.*

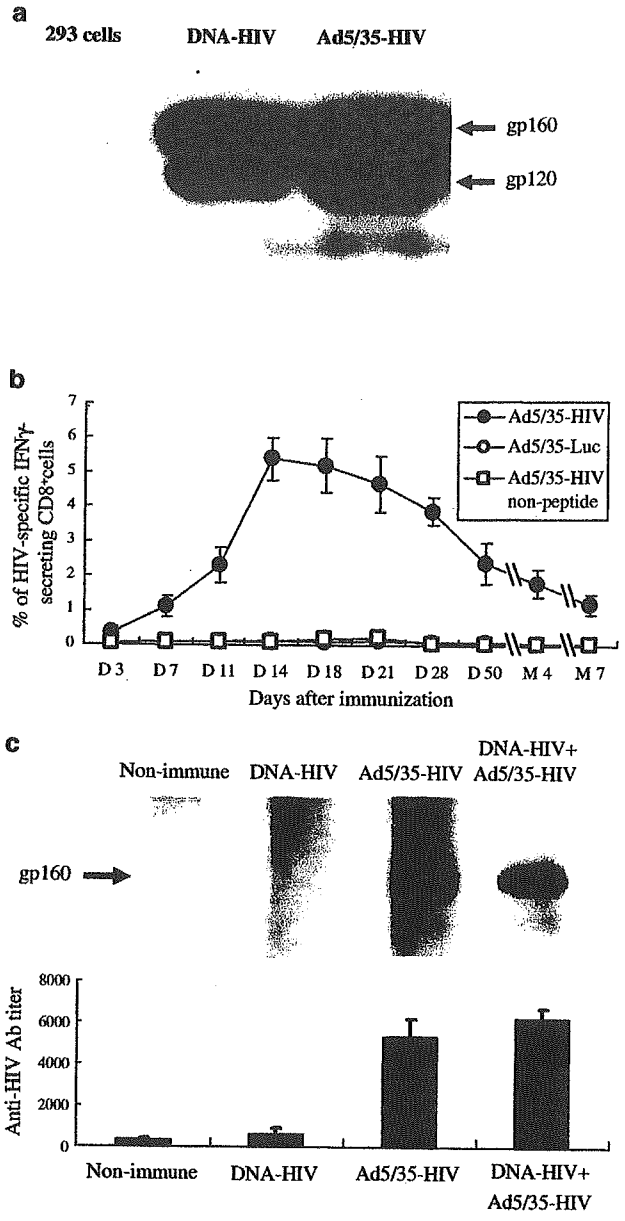

Figure 2 Time course of HIV-specific immune responses in mice. (a) HIV Env protein expression of DNA-HIV vaccine and Ad5/35-HIV on HEK293 cells was confirmed by Western blotting using an HIV Env-specific mAb. (b) Time-course study of cellular immune responses after a single i.m. injection of 10^{10} 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-medicated 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	$8\times10^{8}\pm35$	0.1±0.1	0.1±0.1	$1\times10^{9}\pm65$	0.0±0.0	0.0±0.0
DNA-Empty	0.1±0.1	0.1±0.1	$2\times10^{9}\pm45$	0.0±0.0	0.0±0.0	$8\times10^{8}\pm32$	0.0±0.0	0.0±0.0
Ad5/35-Luc	0.2±0.1	0.2±0.2	$2\times10^{9}\pm25$	0.0±0.0	0.0±0.0	$4\times10^{8}\pm46$	0.0±0.0	0.0±0.0
DNA-HIV	0.7±0.1	1.0±0.3	$6\times10^{6}\pm42$	0.4±0.2	0.6±0.1	$5\times10^{7}\pm51$	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	$2\times10^{3}\pm34$	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.

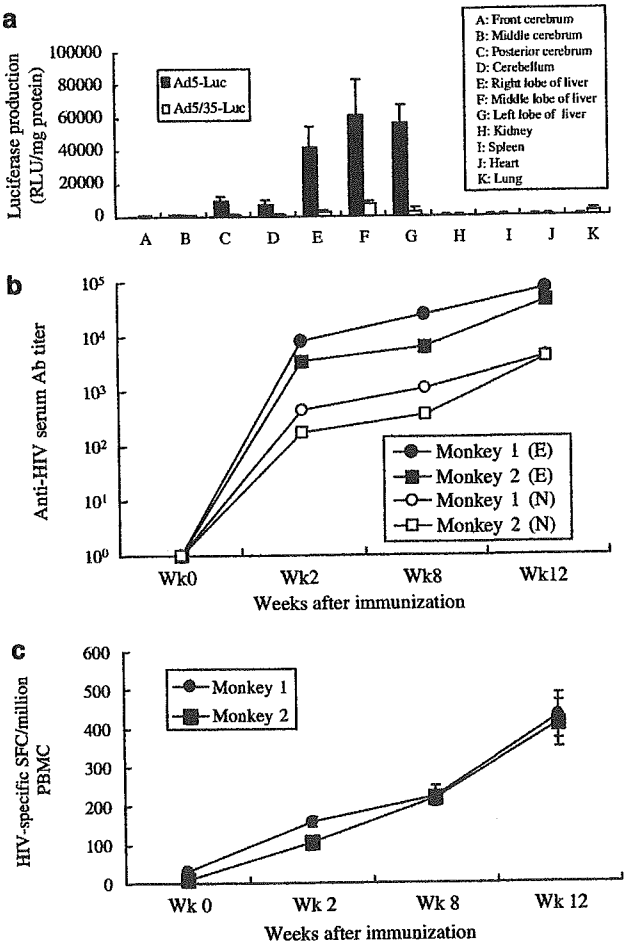

Figure 3 Biodistribution and HIV-specific immune responses in rhesus monkeys. Rhesus monkeys (2 monkeys/group) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc vector. The luciferase activity in the organs of the monkey (expressed in RLU) was examined 3 days after administration (a). Rhesus monkeys were immunized i.m. with 10^{11} 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 ND_{50}/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 10^{10} or 10^{11} vp of Ad5-Luc. After 8 weeks, these animals were immunized with 10^{10} 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 parencymal 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

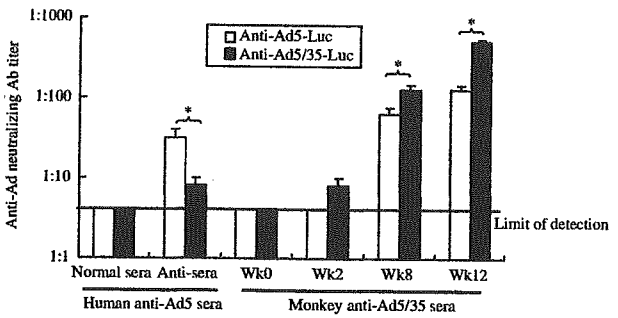

Figure 4 *Effect of pre-existing antiviral immunity. Ad5-Luc and Ad5/35-Luc vectors were incubated with an equal volume of serially diluted normal human sera (No. 2, anti-Ad5 neutralization titer <1:4), human antisera (No. 2, anti-Ad5 neutralization titer = 64), or monkey antisera from Figure 3b and 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
			Ad5/35-HIV (10^{10} vp/mouse)	5.1±0.2
Low dose	Ad5-Luc (10^{10} vp/mouse)	1:102	Ad5-HIV (10^{10} vp/mouse)	2.3±0.4 *
			Ad5/35-HIV (10^{10} vp/mouse)	4.6±0.6 *
High dose	Ad5-Luc (10^{11} vp/mouse)	1:248	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.