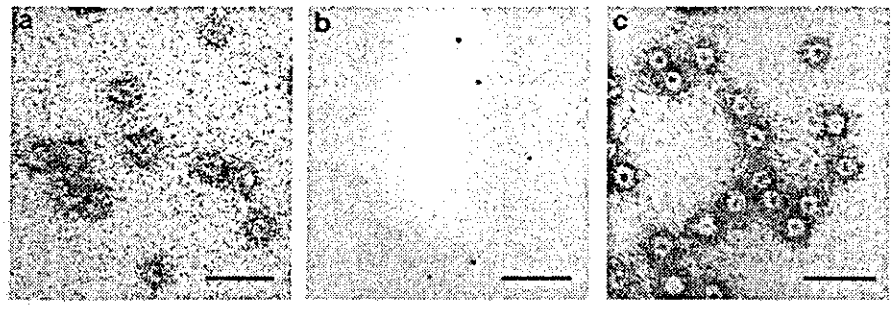

Figure 1 Electron micrographs of HEV-VLPs: (a) purified HEV-VLPs before treatment; (b) disassembled HEV-VLPs after treatment of VLPs with EGTA and DTT; and (c) refolded HEV-VLPs in the presence of $CaCl_2$, DMSO and DNA. Bars represent 50 nm.

human acute hepatitis by fecal–oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has recently been reported that overexpression of a part of open reading frame 2 (ORF2) in a baculovirus expression system results in the assembly of this protein into a VLP.[10] We have also reported that VLPs carrying foreign epitopes elicit strong mucosal and systemic immune responses to both the VLPs and exogenous epitopes without the requirement of any kind of adjuvant when orally administered to mice.[11]

Since infection with human immunodeficiency virus (HIV) most likely occurs through exposure of mucosal tissue to the virus, HIV-specific immune responses at mucosal sites are critical for the initial control of infection. Therefore, a nonreplicating vaccine vector that elicits mucosal immunity by oral administration would be a powerful HIV vaccine. In the present study, we found that unrelated plasmid constructs can be encapsulated into HEV-VLPs and delivered to the intestinal mucosa by oral administration. HIV DNA vaccine-loaded HEV-VLPs can elicit mucosal and systemic cellular as well as humoral immune responses by oral administration.

Results

In vitro refolding of VLPs

The HEV-VLPs produced by a recombinant baculovirus system were disassembled by the removal of calcium ions (Figure 1b). When calcium ions were supplemented to the disrupted VLPs in the presence of plasmid DNA, the DNA was encapsulated into the refolded VLPs (Figure 1c). No significant morphological difference due to the VLP disassembling–refolding process was observed under an electron microscope.

Density shifts of VLPs and amount of plasmid DNA after DNA encapsulation

Plasmid DNA encapsulation in the refolded VLPs was confirmed by CsCl equilibrium gradient centrifugation. VLP density is greater when loaded with a DNA plasmid. A heavier density gradient peak was present only when DNA was incorporated into the VLPs (Figure 2d). A single lighter density peak was produced for VLPs alone (Figure 2a), refolded VLPs (Figure 2b) and intact VLPs in the presence of plasmid DNA (Figure 2c). Despite the various sizes of plasmid DNA used for

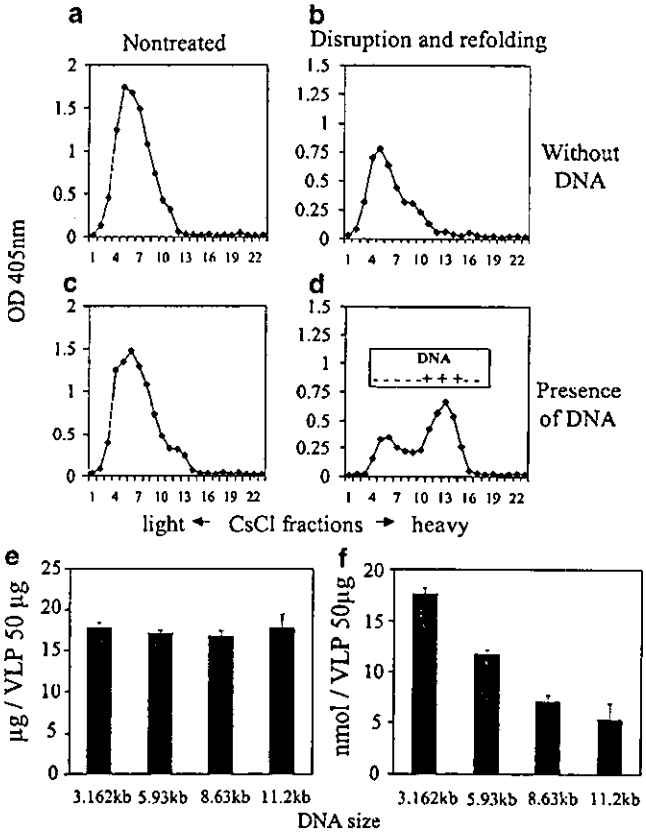

Figure 2 CsCl gradient profiles of intact and refolded VLPs. No DNA added: (a) intact; (b) refolded. DNA added: (c) intact; (d) refolded. The amount of DNA encapsulated in VLPs is expressed as μg (e) and molality (f) per 50 μg VLP protein.

encapsulation, the amounts of plasmid in VLPs were almost the same (17–19 μg per 50 μg of HEV-VLPs) (Figure 2e and f). A solution with a high concentration of plasmid DNA showed high viscosity, and VLPs including DNA were not obtained for general use in experiments. Based on these results, we used this amount (1 mg/ml) as the optimal concentration (data not shown).

Gene transfer by HEV-VLPs

Initially, four cell lines derived from mice, rabbits, monkeys and humans were studied for their ability to

transfer genes *in vitro*. The fluorescence of GFP-expressing cells was observed under a fluorescence microscope. Although the percentages of fluorescence-positive cells were not so high (11.2% of NIH3T3 cells, 19.6% of RK-13 cells, 21.0% of COS-7 cells and 20.1% of HepG2 cells), all of the cell lines used in this study showed positive reactions (Figure 3). In contrast, no fluorescence-positive cells were observed when the cells were incubated with plasmid DNA alone or intact VLPs in the presence of plasmid DNA (data not shown). We next tried gene transduction *in vivo*. Mice that had orally received a vaccine of DNA expressing HIV env gp120 of the NL432 strain (pJWNL432) that was encapsulated in VLPs were killed 2 days after immunization, and the expression of HIV env protein in the digestive tract was examined. HIV env protein was found in epithelial cells of the small intestine by immunohistochemistry (Figure 4), indicating that the HEV structure necessary for the entry of HEV into target cells had been preserved in refolded VLPs and that the DNA encapsulated in HEV-VLPs had been delivered to intestinal tissues.

Systemic and mucosal HIV-specific humoral immune responses in mice that had orally received a vaccine of HIV DNA encapsulated in VLPs

Mice were orally or subcutaneously immunized four times at 1-week intervals with pJWNL432 either naked or encapsulated in HEV-VLPs. The serum levels of HIV env-specific IgG antibodies in mice that had received loaded VLPs were significantly higher than those in mice that had received naked DNA ($P<0.05$ at 12 wpi, Figure 5a and e). Moreover, specific IgA was detected at high levels in sera of mice that had received loaded VLPs but not in sera of mice that had been immunized subcutaneously ($P<0.05$ at 12 wpi, Figure 5b and f). HIV env-specific IgA was only detected in fecal extracts of mice that had orally received pJWNL432-encapsulated HEV-VLPs (Figure 5d and h). No specific IgG was detected in any of the fecal samples (Figure 5c and g). The levels of HIV env-specific IgG antibodies detected in sera from subcutaneously and orally immunized mice were the same (Figure 5a and e). HEV-specific IgA was detected in both sera and fecal extracts of mice that had been orally administered VLP but not in sera or fecal extracts of mice that had been immunized subcutaneously (Figure 5j and l). Both orally and subcutaneously immunized mice showed HEV-specific IgG in sera (Figure 5i) and fecal extracts (Figure 5k).

Elicitation of HIV-specific cytotoxic T lymphocytes at systemic and mucosal sites by oral administration of a vaccination of HIV DNA encapsulated in VLPs

Cytotoxic T lymphocyte (CTL) responses in the spleen, mesenteric lymph nodes (MLN) and Payer's patches (PP) were investigated at 5 weeks after the first immunization. Mice that had orally received pJWNL432 encapsulated in HEV-VLPs showed HIV env epitope-specific CTL responses in the spleen, MLN and PP, whereas cells from the same tissues in mice that had received naked DNA vaccine did not show any CTL activity (Figure 6a). The P18 peptide is a dominant HIV env CTL and Th cell epitope in BALB/c mice and is restricted to the H-2D^d allele. These effector cell functions derived from our experiments were inhibited by either anti-CD8 or -H-2D^d monoclonal antibody (mAb) (Figure 6b,c), indicating that oral immunization of mice with a vaccine of HIV env DNA-encapsulated HEV-VLPs elicited CD8$^+$ and MHC class I-restricted CTLs both locally and systemically.

Discussion

A large number of pathogens gain access to the human body via mucosa such as oral, nasal or genital mucosa. The best defense against these predominantly mucosal

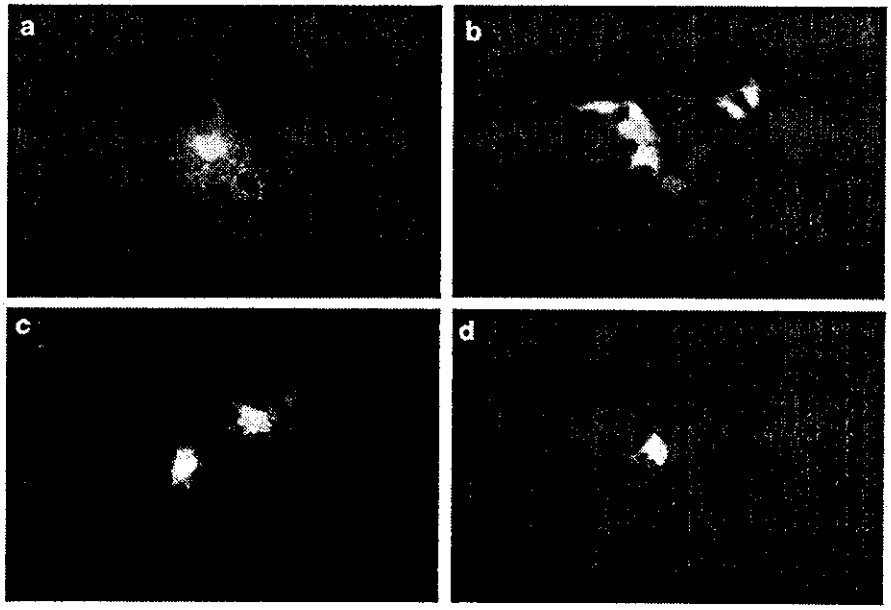

Figure 3 Expression of GFP in cells transfected with plasmid DNA encapsulated in HEV-VLPs: (a) NIH/3T3 cells (mouse); (b) RK-13 cells (rabbit); (c) COS-7 cells (monkey); and (d) HepG2 cells (human).

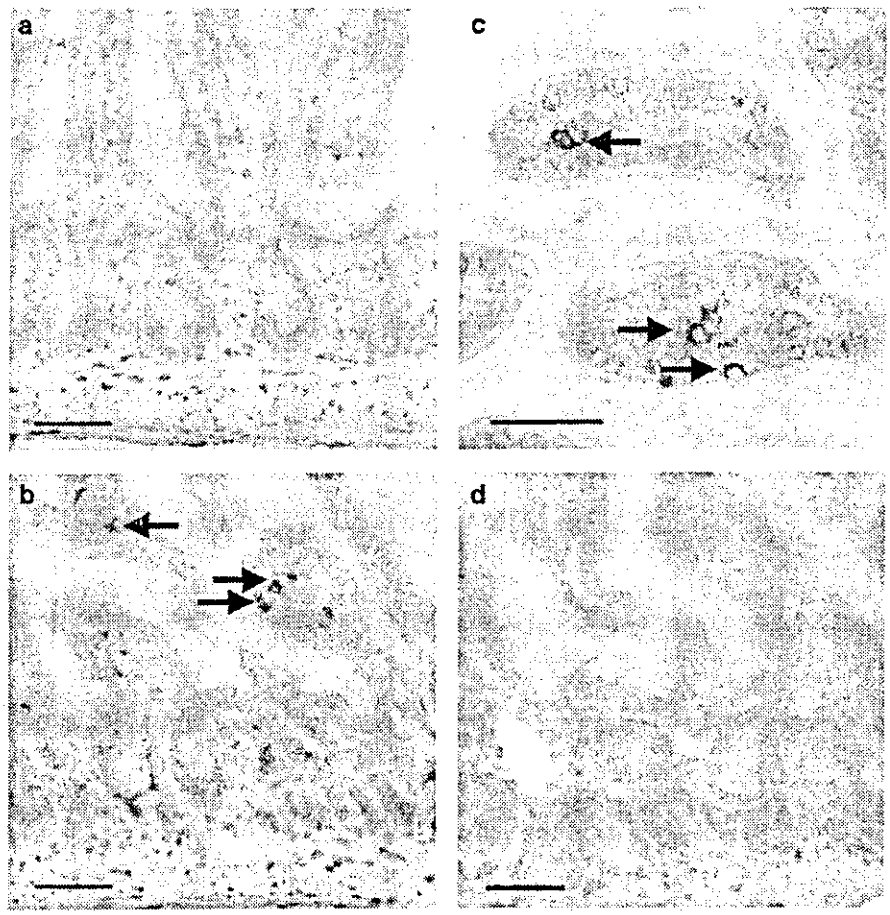

Figure 4 Immunostaining of serial sections of small intestine tissue from mice 2 days after oral administration of pJWNL432-encapsulated VLPs. HIV env proteins were observed in epithelial cells (arrow) (b, c), and control mAb did not show any positive reactions (d). Control mice were also administered pJWNL432 without VLP encapsulation (a). Bar marker represents 50 μm.

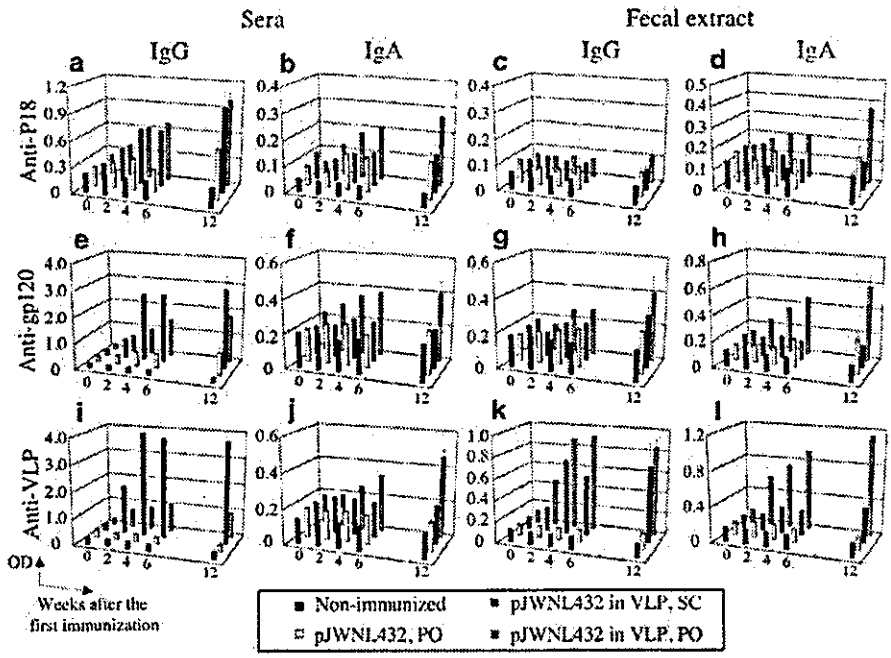

Figure 5 IgG (a, c, e ,g, i and k) and IgA (b, d, f, h, j and l) levels in sera (a, b, e, f, i and j) and fecal extracts (c, d, g, h, k and l) of immunized mice. Mice were orally (■) or subcutaneously (□) administered pJWNL432 encapsulated in VLP or naked (▩). Symbols indicate HIV env-specific antibody levels. Background levels to HIV env in nonimmunized mice (▩) are also shown. The IgG and IgA antibody levels are expressed as OD at dilutions of 1:100 and 1:2 for serum and fecal extracts, respectively. The mean OD values ±s.e.s were obtained from five mice/group.

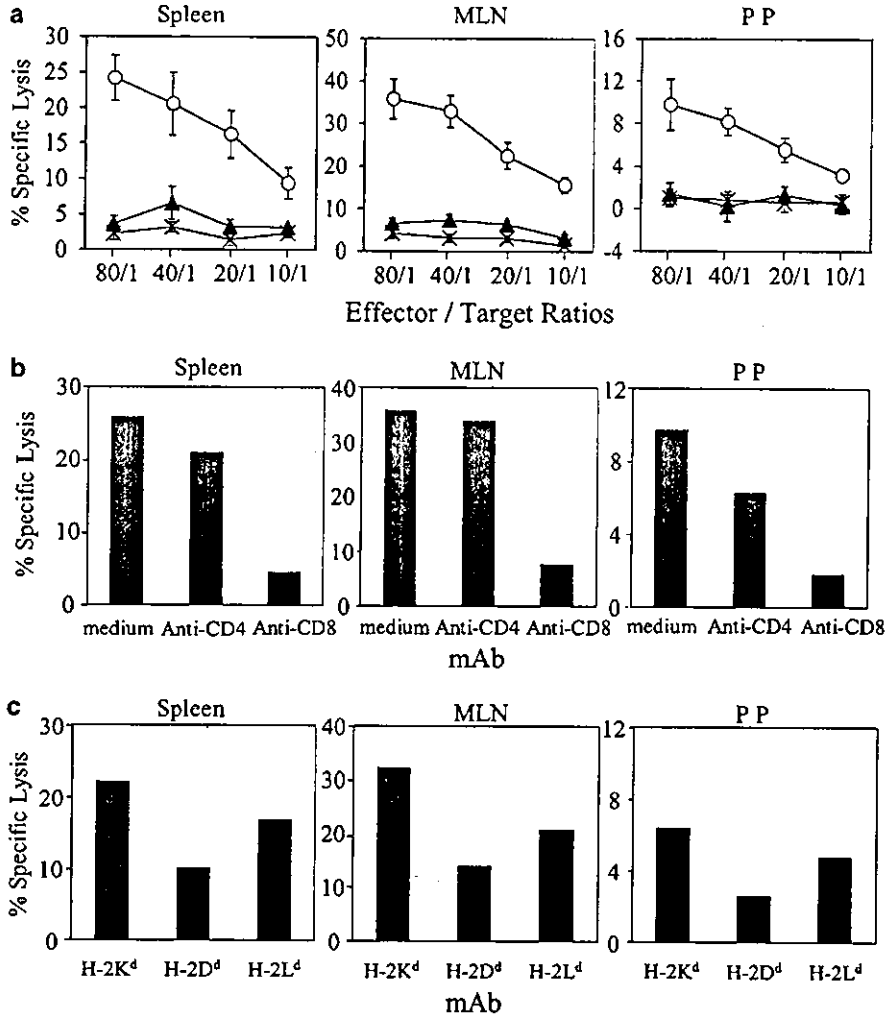

Figure 6 Spleen, MLN and PP cells from mice orally administered pJWNL432-encapsulated VLPs elicited CTL. (a) Mice were orally administered pJWNL432 encapsulated in VLPs (circles) or naked (triangles). Results for nonimmunized controls are also shown (×). (b) Effector cells obtained from the spleen, MLN and PP cells of mice orally administered pJWNL432-encapsulated VLPs are mediated CD8+ cells. Lytic activities of effector cells were assessed in the presence of anti CD4 mAb, anti-CD8 mAb or medium. Effector:target ratio was 80:1. (c) HIV env-specific lysis was restricted by MHC class I. Effector cells were examined for P18-specific lytic activities in the presence of anti-H-2K^d, anti-H-2D^d or H-2L^d mAb. The percentage of P18-specific lysis was calculated as (% lysis of target cells labeled with P18)−(% lysis of target cells labeled with control peptide). Each value is the mean percentage of the specific lysis values obtained from five mice.

pathogens is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Recent evidence has shown that DNA vaccination can confer protection against a number of infectious agents, including viruses and bacteria, although peripheral immunization with naked DNA is less than optimal for stimulating mucosal immunity.[12,13] In fact, it is quite difficult to induce both mucosal and systemic immune responses by oral administration of naked DNA. This study demonstrated that an orally administered DNA vaccine encapsulated in an orally transmissible virus-derived VLP induced both mucosal and systemic immunity.

The delivery of a DNA vaccine for induction of mucosal immune responses is usually achieved by gene transfer to the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[5,14] Despite its obvious convenience, oral administration is rarely successful, since it is quite difficult to protect plasmid DNA from the environment in the digestive tract. The efficacy of orally delivered DNA vaccine to NALT is improved by encapsulating plasmid DNA in poly (lactide-coglycolide) (PLG) microparticles for protection against the gastric environment.[15,16] The immune responses to particle-borne DNA immunizations by means such as utilization of a gene gun or PLG differ from those to DNA immunizations without particles.[13] It is thought that the microparticles are actively taken up by cells such as macrophages or M cells of PP of the small intestine and thus facilitate the presentation of antigens to local immune systems.[15,17] This mechanism is the same as that of gene gun immunization of a DNA vaccine, that is, phagocytic cells such as macrophages or dendritic cells take up plasmid DNA delivered by a gene gun. The delivered gene is expressed only in these cells.[18] Similarly, only mucosal immunity was induced in mice by oral administration of DNA-encapsulated PLG microparticles.[15,16] It is likely that the mechanism underlying immune recognition of

HEV-VLP infection is similar to that of direct intramuscular or subcutaneous DNA immunization without the use of particles. Protein expressed by HEV-VLP-infected cells is recognized by the immune surveillance system, resulting in the elicitation of Ag-specific immune responses. We showed in this study that genes could be expressed in epithelial cells in the small intestine after delivery by HEV-VLPs (Figure 4). It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine, because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[19] The Ag-expressing cells might be recognized by intraepithelial lymphocytes or submucous antigen-presenting cells by the same mechanism as that in the case of general virus infection.

An HEV-VLP has several advantages as a vector of DNA. Firstly, in our experience, large amounts can be easily obtained from standard cultivation protocols compared with the amounts of other VLPs obtained. The yield of purified HEV-VLPs collected from a culture supernatant of 50–100 μg/ml is more than 100 times greater than that of other VLPs. Secondly, the outcome of gene delivery in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious route and target cells.[10,20] Thirdly, HEV-VLPs are stable at room temperature. Fourthly, anti-HEV immune responses had no effect on DNA administration in the present study, and this might be related to the neutralizing antibody for preventing infection with HEV. Neutralizing antibodies to HEV for inhibiting infection have not yet been found. This is also the case for HCV. The mechanism by which HEV is eliminated by antibodies is thought to be antibody-dependent cell-mediated cytotoxicity (ADCC). The effect of induction of immune responses to DNA vaccine in our system is not clear. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLP can be preserved without the requirement of any particular equipment. Finally, we have reported that an HEV-VLP can carry foreign amino-acid sequences as a part of the ORF2 protein exposed on the particle surface without any morphological or biological alteration.[10] Liposomal vectors resembling retroviral envelopes endowed with targeting molecules for gene delivery have been reported. The vicronectin receptor, $\alpha_v\beta_3$-integrin, is commonly upregulated on malignant melanoma cells, and liposome carrying an Arg-Gly-Asp (RGD) integrin-binding motif has been used for a system to deliver DNA to these tumor cells.[21] It has also been reported that targeting DNA to M cells by intranasal administration for the induction of mucosal and systemic responses can be achieved by formulating DNA with polylysine linked to viral adhesion.[22] It may be possible to design chimeric ORF2 proteins carrying these targeting molecules to retarget HEV-VLP to particular cell types.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[23] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for the vaccine administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[24] Trials using DNA vaccines for infectious and malignancy diseases have also been conducted.[25] The results of the present study suggest that oral administration of DNA vaccine encapsulated in oral transmissible virus VLPs, HEV-VLPs, is effective for inducing both humoral and cellular immunity locally as well as systematically. HEV-VLPs might be useful not only for vaccination but also as a vector in human gene therapy.

Materials and methods

Mice

BALB/c female mice were purchased from Clea Japan (Tokyo, Japan) and were housed in the Laboratory Animal Center of Mie University School of Medicine during the experimental period.

Peptide synthesis

The peptides used in this study were the HIV env CTL epitope (HIV 308-322, RIQRGPGRAFVTIGK; P18)[26] and a control peptide (HCV nonstructural protein 5 CTL epitope MSYSWTGALVTPCAAE; P17).[27]

Plasmid DNA

A highly efficient mammalian expression vector, pJW4303,[28] was used for efficient expression of HIV env gp120 of the NL432 strain.[29] Various sizes of plasmid DNA were also used for the *in vitro* packaging experiment (3.162 kb: pUC118; 5.93 kb: pJW322; 8.63 kb: pJWSIVenv; 11.2 kb: pABWN).

Production and purification of HEV-VLPs

HEV-VLPs were produced and purified by previously described methods.[10,11] Briefly, Tn5 cells maintained in Excel 405 serum-free medium (JRH, KS) were infected with the recombinant baculovirus expressing HEV-ORF2 at an m.o.i. of >5 and cultured for 6 days. The supernatant was harvested and the recombinant baculovirus in the supernatant was pelleted by ultracentrifugation at 10 000 *g* for 30 min at 4°C. The VLPs in the supernatant were collected by further ultracentrifugation at 100 000 *g* for 2 h at 4°C. Pelleted VLPs were then resuspended in 10 mM potassium-[2-(*N*-morpholino) ethanesulfonic acid] (MES) buffer (pH 6.2) and purified on a CsCl equilibrium density gradient. The purified HEV-VLPs were spun down and resuspended in potassium-MES buffer and kept at 4°C.

DNA packaging

Plasmid DNA was encapsulated into HEV-VLPs according to a previously described procedure.[30] Purified VLPs (50 μg) were disrupted by incubation in 180 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA and 20 mM dithiothreitol. Following 30 min of incubation at room temperature, 200 μg (20 μl) of each plasmid in 50 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl was added. The disrupted VLP preparation was refolded by incubation for 1 h with increasing concentrations of $CaCl_2$ up to a final concentration of 5 mM. VLPs were pelleted by ultracentrifugation and resuspended in 10 mM potassium-MES buffer (pH 6.2). At each step, the VLP structure formation was confirmed by electron

microscopy after negative staining, as described previously.[11] To estimate the amounts of encapsulated plasmid DNA, refolded and purified VLPs were treated with 10 IU benzonase (SIGMA-ALDRICH, Irvin, UK) for 1 h at 20°C to remove DNA on the surfaces of VLPs and disrupted with EGTA (1 mM). Absorbance of the supernatant was measured for detection of plasmid DNA contents.

Density analysis of refolded VLPs

Refolded VLPs were separated on a CsCl equilibrium density gradient and fractioned into 0.2 ml aliquots. HEV-VLPs in each fraction were detected by ELISA as previously described,[10] as well as DNA contents.

Gene transfer in mammalian cells

Four cell lines (NIH/3T3 (mouse), RK13 (rabbit), COS-7 (monkey), HepG2 (human)) were used in transfection experiments. Sterilized coverslips were placed in six-well plates, and 5×10^5 cells per well were seeded in the plates. After overnight culture, cells were washed twice with a medium, and about 1 μg of VLP-encapsulated EGFP expression vector (BD Bioscience Clontech, CA, USA) diluted with 0.5 ml medium was added. After 2 h of incubation at 37°C, VLPs were removed. Cells were then incubated for 48 h at 37°C. At the end of the culture period, cells were removed from the culture medium and washed three times with PBS. Coverslips were then mounted onto microscope slide glasses. Fluorescence of the GFP-expressing cells was observed under a fluorescence microscope.

Immunization

Mice were orally immunized four times with 50 μg protein of HEV-VLP/DNA (pJWNL432) complex or 20 μg naked pJWNL432 DNA in 100 μl of potassium-MES buffer at 1 week intervals.

Immunohistochemical analysis

At 2 days after oral immunization, the mice were killed and tissues were collected. Cryostat sections were air-dried and incubated in 0.5% HIO_4 for 10 min to quench endogenous peroxidase activity. The sections were further pretreated with chicken anti-mouse IgG antibody (Chemicon International, Inc., CA, USA) to prevent nonspecific reactions of a secondary antibody. The sections were then incubated with an HIV env-specific mAb (HIV-1 IIIB gp120 mAb (902)), which was obtained through the AIDS Research and Reference Reagent Program,[31] for 30 min at 37°C. The bound antibodies were visualized with a biotinated secondary antibody, HRP-labeled avidin–biotin complex (ABC-peroxidase staining kit, Elite Vector Lab. Inc., CA, USA) and 3.3′-diaminobenzidine tetrachloride with 0.01% H_2O_2. Sections were slightly counterstained with hematoxylin. An mAb (A1/3D1, ANOGEN, Canada) against hepatitis C virus core, which is same isotype to 902, was used as a control.

ELISA

Serum and fecal samples were collected at 0 (preimmunization), 2, 4, 6 and 12 weeks after the first immunization. Feces were suspended in ice-cold PBS at 200 mg/ml, and the centrifuge supernatant was used as fecal extract. Culture plates (96-well) were coated with purified HEV-VLPs or synthesized oligopeptides (P18) at a concentration of 10 or 100 μg/well, respectively, overnight at 4°C followed by 30 min of blocking with PBS containing 0.1% FBS and 0.05% Tween 20. To determine the anti-HIV env gp120 antibody responses, CV-1 cells were seeded in 96-well plates and infected with recombinant Sendai virus expressing HIV env gp120 of NL432 strain (SeV gp120),[32] and then the plates were incubated at 37°C. At 3 days after infection, plates were washed and fixed with PBS containing 10% formalin for 10 min. Test samples were added to each well and incubated at room temperature for 1 h. For detection of anti-HIV env gp120 antibody, test samples were reacted with wild-type Sendai virus-infected CV-1 cells before addition to the wells to eliminate the nonspecific antibody. Biotin-labeled anti-mouse IgG (Vector, CA, USA) or IgA (CALTAG, CA, USA) was used as the detection antibody. Following 1 h incubation, the plates were washed and further incubated with avidin-HRP (Vector, CA, USA). The reaction was developed using an ABTS substrate (Roch Diagnostic, Mannheim, Germany).

Generation of CTL effector cells

Effector cells were derived from spleen, MLN and PP cells as precursor CTLs. Aliquots of 5×10^6 spleen cells were co-cultured with 2.5×10^6 mitomycin C-treated autologous spleen cells labeled with a peptide at 37°C in a CO_2 incubator. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

Target cells, A20.2J cells (2×10^6), were incubated at 37°C in a 5% CO_2 atmosphere with 10 μg/ml of P18 or control peptide for 16 h. The target cells were then washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as ((experimental release−spontaneous release)/(100% release−spontaneous release)) × 100. All the experiments were performed at least four times, and each experimental group consisted of five mice.

Blocking of cytolysis

^{51}Cr-labeled target cells (10^6 cells) were preincubated at 4°C for 1 h with anti-H-2 K^d, D^d or L^d mAb (Meiji Institute of Health Science Ltd., Tokyo, Japan) (1 μg/ml), and effector cells were then added. In a separate experiment, effector cells (10^7 cells) were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) (10 μg/ml) at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's U test and Kruskal–Wallis test. Values are expressed as means ± s.d.s. A 95% confidence limit was taken as significant ($P < 0.05$).

Acknowledgements

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Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles

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Abstract

Hepatitis E virus (HEV) is an important causative agent of enterically-transmitted hepatitis. Successful vaccine development is crucial in controlling global HEV infection. HEV capsid protein, with 111 amino acids truncated at the N-terminus, was efficiently expressed in the baculovirus expression system [J. Virol. 71 (1997) 7207–13]. Expressed protein spontaneously assembled into virus-like particles (VLPs) and was released into culture medium. When cynomolgus monkeys were orally inoculated with 10 mg of purified rHEV VLPs, serum IgM, IgG, and IgA responses were observed. All these antibody responses were obtained without adjuvants. When the monkeys were challenged with native HEV by intravenous injection, they were protected against infection or developing hepatitis. These results suggested that recombinant HEV (rHEV) VLPs can be a candidate for the oral hepatitis E vaccine.

Keywords: Hepatitis E virus (HEV); Virus-like particles (VLPs); Oral immunization; Vaccine

1. Introduction

Hepatitis E is an acute viral hepatitis caused by infection with hepatitis E virus (HEV) that was first recognized when a large water-borne hepatitis outbreak occurred in India in 1955 [1]. Subsequently, water-borne outbreaks were reported in many developing regions, including Nepal, Burma, China, Middle Asia, the Middle East, Africa, and Mexico, where sanitary conditions are not well maintained [2,3]. HEV is transmitted via an oral-fecal route, often by contaminated drinking water. Hepatitis E affects predominantly young adults [4,5], and the mortality rate is very high, up to 15–20%, in infected pregnant women [6,7]. In developed countries, most cases have been imported. However, recent studies revealed that hepatitis E occurred in patients who had never been abroad [8–10]. Similar viruses to HEV have been isolated from pigs, suggesting that hepatitis E may be a zoonosis [11–13].

HEV has been identified as a 27–34 nm icosahedral particle with an approximately 7.5 kb positive sense RNA genome [14–18]. The HEV genome encodes three open reading frames (ORFs), of which ORF2 is thought to encode the capsid protein. When ORF2, with 111 amino acids truncated at its N-terminus, is expressed in a baculovirus expression system, it spontaneously assembles into virus-like particles (VLPs) [19]. Electron cryomicroscopy shows that these VLPs are formed with 60 copies of a 54 kDa protein arranged in T = 1 symmetry [19–21]. The particles possess antigenicity similar to that of authentic HEV particles and, consequently, they appear to be a good antigen for the sensitive detection of HEV-specific IgG and IgM antibodies. Furthermore, the VLP may be the most promising candidate yet for an HEV vaccine, owing to its potent immunogenicity [19]. Although vaccination is an effective method of infection control for conventional infectious agents, no practical cell culture system to allow the growth of HEV has been established yet, making it difficult to develop a vaccine.

In our previous study, mice orally immunized with purified recombinant HEV (rHEV) VLPs developed serum IgM, IgG, and IgA as well as the fecal IgA antibody responses without an adjuvant [20]. A similar line of the study has been performed with recombinant Norovirus (rNV) VLPs. Oral immunization with rNV VLPs has shown to induce not only systemic but also mucosal immune response in mice, demonstrating that rNV VLPs are an excellent model

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to study the oral delivery of antigen, and they are a potential mucosal vaccine for NV infection [22]. The safety and immunogenicity of rNV VLPs have been evaluated with healthy volunteers [23].

Because mice are not susceptible to HEV, a protection assay cannot be performed with them. In contrast, macaques (rhesus and cynomolgus monkeys) are sensitive against HEV and are useful models for the study of hepatitis E [24]. When macaques are inoculated with human HEV, they develop acute hepatitis with biochemical, histopathological, and serological markers characteristic of the disease in humans [25,26]. In this study, we demonstrated that oral administration of rHEV VLPs in monkeys-induced serum IgM, IgG, and IgA, anti-HEV antibody responses, and that these monkeys were protected from infection or from developing disease when challenged with native infectious HEV.

2. Materials and methods

2.1. Primates

Cynomolgus monkeys (*Macaca fascicularis*), born and grown in the Tsukuba Primate Center for Medial Science, National Institute of Infectious Diseases (NIID), were used for the studies. Serum alanine aminotransferase (ALT) values of the animals were normal and stable, and anti-HEV antibody was negative by enzyme-linked immunosorbent assay (ELISA) [29]. Primates were individually housed in BSL-2 facilities. All monkey experiments were reviewed and carried out according to "Guides for animal experiments performed at NIID" under the codes 990058 and 000019.

2.2. Fecal specimen and challenge virus stock

A fecal specimen containing infectious HEV was collected from a patient who developed acute hepatitis in India in 1998. This specimen was suspended in 10 mM phosphate buffered saline (PBS), and a 10% suspension was prepared. The suspension was clarified by centrifugation at 10,000 × *g* for 20 min, and the supernatant was passed through a 0.45 μm membrane filter (Millipore, Bedford, MA) and stored at −20 °C. This suspension was positive for HEV antigen by an enzyme-linked immunosorbent assay developed in this laboratory and positive also for HEV RNA by reverse-transcription polymerase chain reaction (RT-PCR).

To prepare a challenge virus stock, a 4-year-old male cynomolgus monkey was inoculated intravenously with 2 ml of the filtrate, and stool samples were collected daily after inoculation. Stools collected from 10 to 40 days after injection were positive for HEV antigen by ELISA and HEV-RNA by RT-PCR, respectively. The monkey stool collected at day 21 after inoculation was diluted with PBS to prepare a 10% suspension, and clarified by centrifugation and filtration as described above. The infectivity was determined, and the virus was stored as the challenge virus.

2.3. Infectivity of HEV

Three monkeys were inoculated intravenously with 2 ml of 1:100, 1:1000, and 1:10,000 dilutions of the challenge virus, respectively. Sera were collected weekly before and after the inoculation and used to determine ALT values and to detect HEV antigen. ALT and HEV antigen in serum were monitored to determine the infectivity.

2.4. Preparation and purification of rHEV VLPs

The bile specimens were collected from rhesus monkeys which was injected with a pool of acute-phase stool specimens from patients with sporadic non-A, non-B hepatitis in Myanmar in 1986 [27]. The complete nucleotide (nt) sequence indicated that HEV included in the bile was genotype I HEV [28]. The molecular cloning and construction of a recombinant baculovirus Ac5480/7126 harboring the capsid protein gene lacking 111 amino acids at the N-terminal were described previously [19–21,29]. The rHEV VLPs were prepared using Tn5 cells (High Five™, Invitrogen, San Diego, CA, USA) infected with Ac5480/7126 at a multiplicity of infection of 10. Following 7 days of incubation at 26.5 °C, intact cells and cell debris were removed from the culture medium, and the rHEV VLPs were concentrated by centrifugation for 2 h at 100,000 × *g* in an SW28 rotor (Beckman Instruments Inc., Fullerton, CA, USA) and purified by isopycnic binding in CsCl gradient. A visible band containing rHEV VLPs was collected, and the rHEV VLPs were diluted five times with phosphate buffered saline solution(−). The rHEV VLPs were pelleted at 100,000 × *g* and then resuspended in PBS(−). Purity of the rHEV VLPs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy after negative staining, as described previously [19].

2.5. Antibody and antigen ELISAs

Serum IgG, IgM, and IgA and fecal IgA antibodies to HEV were detected by antibody ELISAs as previously described [29]. Antigen capture ELISA was used to detect HEV antigen. Briefly, duplicate wells of flat-bottom 96-well polystyrene microplates (Dynex Technologies Inc., Chantilly, VA) were coated with 100 μl of a coating buffer (0.1 M carbonate–bicarbonate buffer, pH 9.6) containing 1:10,000 diluted serum from either non-immunized (normal) rabbit or rHEV VLP-immunized rabbit. The coating was performed at 4 °C overnight. Unbound antibodies were removed, and the wells were washed twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T), and then the blocking was carried out at 37 °C for 1 h with 150 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. When antigen was to be detected in serum, the serum was diluted 1:200 with PBS-T. When the antigen in stool samples was to be detected, the stool was diluted and 10% suspension was prepared with PBS-T containing 1% skim milk. Either 100 μl

of the serum or the same volume of the 10% stool suspension was added to the wells and incubated for 1 h at 37 °C. After the wells were washed five times with PBS-T, 100 μl of hyperimmune serum from guinea pig (1:10,000 dilution with PBS-T containing 1% skim milk) was added to the wells and the plate was incubated for 1 h at 37 °C. The plate was washed five times with PBS-T, and then horseradish peroxidase-conjugated goat anti-guinea pig IgG antibody (1: 2000 in PBS-T containing 1% skim milk) (Cappel, Durham, NC) was added to each well. After incubation for 1 h at 37 °C, the plate was washed five times with PBS-T and 100 μl of substrate *o*-phenylenediamine and H_2O_2 was added. The plate was left for 30 min at room temperature, and then the reaction was stopped with 50 μl of 4N H_2SO_4. After 10 min, the absorbance at 492 nm was measured with a microplate reader (Molecular Devices Corp., Tokyo, Japan).

2.6. Liver enzyme level

ALT value of infected monkeys was monitored weekly by standard methods (Anilytics, Gaithersburg, MD). The geometric mean of ALT values during the pre-inoculation period of each animal was used as the normal ALT value, and two-fold or greater increase at the peak was considered to be biochemical evidence of hepatitis.

2.7. Detection of HEV RNA by RT-PCR

Total RNA was extracted with RNAzol reagent (Tel-test Inc., Friendswood, TX) using 100 μl of the serum or 10% fecal suspension. The RNA was purified with Oligotex-dT30 (Roche Diagnostic Systems, Tokyo, Japan) according to the manufacturer's protocol and converted into cDNA as described previously [30]. The cDNA was subjected to polymerase chain reaction with ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) with an external sense primer HEV-D4 (5′-TGTAGAGAATGCTCAGCAGGATAA-3′, nt 6391–6414) and an antisense primer HEV-U4 (5′-TAACTCCCGAGTTTTACCCACCTT-3′, nt 7103–7126) using the GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension reaction at 72 °C for 60 s followed by final extension at 72 °C for 7 min. The nested PCR was done with an internal sense primer HEV-D5 (5′-CTGCCGAGTATGACCAGTCCACTTA-3′, nt 6576–6600) and an internal antisense primer HEV-U3 (5′-TTAAGGCGCTGAAGCTCAGCGA-3′, nt 7077–7098) under the same conditions, generating a 523 nt fragment.

2.8. Immunization schedule

First, we inoculated two monkeys with each receiving 2 ml of 10% fecal suspension containing infectious HEV at day 96 before the injection (day −96) (M4200 and M4201, Fig. 1). These monkeys recovered from hepatitis E and were used as the serum IgG-positive control. Next, two other monkeys were each orally administered 10 mg of the VLPs on days 0, 7, 21, and 36. The fifth immunization was performed at day 80 (M4364 and M4365, Fig. 1). The third set of two monkeys were orally fed with PBS under the same schedule as the second set of monkeys, and these monkeys were used

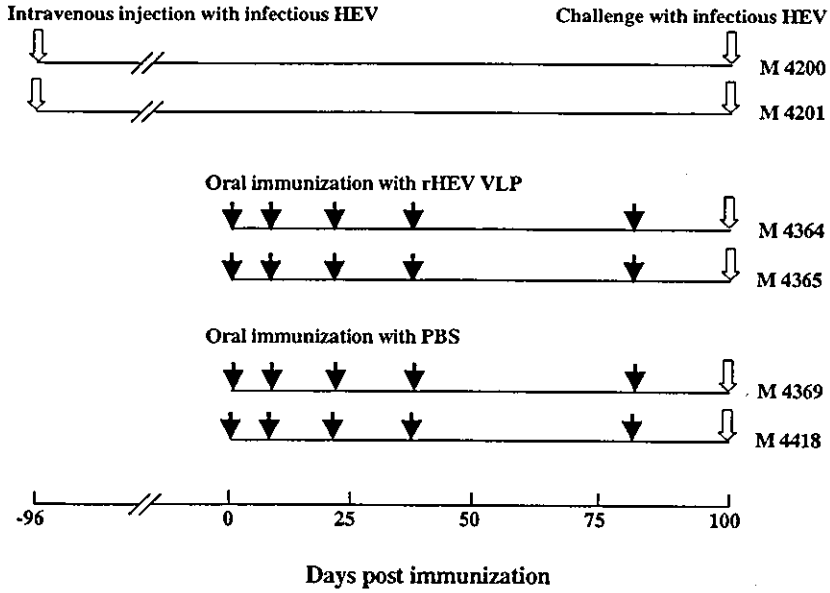

Fig. 1. Immunization schedule. Two monkeys (M4200 and M4201) were inoculated with 2 ml of the 10% stool suspension from an acute HEV patient on day −96 and served as serum antibody-positive controls; two monkeys (M4364 and M4365) were inoculated with 10 mg rHEV VLPs on days 0, 7, 21, 36, and 80; two monkeys (M4369 and M4418) received phosphate buffered saline as antibody-negative controls. On day 100, all of these six monkeys were challenged with 2 ml of the challenge virus. Monkey numbers are indicated on the right side of the figure.

as an antibody-negative control (M4369 and M4418, Fig. 1). On day 100, all of these six monkeys were challenged by intravenous injection each receiving 2 ml of challenge virus stock (Fig. 1).

3. Results

3.1. Titration of challenge virus

Three monkeys were inoculated intravenously with 2 ml of 1:100, 1:1000, and 1:10,000 dilutions of the challenge virus, respectively. Sera were collected weekly before and after the inoculation and used to determine ALT values and to detect HEV antigen (Fig. 2). Elevation of ALT was observed in all monkeys, reaching peaks at days 28 and 24 in the monkeys receiving either the 1:100 or 1:1000 dilution and at day 30 in the monkey receiving the 1:10,000 dilution (Fig. 2A). HEV antigen was also detected in the three monkeys, reaching the maximum amount at 2 weeks in the case of 1:100 dilution and at 3 weeks in the cases of 1:1000 and 1:10,000 dilution (Fig. 2B). These results demonstrated that viremia occurred in all monkeys and that all had developed type E hepatitis. Even the 1:10,000 dilution of the suspension was capable of infecting a subject monkey, indicating that the titer of the challenge virus was $>5 \times 10^3$ monkey infectious dose (MID50) per ml of the suspension. These results also indicated that viremia precedes the clinical symptoms associated with ALT elevation, an observation previously described in hepatitis E [31].

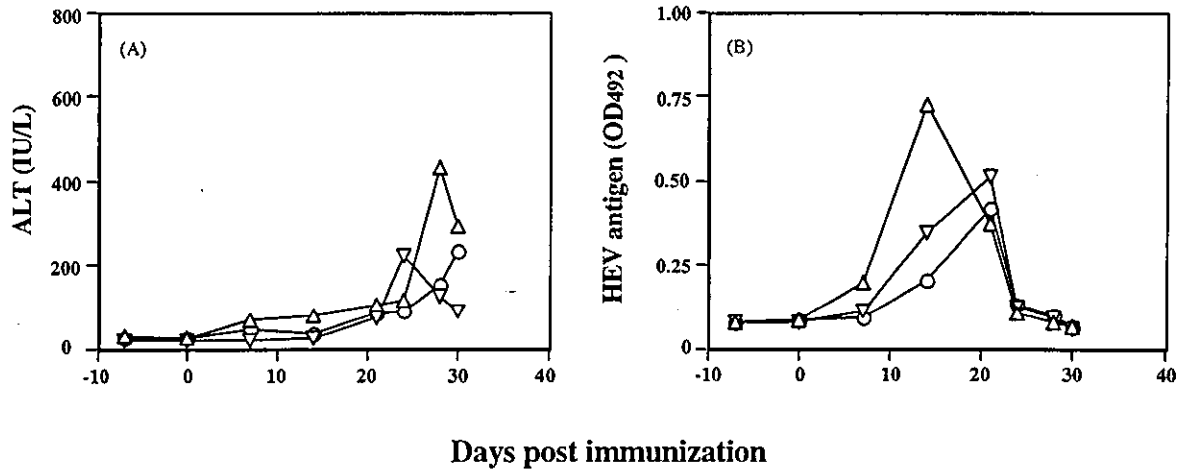

Fig. 2. MID50 of the challenge virus. Three serial 10-fold dilutions (1:100 (△); 1:1000 (▽); and 1:10,000 (○)) of the challenge virus were injected intravenously to monkeys. Sera were collected weekly before and after the inoculation. Elevation of alanine aminotransferase (ALT) was determined (A). HEV antigen was detected by the antigen ELISA (B).

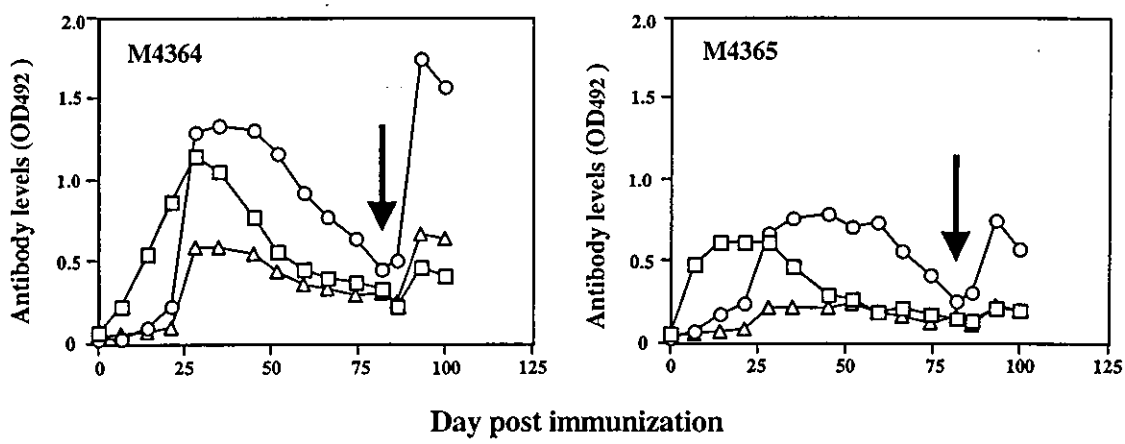

Fig. 3. Oral immunization with rHEV VLPs induces serum IgM, IgG, and IgA antibodies in monkeys. Two monkeys (M4364 and M4365) were orally immunized with rHEV VLPs, and serum samples were collected at 1 week intervals. Antibody was detected by the antibody ELISA. OD values of the HEV IgM (□), IgG (○), and IgA (△) antibodies were indicated. The arrows mean the day when the booster injection was performed.

3.2. Kinetics of serum IgM, IgG, IgA, and fecal IgA responses

To evaluate the potential of the rHEV VLPs as an oral immunogen, two cynomolgus monkeys (M4364 and M4365) were immunized orally with 10 mg rHEV VLPs on days 0, 7, 21, and 36. All immunizations were carried out without adjuvant, and the serum antibody responses were monitored by an antibody ELISA (Fig. 3). All of the pre-immune sera taken prior to the first immunization were negative for IgM, IgG, and IgA. The pattern of serum antibody responses was very similar in the two monkeys, although the antibody level was different; M4364 was higher than M4365 in the three antibody levels. A serum IgM response was observed 1 week post-immunization and reached a maximum at 3–4 weeks. Serum IgG and IgA response was detected at 2 weeks and reached a maximum at 4–5 weeks. These data demonstrated that the rHEV VLPs are immunogenic when delivered orally and that an adjuvant is not required for the induction of serum IgM, IgG, or IgA. Although the antibody titers decreased rapidly after reaching the peak values, IgG and IgA antibody increased very rapidly and reached high titers in 7 days when a boost immunization was performed at day 80 (arrows in Fig. 3). The result indicated that tolerance was not induced. There was no fecal IgA response in either monkey (data not shown). Two monkeys (M4369 and M4418) orally immunized with PBS were negative for IgM, IgG, and IgA throughout the experiment (data not shown).

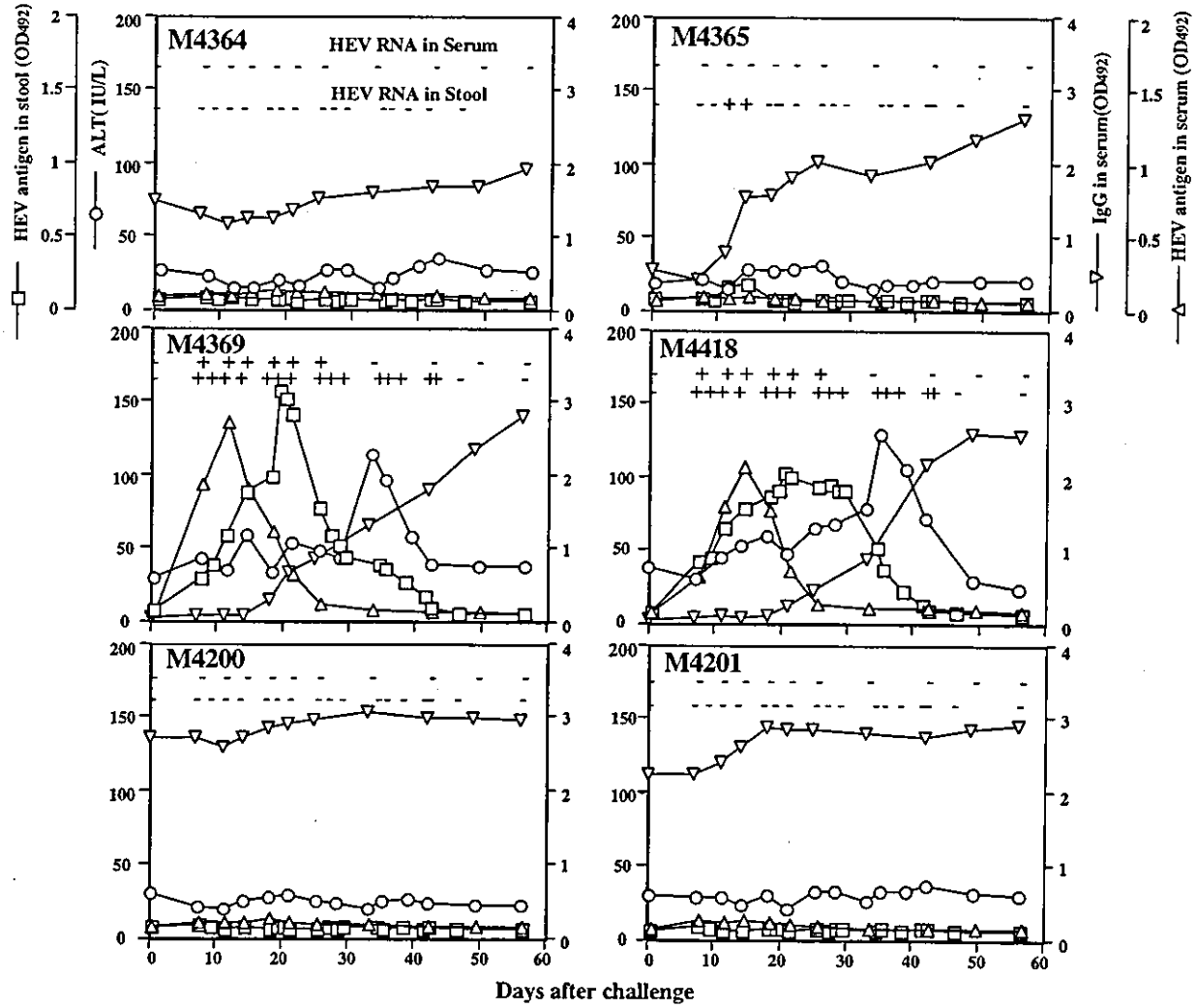

Fig. 4. Kinetics of biochemical, serological, and virological markers after the challenge. Monkeys recovered from hepatitis E (M4200 and M4201), monkeys orally immunized with HEV VLPs (M4364 and M4365), and monkeys for HEV-antibody negative (M4369 and M4418) were challenged with infectious HEV at day 0. HEV antigen in the serum (△), HEV antigen in the stool (□), IgG antibody in the serum (▽), and ALT elevation (○) were determined. HEV RNA in the sera and stool was monitored by RT-PCR. (+) positive; (−) negative.

3.3. Challenge of monkeys with native HEV

We next addressed whether the monkeys immunized with rHEV VLPs are protected from the challenge by native HEV. Three weeks after the final immunization (day 100), two orally immunized monkeys (M4364 and M4365) were intravenously injected with 2 ml (>1.0×10^4 MID50) of the challenge virus. Two monkeys that had recovered from hepatitis E infection (M4200 and M4201) and two HEV-antibody negative monkeys (M4369 and M4418) were similarly injected and used as controls (Fig. 4).

Both of the HEV-antibody negative monkeys showed a pronounced increase in ALT levels at 5 weeks post-challenge. HEV antigen in serum, a marker of viremia, was observed at 1 week post-challenge. Viral RNA in sera appeared simultaneously and correlated well with the presence of antigen in sera. The viral RNA also appeared in stool specimens at 1 week post-challenge, although the peaks of HEV antigen were slightly delayed when compared with those in matched sera, indicating that efficient HEV infection occurred. IgG antibody in serum was detected at 3 weeks post-challenge and reached a maximum at 6–8 weeks. Fecal IgA was not detected.

In contrast, none of the markers indicating viral infection was observed in the HEV-antibody positive monkeys, M4201 and M4200, demonstrating that these two monkeys were protected from infection even when an amount 100 times greater than MID50 HEV was administered. Fecal IgA was not detected in these monkeys.

Interestingly, neither viremia nor ALT elevation was observed in the orally immunized monkeys, M4364 and M4365. In addition, neither HEV antigen nor HEV-RNA was detected in stool from M4364. Therefore, this monkey was completely protected from HEV infection. However, both HEV antigen and the viral RNA were detected in stool from M4365, although the titer was very low (0.172 OD492) and the RNA was detectable for only 3 days. These results indicated that infection occurred in this monkey, but it did not develop the disease. The increased IgG antibody titer during 2–3 weeks after the challenge was additional evidence of infection. Fecal IgA was not detected in any of the orally immunized monkeys.

4. Discussion

A vaccine against HEV could help to prevent epidemics and sporadic cases of hepatitis E in developing countries and afford protection to travelers to those regions. The feasibility of a hepatitis E vaccine was demonstrated by induction of the serum antibodies to HEV in experimentally infected cynomolgus monkeys [32–34]. Epidemiological studies have also provided evidence that IgG antibody is capable of protecting against HEV infection and demonstrated that HEV IgG persists for a long time after infection [35]. Because no cell culture system that permits the propagation of HEV has been established and the amount of the virus recovered from natural infections in human or experimentally infected primates is relatively small, the use of a recombinant protein may be extremely useful for development of an HEV vaccine. We expressed HEV capsid protein, with 111 amino acids truncated at the N-terminus, using a baculovirus expression system. The truncated 58 kDa protein was further processed to 54 kDa proteins through a post-translational cleavage probably mediated by host cellular protease(s) [19,20]. The rHEV VLPs are easy to prepare and can be purified in large quantities, with a yield of approximately 1 mg per 10^7 insect cells. The VLPs possess antigenicity similar to that of authentic HEV particles and, consequently, they were good antigen for the sensitive detection of HEV-specific IgG and IgM antibodies. In addition, the VLPs were immunogenic when they were given to mice orally [20].

In this study, we evaluated the ability of rHEV VLPs to induce neutralizing antibodies to HEV when administered orally to cynomolgus monkeys. Our experiments demonstrated that the rHEV VLPs-induced strong immune responses by oral immunization in monkeys. Oral immunization offers many advantages over parenteral immunization, including easy delivery, greater acceptability by recipients, reduction in the required purity, and reduction in the number of trained personnel needed to administer injections, all of which ultimately reduce production costs. We also found that even without adjuvants serum antibody could be induced. The dose of the immunization, 10 mg of rHEV VLPs per 4 kg monkey, was determined based on the immunization of mice, where 50 μg of rHEV VLP was administered to 20 g weight of mice [20]. The suitability of dose and purity of the antigen must be carefully evaluated for future human clinical testing.

Immunological tolerance is of major concern for oral immunization, since the systemic immunological unresponsiveness often occurs after feeding a soluble antigen. Such unresponsiveness also can be induced by feeding the antigen prior to parenteral immunization. However, our experiments demonstrated that oral immunization with the rHEV VLPs did not induce oral tolerance because the antibody titers of IgG and IgA continually increased after each oral immunization, and serum IgA and IgG antibodies increased rapidly and reached high titers after a boost immunization. This is presumably because rHEV VLPs were administered as particles and they were stable at low pH. Perhaps the capsid protein was folded in a structure that avoided oral tolerance. Similar observation has been reported in the experiment using recombinant Norwalk virus VLP [22].

No fecal IgA response was observed in monkeys following oral administration, which is different from the results obtained in mice immunized with rHEV VLPs. The reason is unknown at the moment. Although the cynomolgus monkey is a useful animal for studying HEV infection, many differences are found from the infection in humans. For example, the animals could not be infected with HEV by the oral route [36], and fecal IgA was detected in humans but not in monkeys post-HEV infection (data not shown). It will

be interesting to determine whether the mucosal immune response occurs in humans after oral administration with rHEV VLPs.

We evaluated infection by intravenous challenges with infectious HEV, and acute hepatitis, viremia, and shedding of the virus in feces were detected in all non-immunized animals. By contrast, orally immunized monkeys were protected against HEV infection (M4364) or against developing hepatitis E (M4365), clearly indicating that the serum antibodies induced by oral immunization have the ability to neutralize infectious HEV. In the case of M4365, the antibody titer was much lower than that of M4364 and a small amount virus shedding was observed in the feces a short time after the challenge. This is probably because some viruses escaped neutralization. In this study, the challenge virus was administered intravenously $>10^4$ MID50 of HEV. This titer may be much higher than that found in naturally occurring HEV infections, suggesting that the efficacy of protection depends on the antibody level and challenge virus doses.

In summary, oral immunization with rHEV VLPs without an adjuvant elicited a neutralization antibody that protects monkeys from either infection or developing the disease. The rHEV VLPs can be an oral hepatitis E vaccine.

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In Vitro and In Vivo Gene Delivery by Recombinant Baculoviruses

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Although recombinant baculovirus vectors can be an efficient tool for gene transfer into mammalian cells in vitro, gene transduction in vivo has been hampered by the inactivation of baculoviruses by serum complement. Recombinant baculoviruses possessing excess envelope protein gp64 or other viral envelope proteins on the virion surface deliver foreign genes into a variety of mammalian cell lines more efficiently than the unmodified baculovirus. In this study, we examined the efficiency of gene transfer both in vitro and in vivo by recombinant baculoviruses possessing envelope proteins derived from either vesicular stomatitis virus (VSVG) or rabies virus. These recombinant viruses efficiently transferred reporter genes into neural cell lines, primary rat neural cells, and primary mouse osteal cells in vitro. The VSVG-modified baculovirus exhibited greater resistance to inactivation by animal sera than the unmodified baculovirus. A synthetic inhibitor of the complement activation pathway circumvented the serum inactivation of the unmodified baculovirus. Furthermore, the VSVG-modified baculovirus could transduce a reporter gene into the cerebral cortex and testis of mice by direct inoculation in vivo. These results suggest the possible use of the recombinant baculovirus vectors in combination with the administration of complement inhibitors for in vivo gene therapy.

The baculovirus *Autographa californica* nuclear polyhedrosis virus is an insect virus possessing a large double-stranded circular DNA genome packaged into a rod-shaped capsid (22). Due to the very strong polyhedrin promoter, baculoviruses have been used as a tool to produce high levels of recombinant protein in insect cells. Several years ago, baculovirus was shown to infect hepatic cell lines and express foreign genes under the control of mammalian promoters (5, 10). We have subsequently shown that recombinant baculoviruses transduce foreign genes into additional cell lines, including those of nonhepatic origin (38). Therefore, baculovirus is now recognized as a useful viral vector not only for abundant expression of foreign proteins in insect cells, but also for gene delivery to mammalian cells (18, 33, 36).

Recently, enhanced gene transfer efficacy was observed in a variety of cell lines with recombinant baculoviruses possessing either other viral envelope proteins, such as vesicular stomatitis virus envelope G protein (VSVG), or excess amounts of its envelope glycoprotein, gp64, on the virion surface (3, 41). Although modification of the virion surface enhanced the efficiency of gene transduction into various cultured cell lines, in vivo gene delivery with recombinant baculoviruses is still unsatisfactory. One obstacle is the inactivation of baculovirus by serum complement (11, 35). In vivo foreign gene transfer with baculovirus vectors into rabbit endothelial cells lining the artery through collar-mediated delivery (1), mouse skeletal muscle cells in the quadriceps by intramuscular injection (32), neural or choroid plexus cells in the rodent brain by intracranial injection (19, 37), and mouse retinal pigment epithelial cells following subretinal injection (8) has been achieved. Few reports exist, however, demonstrating the efficient transfer of genes via baculovirus vectors into internal organs that are directly exposed to serum complement. A recombinant baculovirus possessing decay-accelerating factor, an inhibitor of the various pathways of the complement system, allowed enhanced gene transfer into neonatal rat liver. The level of foreign gene expression, however, was not high, and gene transduction into adult rat liver was not successful (12).

In this study, we examined the efficiency of in vitro and in vivo gene transfer by recombinant baculoviruses possessing rhabdovirus envelope proteins. Recombinant viruses efficiently transferred reporter genes not only into primary neural and osteal cells in vitro but also into the cerebrums and testes of mice in vivo. Addition of a complement inhibitor conferred resistance against serum inactivation of the baculovirus vectors in vitro. The possible application of such a baculovirus vector for future in vivo gene therapy will be discussed.

MATERIALS AND METHODS

Virus construction. Recombinant baculoviruses were constructed as described previously (41). The AcRVG-CAluc and AcRVG-CAGFP viruses possess rabies virus G glycoprotein (RVG) under the control of the polyhedrin promoter as well as the luciferase and green fluorescent protein (GFP) genes under the control of the CAG promoter (27). The RVG gene was excised from pUCRVG (43) by digestion with *Eco*RI and *Hin*dIII, filled in with Klenow enzyme, and cloned into the *Bam*HI site of pAcYM1 (19) after addition of a *Bam*HI linker.

To construct the transfer vector pAcRVG-CAG, the CAG cassette was excised from pCAGGS (27) by *Sal*I and *Bam*HI digestion, filled in with Klenow enzyme, and inserted into the *Eco*RV site of pAcRVG in the opposite direction from the polyhedrin promoter. The luciferase and GFP genes were cloned into the *Bgl*II site of pAcRVG-CAG after addition of a *Bcl*I linker. The correct orientation and

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sequence of each transfer vector construct were confirmed by PCR and sequencing. Sf9 insect cells were cotransfected with the transfer vector and baculovirus DNA (Baculo Gold linearized DNA; Pharmingen, San Diego, Calif.). Following homologous recombination, recombinant baculoviruses were isolated and purified as described previously (20). Recombinant baculoviruses AcVSVG-CAluc, Ac64-CAluc, and AcGFP-CAluc, possessing the luciferase gene under the control of the CAG promoter, and VSVG, gp64, and the GFP gene under the control of the polyhedrin promoter were described previously (41). The infectious titers of the recombinant baculoviruses were determined by plaque assay with Sf9 cells.

Expression of foreign genes in insect and mammalian cells. Expression of RVG protein in insect cells infected with either AcRVG-CAluc or AcRVG-CAGFP was analyzed by immunofluorescence and Western blot analysis. Cell extracts and purified viruses were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). An anti-rabies virus rabbit polyclonal antibody (43) was used to detect RVG protein, which was visualized with the alkaline phosphatase assay method as described previously (34). Immunofluorescence analysis was carried out as described previously (45), with cell fixation performed with 4% paraformaldehyde. Expression of either luciferase or GFP proteins in mammalian cells infected with the recombinant baculoviruses was then examined by either the luciferase assay method or fluorescence microscopy, as described previously (41).

Cell cultures and infection with recombinant baculoviruses. The mammalian neural cell lines BC3H1 (mouse brain, smooth muscle-like tumor), NB41A3 (mouse brain, neuroblastoma), SK-N-MC (human brain, neuroblastoma), IMR32 (human brain, neuroblastoma), and PC-12 (rat adrenal gland, pheochromocytoma) as well as the human hepatoma cell line HepG2 were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). BC3H1, SK-N-MC, IMR32, and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) containing 2 mM L-glutamine, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% (vol/vol) heat-inactivated fetal calf serum (FCS). PC-12 and NB41A3 cell lines were cultivated in RPMI 1640 (Gibco Laboratories) with 10% FCS and Ham's F-12 medium (Gibco Laboratories) supplemented with 2.5% FCS and 15% horse serum, respectively. Recombinant baculoviruses were inoculated into 10^5 cells in 24-well plates at a multiplicity of infection (MOI) of 50 by a 1-h incubation, facilitating viral adsorption. After washing and the addition of fresh medium, the cells were incubated at 37°C for 24 h.

Primary cell cultures and infection with recombinant baculoviruses. All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University). Primary cerebellar and hippocampal cultures were prepared from Wistar ST rats at embryonic day 19 (CLEA Japan, Tokyo, Japan). Briefly, the whole cerebellum or hippocampus, including the region of the cerebral cortex connecting to the hippocampus, was dissected out from rat brains and treated with 0.1% trypsin. Trypsin-treated tissues were dissociated in 0.05% DNase I and resuspended in seeding medium. Cerebellar cultures were maintained in Dulbecco's modified Eagle's medium-F12 medium (Gibco Laboratories) containing 0.5% FCS, putrescine (100 μM), sodium selenite (30 nM), L-glutamine (4 mM), triiodothyronine (0.5 μg/ml), progesterone (5 nM), bovine insulin (10 μg/ml), transferrin (100 μg/ml), and gentamicin (10 μg/ml). The mixture of cerebral cortex and hippocampal cultures was suspended and grown in neurobasal medium (Gibco Laboratories) containing B27 supplement and L-glutamine (0.5 mM) (Gibco Laboratories).

Cells were seeded at 2×10^5 cells/well on poly-L-ornithine-coated coverslips in 24-well plates. These cultures were infected with either AcVSVG-CAGFP or AcRVG-CAGFP at an MOI of 100 at 8 days after incubation. Primary neuronal cultures grown on coverslips were washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde for 20 min, and permeabilized with PBS containing 0.25% Triton X-100 for 10 min at room temperature. Cells were then double labeled with either rabbit anti-GFP (Molecular Probes, Eugene, Oreg.), mouse anti-Calbindin (Swant, Bellinzona, Switzerland), mouse anti-MAP2 (ICN Biomedicals, Costa Mesa, Calif.), or mouse anti- glial fibrillary acidic protein (GFAP) (Zymed Laboratories, Inc., South San Francisco, Calif.) for 30 min to 1 h at 37°C. After incubation, cells were washed with PBS and incubated with Alexa 568-conjugated anti-mouse or Alexa 488-conjugated anti-rabbit immunoglobulin antibody (Molecular Probes) for 30 min at room temperature. Coverslips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.) and subjected to microscopic observation. Images were acquired on a Zeiss LSM510 confocal microscope (Carl Zeiss Inc., Thornwood, N.Y.). The luciferase activity of cerebellar cultures following infection with Ac64-CAluc, AcRVG-CAluc, or AcVSVG-CAluc at an MOI of 50, 100, and 150 was determined. Luciferase activities were determined 2 days after incubation.

Osteoclast or osteoblast cells were isolated from female ddY mice (Japan SLC, Hamamatsu, Japan) and resuspended in Dulbecco's modified Eagle's medium (Gibco Laboratories) containing 10% FCS as described previously (17, 29). After cultivation in a 48-well plate for 3 days, cultures were infected with either AcVSVG-CAluc or Ac64-CAluc at various MOIs. Luciferase activities were determined 2 days after incubation.

Effects of animal serum and complement inhibitor on baculovirus infectivity. Blood was freshly drawn from animals and healthy volunteers after obtaining informed consent. Sera were isolated by centrifugation at 3,000 × *g* for 10 min at 4°C. Serum complement was inactivated at 56°C for 30 min. To determine the effects of serum complement on the inactivation of baculovirus, 10 μl of either AcVSVG-CAluc or AcGFP-CAluc (2×10^9 PFU/ml) was incubated with 90 μl of either untreated or heat-inactivated serum for 1 h at 37°C. AcGFP-CAluc was also incubated for 1 h at 37°C in the presence of rat or human serum with various concentrations of FUT-175 (6-amidino-2-naphthyl 4-guanidinobenzoate; Torii & Co., Ltd., Tokyo, Japan), a synthetic protease inhibitor that inhibits C1r or C1 esterase (26, 40) and C3 convertase (13). Residual infectivity was determined by inoculation into HepG2 cells. Luciferase activity was determined 24 h after incubation.

Direct injections of recombinant baculovirus into mouse brain and histological analysis. BALB/c mice were obtained from CLEA Japan. Three-week-old female mice were injected in the right lateral ventricle with 10 μl of purified AcVSVG-CAGFP (2×10^9 PFU/ml in PBS containing 0.04% trypan blue) with a 28-gauge KN-386 needle (Natsume Co., Ltd., Tokyo, Japan). To assess GFP expression in the brain, fluorescent stereomicroscopic pictures of the whole brain were obtained 24 h after injection. Brains were rinsed with PBS and observed under a Leica WILD M10 fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). Brain samples were then fixed in 4% paraformaldehyde, cryopreserved in 30% sucrose and 5% glycerol, and frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Cryostat sections (10 μm) were observed under a fluorescence microscope. Sections were further examined by immunohistochemical analysis after staining with either rabbit anti-GFP (Molecular Probes), mouse anti-MAP2 (ICN Biomedicals), or mouse anti-GFAP (Zymed Laboratories) as described above.

Gene transduction by recombinant baculovirus into mouse testis. AcVSVG-CAGFP was delivered into mouse testes via the efferent ductules with an injection pipette, as described by Ogawa et al. (28). Trypan blue (0.02%) was included in the virus suspension to monitor the filling of the seminiferous tubules. Approximately 10 μl of viral suspension (2×10^{10} PFU/ml) was injected, filling approximately 70% of the seminiferous tubules with the viral suspension. Two days after injection, the testes were observed under a fluorescence stereomicroscope.

RESULTS

Construction of recombinant viruses. To examine cell surface expression of foreign envelope proteins, Sf9 cells were infected at an MOI of 1 with the recombinant baculoviruses AcRVG-CAluc, Ac64-CAluc, and AcVSVG-CAluc, containing the RVG, gp64, and VSVG genes, respectively, under the control of the polyhedrin promoter. The cell surface expression of foreign envelope proteins on Sf9 cells was detected by using specific monoclonal antibodies (Fig. 1A). Incorporation of rhabdovirus envelope proteins into the viral particles was evaluated by Western blot analysis of purified viruses. The RVG and VSVG envelope proteins were detected in AcRVG-CAluc and AcVSVG-CAluc particles (Fig. 1B). Incorporation of gp64 protein into Ac64-CAluc was higher than that of either AcVSVG-CAluc or AcRVG-CAluc, as reported previously (41).

Gene delivery into neural cells. To investigate the efficiency of the recombinant baculoviruses for the gene transduction of neural cells, five neural cell lines (BC3H1, NB41A3, SK-N-MC, IMR32, and PC-12) were infected with recombinant baculoviruses at an MOI of 50. Reporter gene expression was analyzed at 24 h postinfection. All of the neural cell lines

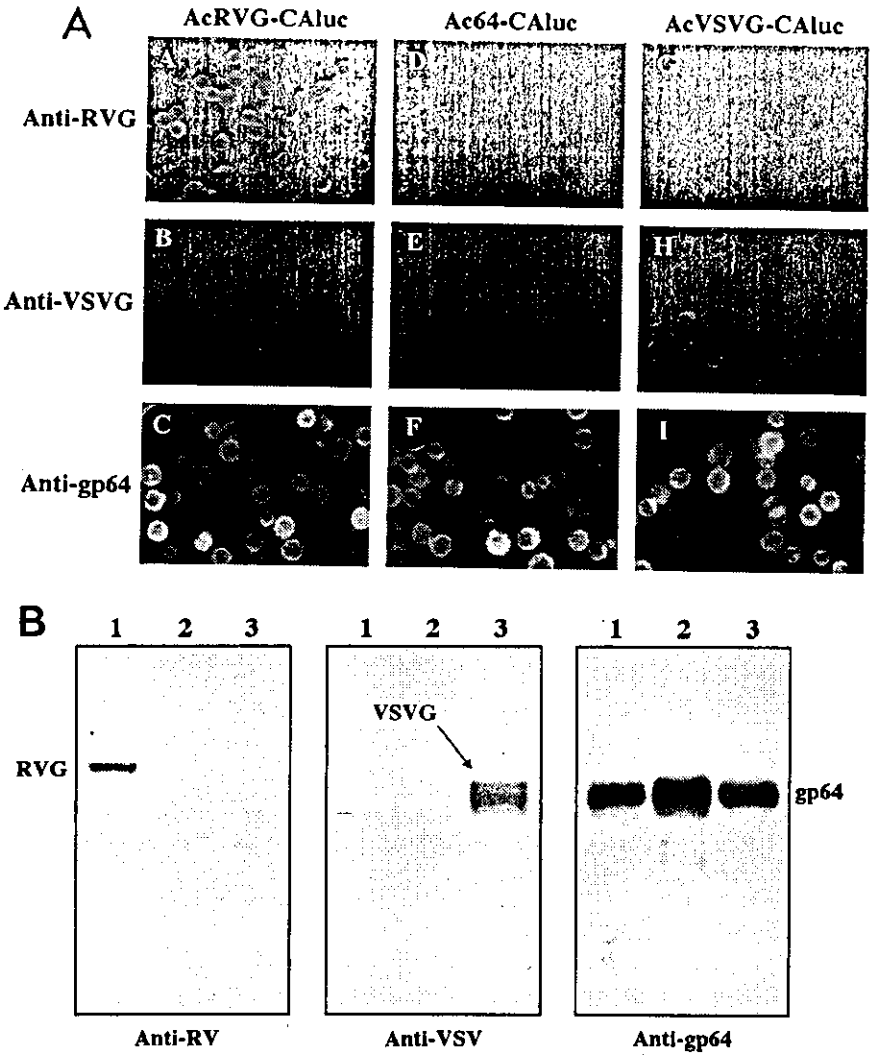

FIG. 1. Expression of viral envelope proteins in insect cells and incorporation of envelope proteins into the recombinant baculoviruses. (A) Sf9 cells were infected with AcRVG-CAluc (A to C), Ac64-CAluc (D to F), or AcVSVG-CAluc (G to I) at an MOI of 1 and harvested 72 h after infection. Expression of RVG (A), VSVG (H), and gp64 (C, F, and I) was examined by immunofluorescence analysis after fixation in 4% paraformaldehyde. (B) The incorporation of RVG, VSVG, and gp64 into the purified virions was examined by Western blotting. Lanes 1, 2, and 3, AcRVG-CAluc, Ac64-CAluc, and AcVSVG-CAluc, respectively.

infected with AcRVG-CAluc or AcVSVG-CAluc exhibited 5- to 1,000-fold higher expression than those infected with either Ac64-CAluc or AcGFP-CAluc, which contain the GFP gene under the control of the polyhedrin promoter (Fig. 2). AcVSVG-CAluc and Ac64-CAluc exhibited higher expression than AcRVG-CAluc and AcGFP-CAluc in HepG2 cells. In PC-12 cells, a rat adrenal pheochromocytoma cell line, AcVSVG-CAluc induced about 50 times higher gene transduction than AcRVG-CAluc. These results indicated that the incorporation of rhabdovirus envelope proteins into baculoviruses can promote gene delivery into neural cell lines.

We next examined the efficiency of gene transfer by recombinant baculoviruses into primary rat cerebellar cells (Fig. 3). While the efficiencies of gene transfer into primary rat cerebellar cells with recombinant baculoviruses were 10- to 100-fold lower than those into mouse and human neural cell lines, these transduction rates were similar to those for rat PC-12 cells. Although AcRVG-CAluc and Ac64-CAluc exhibited lower gene transduction into the PC-12 cell line than AcVSVG-CAluc, AcRVG-CAluc exhibited higher gene transduction into primary rat cerebellar cells than AcVSVG-CAluc and Ac64-CAluc. To examine foreign gene expression in primary neural cells in greater detail, we examined GFP expression in primary cerebellar and hippocampal cultures infected with AcVSVG-CAGFP with the confocal microscope. Immunostaining of GFP-expressing cells with a Calbindin purkinje cell marker, a GFAP glial marker, and a MAP2 neuronal marker confirmed the phenotype of the infected cells. AcVSVG-CAGFP transferred GFP to purkinje cells (Fig. 4A to C), astrocytes (Fig. 4D to F), and pyramidal cells (Fig. 4G to

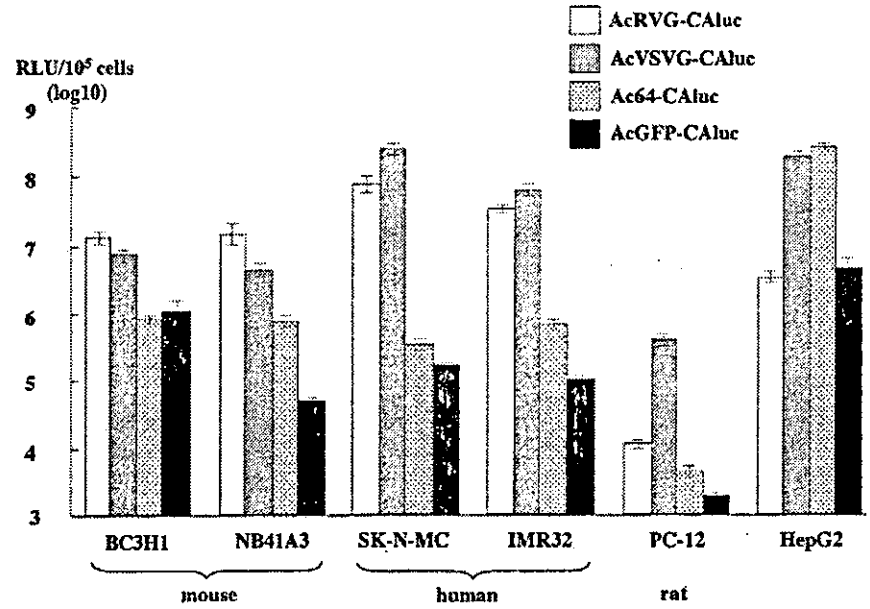

FIG. 2. Expression of luciferase in various neural cell lines infected with recombinant baculoviruses. Neural cells were infected with AcRVG-CAluc, AcVSVG-CAluc, Ac64-CAluc, or AcGFP-CAluc at an MOI of 50. Luciferase expression was determined 24 h after infection. Values for relative light units (RLU) reflect values obtained for the extracts from 10^5 cells. The value for mock-infected cells was subtracted from all values. The results shown are the averages of three independent assays, with error bars representing the standard deviation.

I). A similar level of GFP expression in these primary neuronal cells was demonstrated following infection with AcRVG-CAGFP (data not shown).

Despite comparable gene delivery into human and mouse neural cell lines and primary rat neural cells of the recombinant baculoviruses possessing RVG protein (AcRVG-CAGFP and AcRVG-CAluc) and those possessing VSVG protein, gene transduction into HepG2 and PC-12 cell lines was low. We therefore used AcVSVG-CAGFP and AcVSVG-CAluc for further in vitro and in vivo experiments.

Gene transfer into osteal cells. Recently, the efficient gene transduction of osteogenic sarcoma cell lines by recombinant

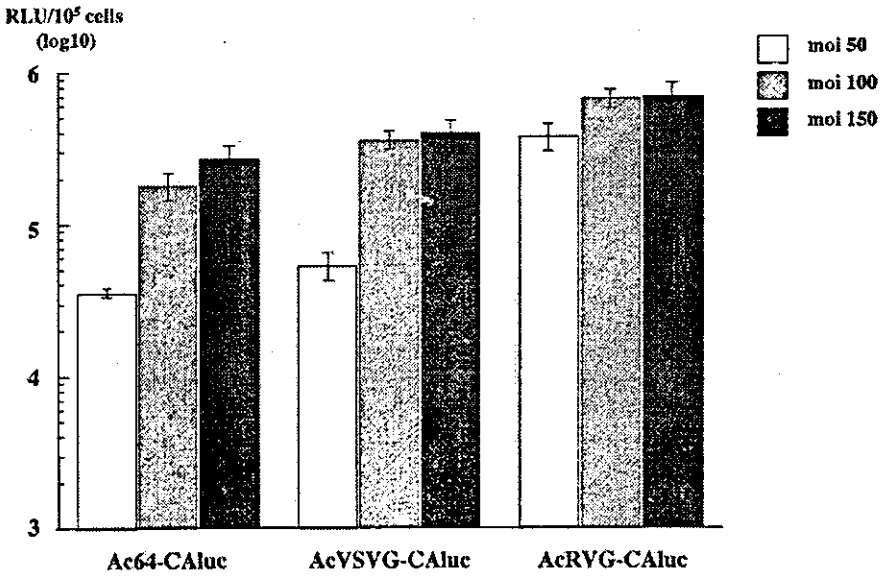

FIG. 3. Expression of luciferase in primary rat cerebellar cells infected with recombinant baculoviruses. Rat cerebellar cells were infected with Ac64-CAluc, AcVSVG-CAluc, or AcRVG-CAluc at MOIs of 50, 100, and 150. Luciferase expression was determined 24 h after infection. Values for relative light units (RLU) reflect values obtained for the extracts from 10^5 cells. The value for mock-infected cells was subtracted from all values. The results shown are the averages of three independent assays, with error bars representing the standard deviation.

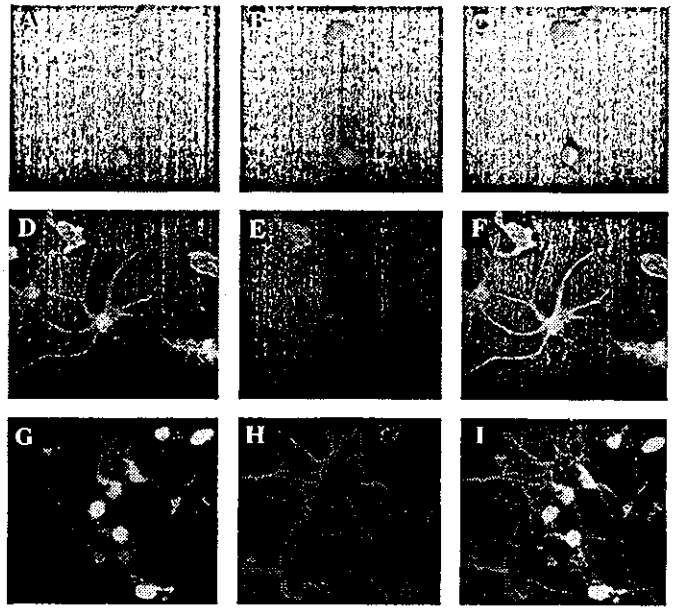

FIG. 4. Gene transduction into primary rat cerebellar cells with AcVSVG-CAGFP. Primary rat cerebellar and hippocampal cultures were infected with AcVSVG-CAGFP. Immunofluorescence was examined by confocal microscopy. (A, D, and G) Anti-GFP immunochemistry. (B) Anti-Calbindin immunochemistry as a purkinje marker. (E) Anti-GFAP immunochemistry as a glial marker. (H) Anti-MAP2 immunochemistry as a neuronal marker. C, F, and I are merged images.

baculoviruses possessing cytomegalovirus or Rous sarcoma virus promoters was reported (39). To determine the efficiency of gene transfer by recombinant baculoviruses into primary osteal cells, primary mouse osteoblasts and osteoclast cells were infected with either AcVSVG-CAluc or Ac64-CAluc (Fig. 5). AcVSVG-CAluc transduced the luciferase gene into osteal cells more efficiently than did Ac64-CAluc, in a dose-dependent manner. For transduction into osteoclast cells, 100- to 1,000-fold more virus than was needed for osteoblast cells was required. It is worth noting that no cytopathic effects were observed even at high MOIs. The necessity for increased viral titers may reflect the size of the cells (osteoclasts are 10- to 20-fold bigger than osteoblasts) or the expression of cellular receptors specific for the recombinant viruses.

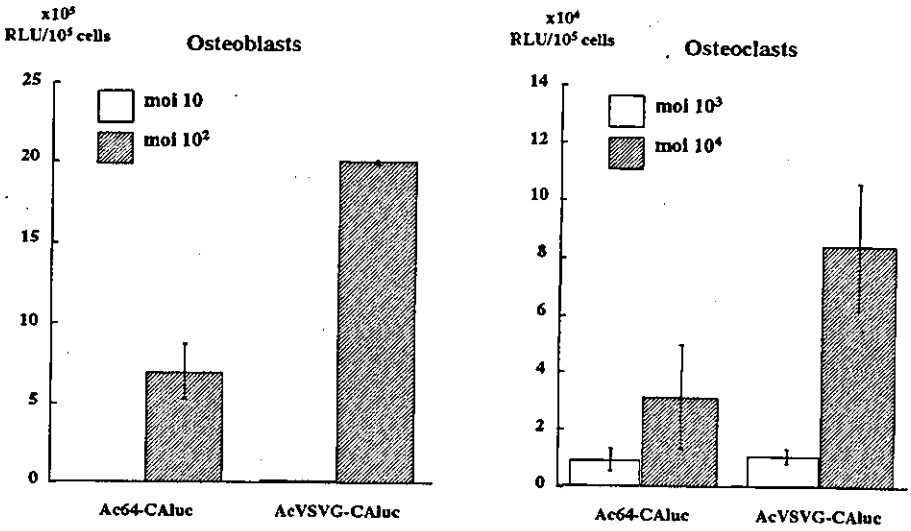

FIG. 5. Gene transduction into primary mouse osteoblast (left) and osteoclast (right) cells with recombinant baculoviruses. Osteal cells (10^5) were infected with either Ac64-CAluc or AcVSVG-CAluc at various MOIs. Following harvest at 24 h after infection, luciferase expression was determined. Values for relative light units (RLU) reflect values obtained for the extracts from 10^5 cells. The value for mock-infected cells was subtracted for all values. The results shown are the averages of three independent assays, with error bars representing the standard deviation.