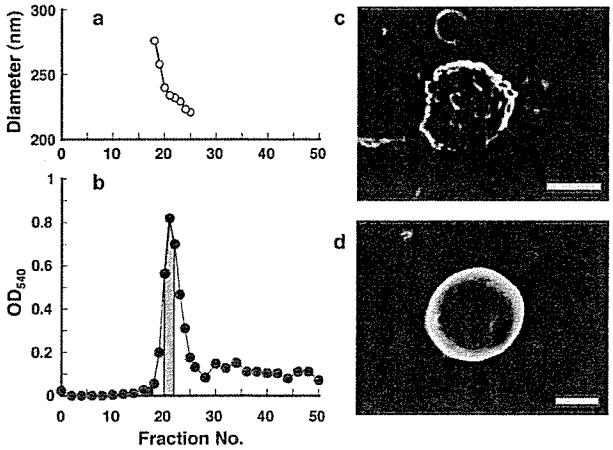

Fig. 1. Purification and Characterization of Recombinant Sendai Virus Particles

SeV-GFP vectors (10 mg protein), partially purified using sucrose step centrifugation, were separated on Sephacryl S1000 columns. The average diameter of the viral particles in each fraction (a) and the optical density (OD) at 540 nm (b) were determined as described in the Materials and Methods. Major peak fractions, indicated by a gray shadow, were collected and used for the perfusion experiment. The average diameter of the viral particles present in this pooled peak fractions was 239.9 nm (polydispersity index 0.083). Recombinant Sendai virus (SeV-EGFP) (c) and naked unilamellar liposomes (d) were fixed with 2.5% glutaraldehyde and trapped on polycarbonate membrane filters (pore size 0.05 μm.). The samples were then dehydrated, dried, coated with osmium and examined as described in the Materials and Methods. Scale bar 100 nm.

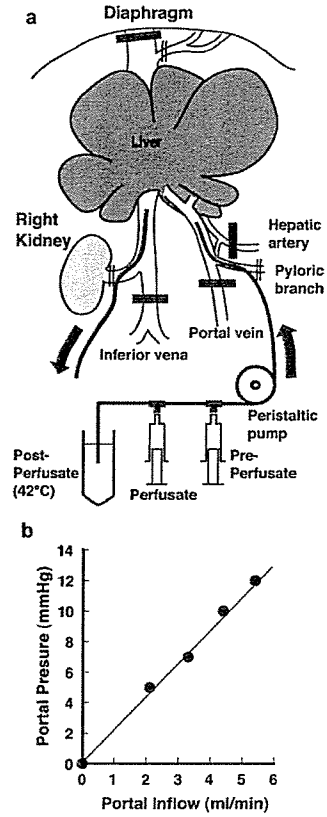

Fig. 2. Portal Pressure Control during Hepatic Perfusion

The distal part of the portal vein, the inferior *vena cava*, the suprahepatic *vena cava* and hepatic artery were clamped just before perfusion as indicated in (a). The preperfusate solution was switched to the perfusate and then to the postperfusate solution using a three-way stopcock. The post perfusate solution was maintained at 42 °C in a water bath. Perfusion inflow was controlled using a peristaltic pump. The relation between the portal inflow and the portal pressure is shown in (b) as the mean of results obtained from two independent experiments.

the particle size distribution of the viruses (data not shown). We also characterized the structure of the recombinant virus using scanning electron microscopy, in comparison with that of the naked liposomes. After the samples had been coated with a 2 nm thick layer of osmium,[25] the viral particle was observed as a microsphere with a complex surface structure and a diameter consistent with that determined by dynamic light scattering (Fig. 1c). On the other hand, the naked unilamellar liposome was observed as a microsphere with a smooth surface (Fig. 1d).

Using these characterized recombinant Sendai virus particles, we optimized the conditions for delivering them by isolated hepatic perfusion in rats. To minimize damage to the hepatic tissue by ischemia, the surgical procedure was arranged to minimize the total perfusion period (Fig. 2a). The animals tolerated the surgical procedure well, without significant irreversible damage in hepatic tissues. One of the key parameters for the successful delivery of Sendai virus vectors by hepatic perfusion is the control of portal pressure. The mean pore size of the hepatic sinusoid is 117 nm (ranging from 40 to 260 nm) under normal portal pressure (7 mmHg).[28] However, the sinusoid is a relatively flexible structure and the mesh size of the sieve can be altered artificially by controlling the portal pressure. Thus, Fraser *et al.* reported that the mean inner diameter of the hepatic sinusoid increases to 193 nm (ranging from 60 to 600 nm) when the vessel is perfused under increased portal pressure (15 mmHg).[28] We controlled portal pressure precisely by regulating the rate of perfusion with a peristaltic pump. As shown in Fig. 2b, we found that the portal pressure correlated linearly with the perfusion rate, from 5 mmHg (average portal pressure of the normal experimental animals) at 2.1 ml/min to 12 mmHg at 5.4 ml/min. We used a portal pressure of 12 mmHg or less, because greater pressure has been defined as clinically significant portal hypertension.[29] We expected that the sinusoids might alter their sieve structure reversibly within this physiological range, allowing the Sendai virus particles to pass through the sieve. Administration under the controlled high pressure is also a key factor in hydrodynamic-based transfection of plasmid DNA through peripheral vein infusion.[30] For example, Zhang *et al.* reported that the efficient plasmid delivery was observed in rats under administration at 24 ml/min,[31] the condition less physiologic compared to our perfusion condition; we could not detect any luciferase activity when 150 μg of pCMV-luc[32] was perfused at 5.4 ml/min (data not shown).

We also optimized the concentration of the vectors in the perfusate to minimize hepatic injury. We first perfused 10^9 plaque-formation units (pfu) of Sendai virus (10^8 pfu/ml in 10 ml) and examined tissue sections prepared 14 h later. As the total number of parenchymal hepatocytes in the rat liver is *ca.* 5 10^8, and as 1 pfu of virus corresponds to about 50 physical viral particles (M. Nakanishi, unpublished observations), this amount of vector should suffice for delivering foreign genes into all the parenchymal hepatocytes. However, perfusion of the vector at 10^8 pfu/ml caused extensive liver necrosis (Fig. 3a). These lesions might have resulted from ischemia caused by microcirculatory dysfunction, because blood clotting in the hepatic vessels was detected in the tissue sections (arrowheads in Fig. 3). As this was not observed

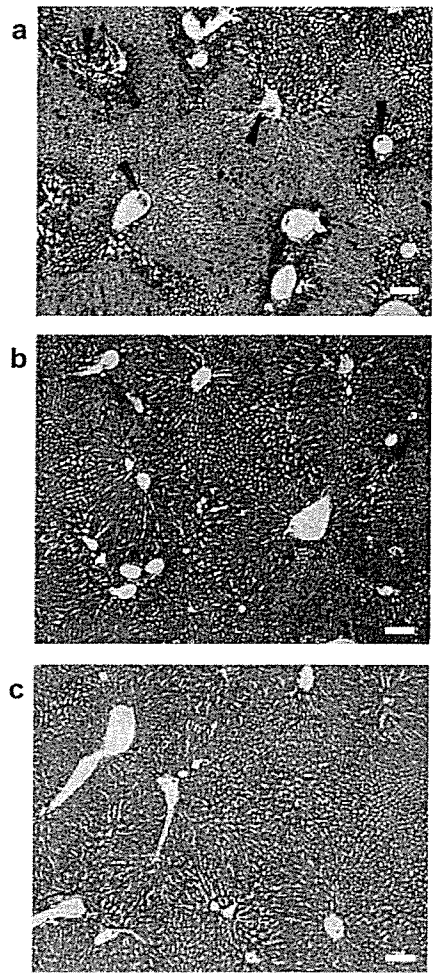

Fig. 3. Histological Analysis of Sections of Hepatic Tissues Perfused Under Various Conditions and Fixed After 14 h

Tissue sections were examined after standard hematoxylin and eosin staining. Perfusion conditions were: perfusion with SeV-EGFP vector at 10^8 pfu/ml and postperfusion at 25 °C (a); perfusion at 10^8 pfu/ml and postperfusion at 42 °C (b); perfusion with 10^7 pfu/ml and postperfusion at 42 °C (c). Arrowheads indicate blood vessels with clotting. Scale bar 100 nm.

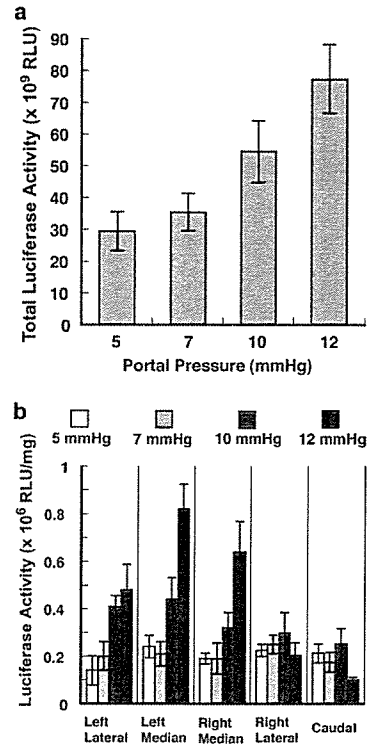

Fig. 4. Luciferase Activity in the Hepatic Tissue Perfused with SeV-luc Vector

SeV-luc (10^8 pfu) was perfused under various portal pressures. After 4 h, the animals were killed and the luciferase activities in the liver were determined as described in the Materials and Methods. Total luciferase activity in hepatic tissue (a) is presented as mean luciferase activities and standard deviations in relative light units (RLU), based on four independent experiments. Specific luciferase activity in each lobe (b) is presented as mean RLU per microgram of protein with standard deviations, based on four independent experiments. Wet weights of the tissues were: left lateral lobe, 3.7 0.4 g; left median lobe, 2.6 0.3 g; right median lobe, 1.1 0.1 g; right lateral lobe, 2.0 0.2 g and caudate lobe, 0.8 0.1 g.

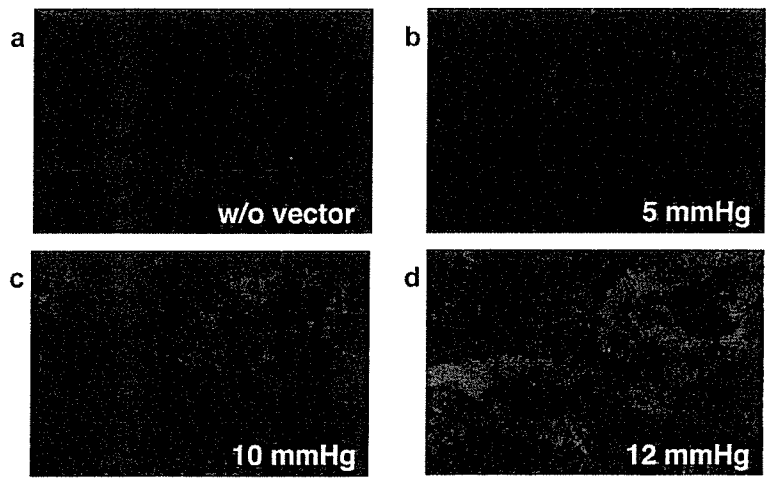

Fig. 5. Levels of Enhanced Green Fluorescent Protein (EGFP) in Hepatic Tissues Perfused with SeV-EGFP Vector (10^7 pfu/ml) at the Following Portal Pressures: 5 mmHg (b), 10 mmHg (c), and 12 mmHg (d)

As a control, lactate-Ringer solution containing no vector was perfused at 12 mmHg in (a). After 12 h, the animals were killed and the expression of EGFP in the right median lobe was examined as described in Materials and Methods.

in the tissue samples perfused without viral particles (data not shown), we believe that the viral particles, or the viral envelope proteins on the vessel surface, entrapped the blood cells just after recirculation to cause clotting. However, extensive postperfusion at 25 °C could not prevent these lesions (data not shown). Perfusion at lower temperatures is desirable to avoid hepatic dysfunction and to maintain the biological function of the vectors in the perfusate,[33] whereas incubation at a higher temperature is essential for stimulating virus-induced membrane fusion[34] and for the rapid clearance of the viral glycoproteins from the cell surface.[35] Therefore, we examined the effect of a brief hyperthermic postperfusion (42 °C for 5 min) and found that this procedure largely prevented the formation of necrotic lesions (Fig. 3b).

We also identified vector concentration as another key factor affecting the hepatic lesion: decreasing the vector concentration to 10^7 pfu/ml reduced the rate of formation of hepatic lesions significantly (data not shown). The combination of a perfusion at lower vector concentration (10^7 pfu/ml) with hyperthermic postperfusion (42 °C for 5 min) prevented these necrotic lesions almost completely (Fig. 3c). Therefore, we used these conditions in the subsequent gene transfer experiments.

Using the isolated hepatic perfusion procedures optimized above, we characterized marker gene expression induced by the recombinant Sendai virus vectors expressing luciferase (SeV-luc) and enhanced green fluorescent protein (SeV-EGFP). First, we examined the effect of portal pressure on the luciferase activity in the hepatic tissue into which 10^8 pfu (10^7 pfu/ml in 10 ml) of the SeV-luc vectors had been perfused under various portal pressures. As shown in Fig. 4a, total luciferase activity recovered from the whole liver tissue increased to 260% when the portal pressure during the perfusion was increased from 5 to 12 mmHg. Significant enhancement of gene expression was observed in three upper lobes (the right median lobe, the left median lobe and the left lateral lobe) and the specific luciferase activity (relative light units per microgram of protein) in these lobes increased to 340% (Fig. 4b). These three lobes, corresponding to 73% of the total liver mass, expressed 92% of the total luciferase activity at a portal pressure of 12 mmHg. On the other hand, luciferase activities in the lower lobes did not respond to this portal pressure significantly (Fig. 4b). Thus, at least in the upper major lobes, a moderate portal pressure (12 mmHg) enhanced the delivery of the vectors across the sinusoid barrier into the hepatic tissues. This observed unevenness in gene expression may be caused by inevitable technical difficulty of the surgical procedure in small animals; de Roos *et al.* reported that the trans-gene expression varied significantly (up to 1000-fold) among experiments when adenovirus vectors were administrated by isolated liver perfusion.[15]

Next, we examined the effect of portal pressure on the spatial distribution of the cells expressing the marker genes in the hepatic tissue. We delivered 10^8 pfu (10^7 pfu/ml in 10 ml) of the SeV-EGFP vectors by perfusion under various portal pressures, then fixed the livers and examined the expression of EGFP in frozen sections using fluorescent microscopy (Fig. 5). When the vectors were delivered at the normal portal pressure (5 mmHg), the expression of EGFP was restricted to the endothelium of the hepatic vessels (Fig. 5b).

Table 1. Tissue Distribution of Luciferase Activity in Animals into Which SeV-luc Was Administered by Isolated Hepatic Perfusion

	Luciferase activity (10^9 RLU/tissue)						
	Liver	Heart	Lung	Kidney	Spleen	Testis	Thymus
5 mm Hg	11.3 2.7	0.01	0.01	0.01	0.01	0.01	0.01
7 mm Hg	13.1 2.5	0.01	0.01	0.01	0.01	0.01	0.01
10 mm Hg	19.5 4.0	0.01	0.01	0.01	0.01	0.01	0.01
12 mm Hg	27.6 3.3	0.01	0.01	0.01	0.01	0.01	0.01

SeV-pluc (10^8 pfu) was perfused under the portal pressures indicated. After 4 h, the animals were killed and the total luciferase activity in each tissue was determined as described in Materials and Methods. The data are presented as means and standard deviations of luciferase activity in relative light units (RLU), based on four animals. 0.01 indicates that the luciferase activity was below the detection limit.

This observation is consistent with the hypothesis that normal hepatic sinusoids prevent the effective access of particles larger than 100 nm in diameter to the parenchymal hepatocytes. However, as the portal pressure was increased, EGFP became detectable in hepatocytes distant from the hepatic vessels (Fig. 5c), and almost all the parenchymal hepatocytes expressed EGFP at the portal pressure of 12 mmHg (Fig. 5d). Increased EGFP expression in the parenchymal hepatocytes was observed in the right median lobe (Fig. 5) as well as in the caudate lobe (data not shown). Thus, a moderate portal pressure of 12 mmHg was sufficient to deliver the Sendai virus-based vectors across the hepatic sinusoid sieve.

We then examined the effectiveness of isolated hepatic perfusion in preventing gene delivery to nonhepatic tissues. We perfused 10^8 pfu of the SeV-luc vector through the portal vein, and examined luciferase activities in hepatic and nonhepatic tissues. As shown in Table 1, activities in nonhepatic tissues (heart, lung, left kidney, spleen, testis and thymus) were below the detection limit, regardless of the perfusion rate (portal pressure), whereas the hepatic tissue expressed this enzyme at least 1000 times greater that of the nonhepatic tissues. We also perfused 10^8 pfu of the SeV-EGFP vectors through the portal vein at 12 mmHg, and found no EGFP-positive cells in nonhepatic tissues examined as above (data not shown). Therefore, we conclude that our perfusion protocol is an effective approach for targeting gene delivery vectors to the hepatic tissues and for preventing undesired systemic vector delivery.

DISCUSSION

Although the liver is one of the major target organs for gene therapy of inheriting metabolic disorders, no practical strategy has been established yet that achieves continuous supplement of therapeutic gene products in this organ. This is partly due to the limitations of current gene transfer vectors that persistent expression of therapeutic genes requires their integration into the host chromosome. Plasmid DNA and adenovirus vectors are not considered as practical candidates in liver-targeted gene therapy because they usually could not insert genes into the host chromosome. Retrovirus, lentivirus- and AAV-vectors can integrate their genome into the host chromosomes, but with low efficiency in nondividing tissue cells (including hepatocytes). Even if we can facilitate random gene integration dramatically, it may cause severe side effects (oncogenesis) instead, as revealed in re-

cent clinical trials using bone-marrow stem cells.

In this context, the Sendai virus vector is unique among the current vector systems. It transcribes mRNA from the cytoplasmic RNA genome, which avoids the possible side effects caused by the random gene integration. Furthermore, we recently develop a novel Sendai virus vector based on a variant virus strain, capable of sustained gene expression without chromosomal integration. By using this novel vector, we observed that strong marker gene expression has been sustained for more than six months in cultured cells without obvious cytotoxicity (Nishimura and Nakanishi, manuscript in preparation). These characteristics strongly suggest that this novel Sendai virus-based vector will becomes a candidate tool for gene therapy targeted to the liver. Nevertheless, application of the Sendai virus vector is limited due to the absence of the appropriate protocol of administration to the liver.

The primary objective of this article is to demonstrate a novel and practical protocol for administrating the recombinant Sendai virus vectors into the liver *in situ*. As the sizes and structures of the various Sendai virus vectors are essentially same regardless their genetic structures, the procedure described in this article is also applicable to the improved vectors. Our isolated hepatic perfusion procedure provides for the first time a protocol for administering Sendai virus vectors efficiently into hepatic tissues *in situ*. Moreover, we have proved that this procedure effectively avoids the delivery of foreign genes into nonhepatic tissues, in particular the gonads. The ethical guidelines for gene therapy prohibit the delivery of foreign genes into the reproductive organs (testis and ovary) and germ cell lineage.[36] Moreover, ectopic unregulated expression of therapeutic genes may cause unexpected side effects. Therefore, delivering the vectors to appropriate target organs and tissues, and restricting the expression of the therapeutic genes to these target sites are fundamental issues for *in vivo* gene therapy.

The vectors we used in this study are prototype Sendai virus vectors retaining full function of viral replication. These vectors are ready to prepare in large-scale and are suitable for examining transient (but strong) gene expression *in situ*. However, due to the death by viremia, they are not suitable for examining the time course of gene expression and for evaluating therapeutic effect of the gene products. This forced us to assay gene expression in the early stage after administration (at 4 h for luciferase and at 12 h for EGFP), thus made it difficult to compare the gene expression with other system. This failure was not due to the surgical procedure but reflected the characteristics of the prototype vector, because all the animals perfused with UV-inactivated virus survived without symptom of the hepatic failure (data not shown). Currently, we are investigating the procedure for large-scale production of novel improved Sendai virus vectors, and will examine their effectiveness using the isolated perfusion protocol described in this article.

Although the hepatic perfusion is not yet established as a standard clinical procedure, regional high-dose administration of melpharan *via* isolated hepatic perfusion was effective for treating nonresectable hepatic metastatic tumors in several clinical trials.[12,13] The morbidity of the operation was reported to be acceptably low in a recent clinical trial for treating patients with nonresectable hepatic metastases of colorectal cancer (2%, or one perioperative death in 50 patients),[37] suggesting that this procedure may become a practical procedure for gene therapy. Therefore, successful hepatic delivery of improved recombinant Sendai viruses promises to become an important step for gene therapy of metabolic diseases.

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RESEARCH ARTICLE

Naked Sendai virus vector lacking all of the envelope-related genes: reduced cytopathogenicity and immunogenicity

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Abstract

Background Sendai virus (SeV) is a new class of cytoplasmic RNA vector that is free from genotoxicity that infects and multiplies in most mammalian cells, and directs high-level transgene expression. We improved the vector by deleting all of the envelope-related genes from the SeV genome and thus reducing its immunogenicity.

Methods The matrix (M), fusion (F) and hemagglutinin-neuraminidase (HN) genes-deleted SeV vector (SeV/ΔMΔFΔHN) was recovered in a newly established packaging cell line. Then, the generated SeV/ΔMΔFΔHN vector was characterised by comparing with single gene-deleted type SeV vectors.

Results This SeV/ΔMΔFΔHN vector carrying the green fluorescent protein gene in place of the envelope-related genes could be propagated to a titer of more than 10^8 cell infectious units/ml. This vector showed an efficient transduction capability *in vitro* and *in vivo*, and the cytopathic effect and induction of neutralizing antibody *in vivo* were greatly reduced compared with those of single gene-deleted type SeV vectors. No activity of neutralizing antibody or anti-HN antibody was seen when SeV/ΔMΔFΔHN was transduced *ex vivo*. Additional introduction of amino acid mutations that had been identified from SeV strains causing persistent infections was also effective for the reduction of cytopathic effects.

Conclusions The deletion of genes from the SeV genome and the additional mutation are very effective for reducing both the immunogenic and cytopathic reactions to the SeV vector. These modifications are expected to improve the safety and broaden the range of clinical applications of this new class of cytoplasmic RNA vector.

Keywords Sendai virus; cytoplasmic RNA vector; gene deletion; reduced cytotoxicity; genotoxicity-free; gene therapy

Introduction

A new class of cytoplasmic RNA vectors is thought to provide advanced transgene expression systems that can overcome recent problems of genetic disturbance caused by presently available vectors. The Sendai virus (SeV) vector, based on SeV (belonging to the genus *Respirovirus* of the family *Paramyxoviridae*), infects and multiplies in most mammalian cells, and directs high-level transgene expression. Its replication is independent of nuclear

functions, and there is no DNA phase during its life cycle, so that the possible transformation of cells due to the integration of vector materials into the cellular genome is not a concern [1]. These properties make SeV vectors very promising for applications to gene therapy (cytoplasmic gene therapy) and vaccination via the expression of therapeutic genes and antigens [2,3]. In particular, a clinical trial protocol for therapeutic induction of angiogenesis, which is for the treatment of critical limb ischemia using an SeV vector carrying the fibroblast growth factor-2 gene [4,5], has been approved by the Ministry of Health, Labour and Welfare (MHLW) of Japan and will begin soon. In addition, cancer treatments including treatments for brain tumors [6] and many types of aggressive tumors [7], and a variety of vaccination protocols such as for the human immunodeficiency virus [8,9], using SeV vectors are planned.

The viral envelope comprises a lipid bilayer derived from the host plasma membrane and two inserted viral glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) proteins. Beneath the envelope is a lining consisting of the matrix or membrane (M) protein. The F and HN proteins are primarily required during the entry of SeV into cells. The F protein is involved in virus penetration, hemolysis and cell fusion [10]. The HN protein mediates the viral attachment to cells by interacting with cell-surface sialic acid containing receptor(s). The HN protein is known to be one of the major targets of the host humoral immune responses against SeV infection, and to induce NK and cytotoxic T lymphocyte responses [11,12]. The M protein promotes vesiculation of the membrane and the release of particles into the extracellular medium without the aid of other viral proteins [13,14]. We previously succeeded in the recovery of high titers of F-gene-deleted (SeV/ΔF) [15], M-gene-deleted (SeV/ΔM) [16], HN-gene-deleted (SeV/ΔHN), and both M- and F-genes-deleted (SeV/ΔMΔF) [17] SeV vectors in addition to other types of SeV vectors [18] by using packaging cell lines that express the respective proteins encoded by the deleted gene(s). All the vectors showed efficient infectivity and transgene expression in various types of cell lines and primary cells *in vitro*. Deletion of the F gene made the SeV vector non-transmissible, deletion of the M gene worked well to render the vector incapable of directing the formation of particles in infected cells, and deletion of the HN gene was expected to reduce the host immune response against the SeV vector. The single-gene-deleted SeV vectors were also rescued and propagated by another group [19], but multiple-genes-deleted SeV vectors have not been propagated at high titer. For the wide-range application of SeV vectors, such as for the treatment of chronic diseases, virus-gene-derived protein expression should be reduced as much as possible. High-level transgene expression from the SeV vector results from the high-level transcription associated with the genome replication that is directed by nucleoprotein (NP), phospho (P) and large (L) proteins [20]. To keep the high-level expression of SeV vectors, the NP, P and L genes should not be removed. Therefore, the most advanced SeV vector, which reduces the viral genome as much as possible, is the M-, F- and HN-genes-deleted SeV vector (SeV/ΔMΔFΔHN) at present. However, the recovery of SeV/ΔMΔFΔHN has hitherto been very difficult because the efficient complementation of all three proteins (M, F and HN) needed to form the virion particle was essential for its propagation.

In this study, we succeeded in the establishment of a packaging cell line that expresses all three envelope proteins, M, F and HN, by using a Cre/*lox*P induction system. Using this cell line, we succeeded in producing the SeV vector deleted for the M, F and HN genes at a titer of more than 10^8 cell infectious units (CIU)/ml. SeV/ΔMΔFΔHN possesses only the NP, P and L genes in its genome. This vector showed efficient transduction capability and reduced cytopathic effect. Importantly, the immune reaction against SeV was also reduced when SeV/ΔMΔFΔHN was used *in vivo* and *ex vivo*.

Materials and methods

Cells and viruses

Monkey kidney cell lines, LLC-MK_2 and CV-1, were maintained in monolayer cultures in minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum and penicillin-streptomycin in the presence of 5% CO_2. The molecular clone of SeV Z strain with attenuated virulence was used as the starting material for genome modifications in this study. The F, M or both M/F gene(s)-deleted SeV vectors were prepared by using LLC-MK_2 cells stably transformed with the F gene (LLC-MK2/F7 [15], M gene (LLC-MK2/F7/M62 [16]), or both M and F genes (LLC-MK2/F7/M#33 [17]). The SeV/ΔMΔFΔHN was prepared in a new packaging cell line (LLC-MK2/F7/M#33/A/HN7; this report). An adenovirus vector, AxCANCre [21], expressing Cre recombinase, was used for the induction of respective protein(s) encoded by these deleted gene(s).

Plasmid construction

For the construction of genomic cDNA of M-, F- and HN-genes-deleted SeV carrying the green fluorescent protein (GFP) gene, LitmusSalINheIhfrag-ΔMΔFGFP that was used to generate the cDNA of SeV/ΔMΔF-GFP (pSeV/ΔMΔF-GFP) containing the GFP and HN genes in the *Sal* I/*Nhe* I site in LITMUS38 (NEB) [17] was utilised. Inverse polymerase chain reaction (PCR) was conducted with primer pairs of 5′-GAGGTCGCGCGTTAATTAAGCTTTCACCTCAAACAAGC-ACAGATCATGG-3′ and 5′-GCATGTTTCCCAAGGGGAGA-GTTAATTAACCAAGCACTCACAAGGGAC-3′ to introduce the *Pac* I site just behind the P gene. The PCR product was digested with *Pac* I and *Dpn* I and then self-ligated; thus both GFP and HN genes were deleted from LitmusSalINheIhfrag-ΔMΔFGFP and generated

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LitmusSalINheIhfrag-ΔMΔFΔHN-Pac I. To insert the GFP gene with end and start signals (EIS) between the P and L genes, PCR was conducted with primer pairs of 5′-CTGCGATCGCGCCCCAAGCAGACACCACCT-3′ and 5′-TACGCGATCGCTGATAATGGTCGTGATCAT-3′ on pSeV18+/ΔF-GFP [15] as a template. The amplified GFP fragment was digested with *Sgf* I and inserted into the *Pac* I site of LitmusSalINheIhfrag-ΔMΔFΔHN-PacI to generate LitmusSalINheIhfrag-ΔMΔFΔHN-GFP. The 5.9 kb *Sal* I- and *Nhe* I-digested fragment containing the GFP gene was substituted for the corresponding fragment of pSeV18+/ΔMΔF-GFP to generate pSeV/ΔMΔFΔHN-GFP. To introduce the mutations of persistent infection into the P and L genes, site-directed mutagenesis was conducted using a QuickChange site-directed mutagenesis kit (Stratagene) with the primer pairs 5′-ctcaaacgcatcacgtctcTtTccctccaaagagaagc-3′ (sense) and 5′-gcttctctttggagggAaAgagacgtgatgcgtttgag-3′ (antisense) for L511F in the P gene, and 5′-gttctatcttcctgacTCtatagacctggacacgcttac-3′ (sense) and 5′-gtaagcgtgtccaggtctataGAgtcaggaagatagaac-3′ (antisense) for N1197S and 5′-ctacctattgagcccctttagttgacGaAgataaagataggcta-3′ (sense) and 5′-tagcctatctttatcTtCgtcaactaaggggctcaataggtag-3′ (antisense) for K1795E in the L gene used on pSeV/ΔMΔFΔHN-GFP as a template. Thus, full-length genomic cDNA of pSeV/PLmutΔMΔFΔHN-GFP was generated and its structure was confirmed by sequencing. For the plasmid expressing the HN protein under the control of the *Cre/lox*P induction system [22] a PCR-generated 1.8 kb fragment containing the HN gene from SeV cDNA was inserted into the *Swa* I site of pCALNdLw [22] to generate pCALNdLw/HN, in which the HN gene was located after the drug-resistant gene sandwiched between *lox*P sequences. Hence, the expression of the HN protein in pCALNdLw/HN-introduced cells, LLC-MK2/F7/M#33/A/HN7, was induced after expressing Cre recombinase from AxCANCre.

Insertion of the SEAP gene

To quantify the expression of a foreign gene carried in the SeV genome, the gene for the secreted form of human placental alkaline phosphatase (SEAP) was inserted upstream of the open reading frame of the NP gene. The SEAP gene with the EIS element [17] was introduced into the *Not* I site located in the non-coding sequence between the start (S) signal and the translation initiation codon (ATG) of the NP gene of pSeV/PLmutΔMΔFΔHN-GFP to generate pSeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP.

Cloning and analysis of packaging cell lines

LLC-MK$_2$/F7/M#33 cells were transfected with pCALNdLw/HN using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instruction. Two weeks after transfection, viable clones of single cell origin in 96-well plates were expanded in 12-well plates, and, when they reached a nearly confluent state, they were infected with AxCANCre at an multiplicity of infection (MOI) of 5 [21,22]. After culturing for 2 days at 32 °C, the cells were recovered and subjected to semiquantitative Western blotting with anti-HN antibody according to a method described previously [16].

M-, F- and HN-genes-deleted SeV vector recovery from cDNA

Preparation of cell lysate containing RNPs and primary virions of SeV/ΔMΔFΔHN-GFP was carried out according to the method described previously [16] with minor modification. Briefly, approximately 1×10^7 LLC-MK$_2$ cells seeded in Ø10-cm dish were transfected with pSeV/ΔMΔFΔHN-GFP and pCAG-plasmids each carrying the NP, P, M, F, HN or L gene (Ban *et al.*, personal communication). The cells were cultured in MEM containing trypsin (7.5 μg/ml). Twenty-four hours after transfection, the cells were overlaid with LLC-MK2/F7/M#33/HN7 cells after induction of M, F and HN proteins by AxCANCre infection at an MOI of 5 and cultured for another 48 h. The cells were harvested and lysed by repeating a freeze/thaw cycle three times in Opti-MEM (Invitrogen). The cell lysate was infected into new LLC-MK2/F7/M#33/HN7 cells after AxCANCre infection. After that, these cells were cultured at 32 °C in MEM containing trypsin for 10 to 20 days. When spread of GFP expression to neighbour cells was seen by fluorescence microscopy, it was considered that viral vectors were recovered in the culture supernatants. Those viral vectors were further amplified by several rounds of propagation. Titers were determined by the proportion of GFP-expressing cells (GFP-CIU) per milliliter [15]. The culture supernatant of the fourth passage was collected and stored at −80 °C, after adding bovine serum albumin (BSA) solution to a final concentration of 1% (w/v), until usage in all the experiments described below.

RT-PCR

Total viral RNA from SeV/ΔMΔFΔHN-GFP was extracted from the supernatant containing viral particles using a QIAamp viral RNA minikit (Qiagen). Reverse-transcription (RT)-PCR was performed in a one-step process using the Superscript RT-PCR system (Invitrogen). RT-PCR amplication was performed with random hexamers and the primer pair 5′-AGAGAACAAGACTAAGGCTACC-3′ (forward primer specific for the P gene) and 5′-TATTCAACCAAAGATCCTGGAACCC-3′ (reverse primer specific for the L gene) probed.

Detection of viral proteins by Western blotting

Analysis of viral proteins by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was

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performed according to the method described previously [16]. LLC-MK_2 cells (1×10^6) in 6-well plates were infected at an MOI of 3 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP or SeV/ΔMΔFΔHN-GFP and incubated in serum-free MEM at 37 °C. Three days after transduction, the cells were recovered and solubilized in a sample buffer (Biolabs) for SDS-PAGE. Proteins separated by SDS-PAGE were transferred to Immobilon-PVDF membranes (Millipore). The membranes were probed with a rabbit polyclonal anti-M antibody [16] or mouse monoclonal anti-F, γ236 [23], and anti-HN, HN-2 [24], antibodies. Secondary antibodies were goat anti-rabbit IgG (Santa Cruz Biotechnology) or goat anti-mouse IgG+IgM (Bioscience) conjugated with horseradish peroxidase. The protein bands were detected by chemiluminescence using ECL Western blotting detection reagents (Amersham Biosciences) following the manufacturer's protocol.

Quantitative analysis of cytotoxicity

Confluent CV-1 cells grown in 96-well plates were infected at an MOI of 0.1, 0.3, 1, 3, 10 or 30 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP or SeV/PLmutΔMΔFΔHN-GFP and incubated at 37 °C in serum-free MEM. The culture supernatants were collected 3 days after transduction and assayed with a cytotoxicity detection kit (Roche) that measures lactase dehydrogenase (LDH) activity released from damaged cells [25].

SEAP assay

Confluent LLC-MK_2 cells grown in 96-well plates were infected at an MOI of 3 with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP or SeV^{18+}SEAP/PLmutΔMΔF-ΔHN-GFP and incubated in serum-free MEM at 37 °C. The culture supernatants were collected every 24 h and assayed for SEAP activities using an SEAP reporter assay kit (Toyobo) with an LAS 1000 image analyser (Fuji Film). Means were calculated from three replicate samples.

In vivo study of M-, F- and HN-genes-deleted SeV vector

Twelve BALB/cA mice (Charles River), 7 weeks old weighing 20–25 g, were used. In half of the mice, submandibular lymph nodes (SMLN) were removed before injection. Mice were anaesthetised with diethyl ether, and 5 μl of SeV/ΔF-GFP (5×10^6 GFP-CIU/head) or SeV/PLmutΔMΔFΔHN-GFP (5×10^6 GFP-CIU/head) was injected into the ear auricle intradermally (n = 3). GFP expression was assessed by examining the ear auricle under a fluorescence stereomicroscope. The surface of the auricle was photographed and the GFP intensity was quantified with NIH image software. Sera of injected mice were collected at weekly intervals and stored at − 80 °C until the measurement of neutralizing and total anti-SeV antibody levels.

Immunohistochemical staining

The auricle was frozen and sliced into 10 μm thick sections with a cryotome (Coldtome CM-502; Sakura Seiki). The frozen sections were pretreated with 0.3% H_2O_2 in methanol. Migrated macrophages and pan-T cells were detected using a rabbit polyclonal anti-CD11b (Santa Cruz) and anti-CD3 (Santa Cruz) antibodies, respectively, followed by using a Histofine SAB-PO(R) kit (Nichirei). Immunopositive cells were visualised with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Ex vivo transduction using the M-, F- and HN-genes-deleted SeV vector

MC57G cells, an isogenic cell line of C57BL/6, were inoculated with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP at an MOI of 3. Two days later, the cells were transplanted subcutaneously in the back of C57BL/6 mice. Sera of transplanted mice were collected at weekly intervals.

Quantitation of neutralizing and total anti-SeV antibodies

To quantify neutralizing antibody against SeV, serial 1:5 dilutions of sera previously inactivated at 56 °C for 30 min were mixed with wild-type SeV carrying the GFP gene (SeV^{18+}GFP (wild)) at 37 °C for 1 h. These virus-serum mixtures were inoculated to LLC-MK_2 cells in 96-well plates and cultured in serum-free MEM at 37 °C for 3 days. The remaining infectivity of SeV^{18+}GFP (wild) was estimated by measuring the fluorescence of GFP with a fluorescence microplate reader (Cytofluor II; Biosearch/Millipore). Results are expressed as percent inhibition by setting the values from the cells infected with SeV^{18+}GFP (wild) alone as 0% and those from the uninfected cells as 100%. Total anti-SeV antibody level in the sera was subsequently measured by a test kit for HVJ (Denka Seiken) according to the manufacturer's protocol. In this test, the quantities of anti-SeV antibody were measured as relative ones represented by OD450 because the absolute quantity of anti-SeV antibody could not be determined. Those were determined using 1:300 dilutions of the sera and measured simultaneously.

Characterization of serum antibodies by Western blotting

The mixture of NP and HN viral proteins which was prepared from infected cells and whole virion proteins were separated by SDS-PAGE and transferred

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to Immobilon-PVDF membranes (Millipore) as described before. Sera collected from transplanted mice were used as the first antibody. Secondary antibodies were goat anti-mouse IgG+IgM conjugated with horseradish peroxidase.

Results and discussion

Establishment of M-, F- and HN-expressing packaging cell line

For the recovery of SeV/ΔMΔFΔHN virion particles from cDNA, the missing M, F and HN genes must be complemented *in trans*. To establish such an M-, F- and HN-expressing packaging cell line, a Cre/*lox*P induction system [22] was employed as has been reported [15,16]. Moreover, we previously established an M/F-expressing packaging cell line (LLC-MK_2/F7/M#33), in which M- and F-genes-deleted SeV (SeV/ΔMΔF) was successfully recovered [17]. Therefore, it was thought that the introduction of the HN gene into LLC-MK_2/F7/M#33 would make this cell line capable of supporting the recovery of SeV/ΔMΔFΔHN. Early-passage LLC-MK_2/F7/M#33 cells were transfected with pCALNdLw/HN. After incubation at 37 °C for 2 weeks, clones were selected and expanded. The HN protein expression was examined by the Western blot analysis of the cellular proteins after infection with the Cre-encoding adenovirus vector, AxCANCre [21], at a MOI of 5. The clones expressing the HN protein were then subjected to two rounds of subcloning, resulting in the final selection of one cell line, LLC-MK_2/F7/M#33/A/HN7.

Construction of a vector with M, F and HN gene deletions

SeV genomic cDNA carrying the GFP gene in place of the M, F and HN genes (SeV/ΔMΔFΔHN-GFP) was constructed (Figure 1A). The GFP gene in SeV cDNA allows us to confirm easily the successful recovery of the SeV/ΔMΔFΔHN vector. Using the established cell line and the plasmid-based reverse genetics technology [26], we propagated SeV/ΔMΔFΔHN-GFP, leading to a titer of 1×10^8 CIU/ml in the culture supernatant. During the reconstitution of SeV/ΔMΔFΔHN-GFP, some degree of cytotoxicity toward the packaging cell lines, which had not been observed with previous SeV vector reconstitutions, was observed (data not shown). The cause of this cytotoxicity is still obscure, but one possibility is that the L protein might be expressed in relative excess and drive the transcription and replication of the vector beyond the capacity of the packaging cells because of the shorter genome size of SeV/ΔMΔFΔHN-GFP. Based on this hypothesis, we prepared the SeV/PLmutΔMΔFΔHN-GFP vector, which has amino acid substitutions in P (L511F) and L (N1197S, K1795E). These substitutions had been identified in SeV strains capable of persistent infection *in vitro* with slightly reduced transcription and replication [27]. As expected, SeV/PLmutΔMΔFΔHN-GFP was propagated without obvious cytotoxicity during reconstitution, leading to a titer of 5×10^8 CIU/ml in the culture supernatant.

Gene structure of the recovered SeV vector

The vector gene structure was confirmed by RT-PCR. The DNA fragment of SeV/ΔMΔFΔHN-GFP from the 5′-terminus of the P gene to the 3′-terminus of the L gene containing the GFP gene was amplified from the vector genome, and the amplified fragment was compared with the corresponding fragments amplified

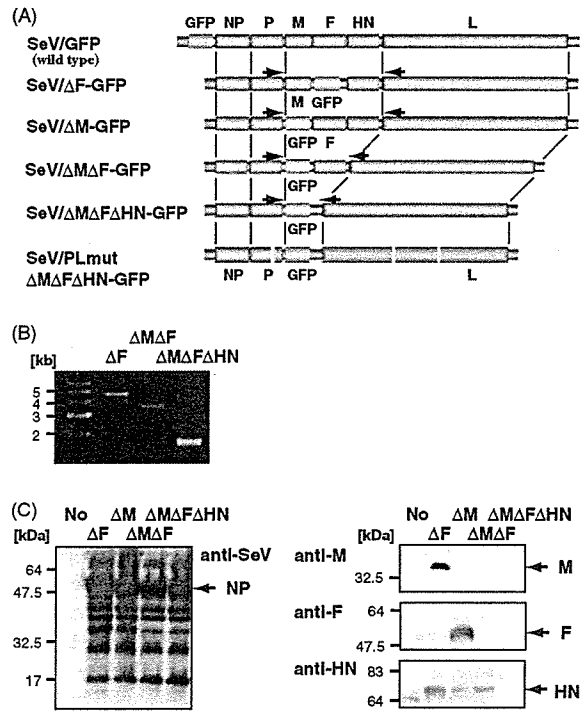

Figure 1. Construction of M-, F- and HN-genes-deleted SeV vector carrying the GFP gene, and confirmation of its structure. (A) The structures of recombinant SeV genomes. The open reading frame of the GFP gene was inserted with the SeV end and start signals (EIS) in the respective positions of the deleted gene(s). The positions of the primers for RT-PCR are shown by arrows. (B) Viral genome structure was confirmed by RT-PCR. The DNA fragment of SeV/ΔMΔFΔHN-GFP (ΔMΔFΔHN) from the 5′-terminal of the P gene to the 3′-terminal of the L gene (containing the GFP gene) was amplified from the vector genome, and the fragment was compared to the corresponding fragments amplified from the vector genomes of SeV/ΔF-GFP (ΔF) and SeV/ΔMΔF-GFP (ΔMΔF). (C) Viral proteins were detected by Western blot analysis. LLC-MK_2 cells were infected with SeV^{18+}GFP (wild), SeV/ΔF-GFP (ΔF), SeV/ΔM-GFP (ΔM), SeV/ΔMΔF-GFP (ΔMΔF) or SeV/ΔMΔFΔHN-GFP (ΔMΔFΔHN) at an MOI of 3. The viral proteins in the cells 2 days after transduction were detected by Western blotting using anti-M, anti-F, anti-HN and anti-SeV (which mainly detects NP protein) antibodies after transferring the proteins to a PVDF membrane

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from the vector genomes of SeV/ΔF-GFP and SeV/ΔMΔF-GFP. Amplification of 1719-bp, 3576-bp and 4773-bp DNAs for SeV/ΔMΔFΔHN-GFP, SeV/ΔMΔF-GFP and SeV/ΔF-GFP, respectively, was expected based on the genome structures. The results of RT-PCR clearly showed that SeV/ΔMΔFΔHN-GFP had the M, F and HN genes deleted from its genome (Figure 1B). In the case of SeV/PLmutΔMΔFΔHN-GFP, the deletion of the M, F and HN genes was examined by RT-PCR as in the case of SeV/ΔMΔFΔHN-GFP, and newly introduced mutations in the P and L parts were also confirmed by determining the sequences of the DNA fragment amplified from the cDNA (data not shown). The absence of the M, F and HN genes was also confirmed by Western blot analysis of the protein expression of LLC-MK_2 cells infected at an MOI of 3 with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP or SeV/ΔMΔFΔHN-GFP. In these analyses, anti-M, anti-F, anti-HN or anti-SeV (which mainly detects the NP protein) antibodies were used. The NP protein, but not M, F or HN proteins, was detected in the cells transduced with SeV/ΔMΔFΔHN-GFP (Figure 1C). Similarly, neither the M nor the F protein was observed in cells transduced with SeV/ΔMΔF-GFP. These results clearly indicated the absence of the envelope genes in these vectors.

Cytopathic effect of SeV is efficiently diminished by M, F and HN gene deletion

Infection with SeV vectors causes a cytopathic effect (CPE) in some types of cells. Therefore, it was important to characterise the newly recovered SeV/ΔMΔFΔHN-GFP and SeV/PLmutΔMΔFΔHN-GFP in terms of CPE. The CPE was investigated in CV-1 cells, which are known to be very sensitive to SeV infection-dependent cytotoxicity [17]. CV-1 cells plated in 96-well plates were transduced with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP and SeV/PLmutΔMΔFΔHN-GFP, respectively, and incubated in serum-free MEM for 3 days. Then, their CPE was quantitatively measured by using Decker's method [25]. The CPE of SeV/ΔMΔFΔHN-GFP was approximately equal to that of SeV/ΔMΔF-GFP. However, that of SeV/PLmutΔMΔFΔHN-GFP was greatly reduced as compared to that of all other types of SeV vectors (Figure 2A). Morphological damage to the cells infected with vectors at an MOI of 20 was also examined under a fluorescence microscope. CV-1 cells transduced with SeV/PLmutΔMΔFΔHN-GFP showed little CPE (Figure 2B). Thus, the combination of the deletion of all envelope-related genes and the amino acid substitutions in P and L was found to be quite effective in reducing the CPE of SeV *in vitro*. In addition to membrane fusion and apoptosis, rapid transcription and replication increase SeV-induced cytotoxicity. The substitutions in P and L that induce slightly reduced transcription and replication [27] brought about additional reduction of CPE.

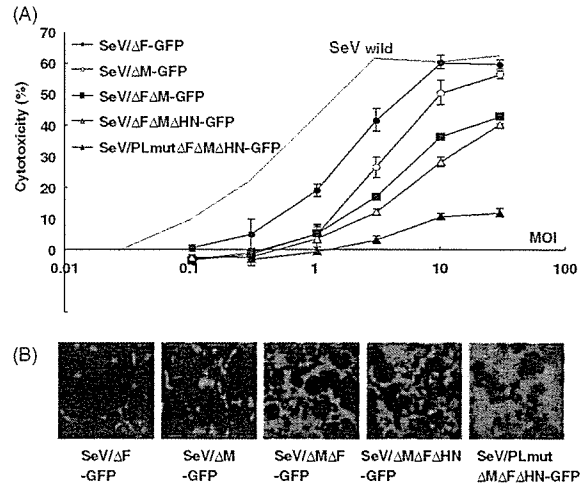

Figure 2. Analysis of SeV infection-dependent cytotoxicity. (A) Quantitative measurement using SeV infection-sensitive cells. CV-1 cells were infected with SeV/ΔF-GFP, SeV/ΔM-GFP, SeV/ΔMΔF-GFP, SeV/ΔMΔFΔHN-GFP or SeV/PLmutΔMΔFΔHN-GFP at an indicated MOI. Cytotoxicity was determined by the quantity of LDH released from damaged cells. The assay was carried out 3 days after infection using the supernatants of cultures in serum-free medium. The percentage of cytotoxicity (%) was calculated using the low control (0%) from the supernatant of uninfected cells and the high control (100%) from the supernatant of cell lysates after treatment with 2% Triton X-100. Cytopathic effect of a wild-type SeV (SeV^{18+}GFP (wild)) was also examined and shown in the thin line. (B) Morphology of CV-1 cells infected with respective SeV vectors at an MOI of 20 on day 3 after infection

Quantitative analysis of foreign gene expression of M-, F- and HN-genes-deleted SeV

To quantify the expression level of a foreign gene(s) carried in SeV/ΔMΔFΔHN, the gene for the secreted form of human placental alkaline phosphatase (SEAP), which is an easily detectable marker of protein production, was inserted upstream of the open reading frame of NP. The thus-generated SeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP vector was used for the SEAP assay. LLC-MK_2 cells were infected at an MOI of 3 with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP [17] or SeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP and the culture supernatants were collected every 24 h. The expression level of SEAP in the culture supernatant of SeV^{18+}SEAP/PLmutΔMΔFΔHN-GFP was rather low as compared to that of SeV^{18+}SEAP/ΔF-GFP, but was similar to that of SeV^{18+}SEAP/ΔMΔF-GFP (Figure 3). This supported the notion that the M protein might participate not only in viral assembly and budding, but also in the control of transgene transcription [28].

Prolonged transgene expression of M-, F- and HN-genes-deleted SeV *in vivo*

We next examined the transduction efficiency of SeV/PLmutΔMΔFΔHN-GFP *in vivo*. SeV/PLmutΔMΔF-

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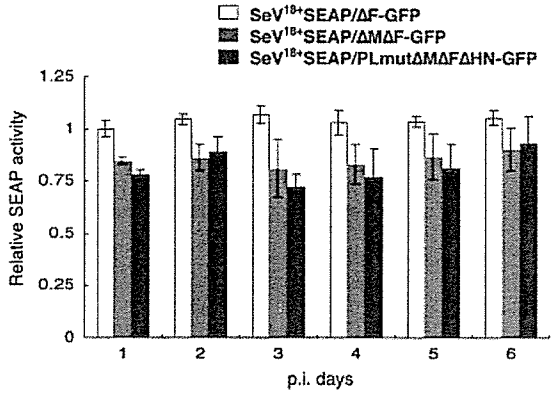

Figure 3. Comparison of expression performance of SeV vectors carrying the SEAP gene. The culture medium of LLC-MK_2 cells was collected every 24 h after infection with SeV^{18+}SEAP/ΔF-GFP, SeV^{18+}SEAP/ΔMΔF-GFP or SeV^{18+}SEAP/ΔMΔFΔHN-GFP at an MOI of 3. SEAP activity was determined as a relative activity by setting the value from the medium of uninfected cells as zero (0) and that from the medium of SeV^{18+}SEAP/ΔF-GFP-infected cells (MOI = 3, 1 day post-infection.) as one (1). Average of three experiments; bar: SD

ΔHN-GFP or SeV/ΔF-GFP was administered intradermally to the ear auricles of BALB/cA mice (5×10^6 CIU/head). We can directly observe the fluorescence from expressed GFP over a period of time through the skin surface of the ear auricle without sacrificing animals. Efficient transduction from both vectors was confirmed and the level of GFP expression was approximately equal between SeV/ΔF-GFP and SeV/PLmutΔMΔFΔHN-GFP (Figure 4A). However, the peak of expression was prolonged in the case of SeV/PLmutΔMΔFΔHN-GFP (Figure 4B). In an additional study, SeV vectors were administered after the removal of submandibular lymph nodes in order to avoid direct transduction to them. In this case, GFP expression derived from SeV/PLmutΔMΔFΔHN-GFP was significantly prolonged (Figure 4B). In fact, both anti-CD11b (macrophage marker) and anti-CD3 (Pan-T cell marker) staining of the frozen sections of the inoculated auricles showed the delayed migration of macrophages and T cells to the site of SeV/PLmutΔMΔFΔHN-GFP administration compared with that of SeV/ΔF-GFP (Figures 5A and 5B). These results clearly indicate that the immunoreaction against SeV/PLmutΔMΔFΔHN-GFP is weak and delayed compared to that against SeV/ΔF-GFP. Efficient transduction and prolonged expression of SeV/PLmutΔMΔFΔHN were also observed in the cases of transduction to the airway epithelial cells and neuronal cells in the brain (data not shown).

Humoral immune reaction of M-, F- and HN-genes-deleted SeV *in vivo*

We next examined the effect of SeV/PLmutΔMΔFΔHN-GFP on the induction of anti-SeV antibodies and

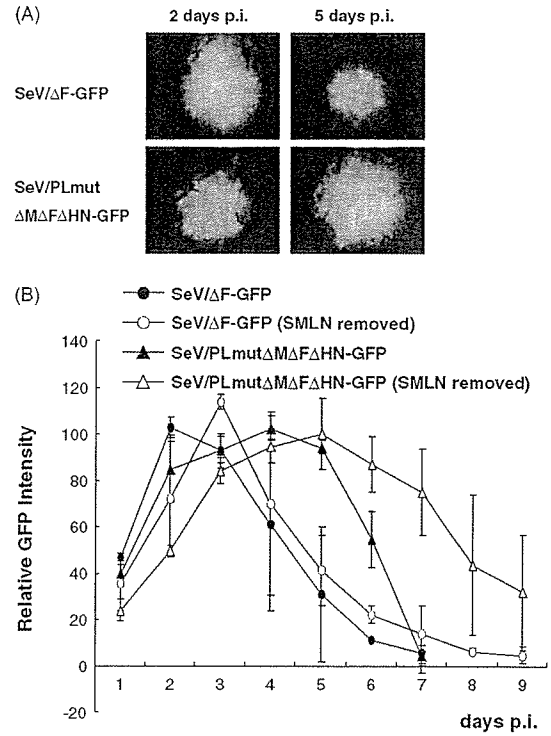

Figure 4. Gene transfer by M-, F- and HN-genes-deleted SeV vector *in vivo*. (A) Gene transfer to the ear auricle. SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP (5×10^6 GFP-CIU/head) was administered to the ear auricle of BALB/cA mice with or without submandibular lymph nodes (SMLN) by a single intradermal injection (n = 3). GFP expression was detected by a fluorescent stereomicroscope from the skin surface of the ear auricle under a fixed condition. (B) Quantitative analysis of GFP fluorescence. The expression of GFP was calculated by multiplying its fluorescence intensity and its area by NIH image software and expressed as relative ones

neutralizing antibodies. SeV/PLmutΔMΔFΔHN-GFP or SeV/ΔF-GFP was administered to BALB/cA mice, and sera of mice were collected weekly for 3 weeks. Phosphate-buffered saline (PBS)-injected mice were used as controls. The level of serum neutralizing antibodies in the mice administered SeV/PLmutΔMΔFΔHN-GFP was reduced to about half of that in the mice administered SeV/ΔF-GFP (Figure 5C). On the other hand, the overall quantity of anti-SeV antibodies was only slightly reduced by using the new SeV vector. The envelope proteins of SeV/PLmutΔMΔFΔHN-GFP particles supplied by the packaging cells may contribute to the induction of both overall anti-SeV antibodies and a certain amount of neutralizing antibodies against the vector.

No neutralizing antibody production after M-, F- and HN-genes-deleted SeV transduction *ex vivo*

We then examined whether the envelope gene deletions reduce the induction of humoral immunoreaction

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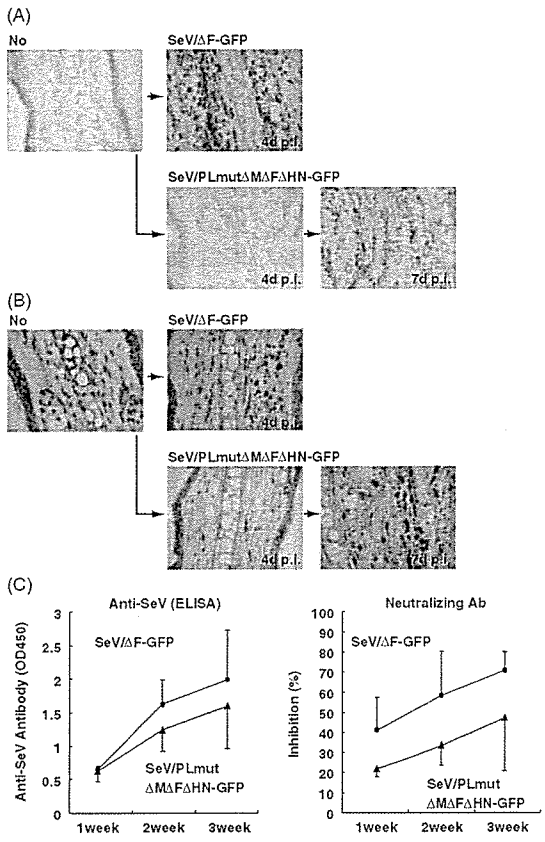

Figure 5. Immune reaction to SeV vectors introduced by *in vivo* transduction. Immunohistochemical analysis of the migrations of (A) macrophages and (B) T lymphocytes. The frozen sections of ear auricles of BALB/cA mice intradermally administered with SeV/ΔF or SeV/PLmutΔMΔFΔHN. (C) Sera from mice administered with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP were assayed for overall anti-SeV antibodies as well as neutralizing antibodies directed against SeV. Neutralizing antibody level is shown as a percent inhibition of SeV^{18+}GFP (wild) infectivity

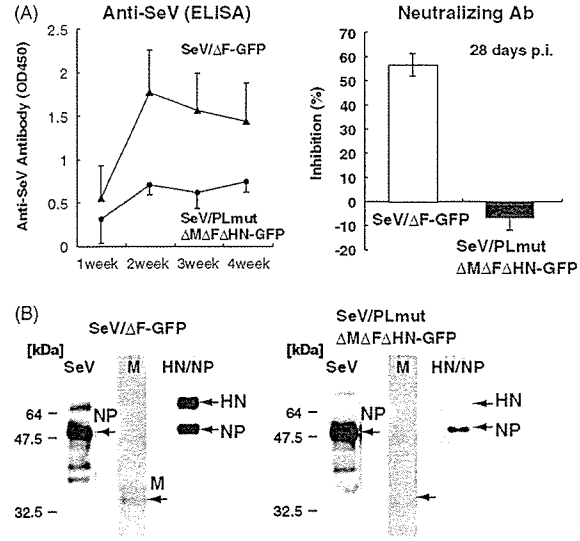

Figure 6. Immune reaction to SeV vectors following transplantation of SeV-infected cells. (A) Serum levels of anti-SeV antibody (left) following transplantation of isogenic cells (1×10^6 cells/head) infected with SeV/ΔF-GFP or SeV/PLmutΔMΔFΔHN-GFP at an MOI of 3 were examined. Neutralizing antibody level was measured in the sera on days 28 after transplantation (right). (B) Serum was analysed by Western blotting. The purified M protein, the mixture of NP and HN viral proteins that were prepared from infected cells, and whole virion proteins were loaded and transferred to a membrane. Western blotting was carried out using sera collected from mice transplanted with isogenic cells infected with SeV vectors as a primary antibody

in the case of *ex vivo* transduction. Isogenic cells (MC57G; 1×10^6 cells) transduced by SeV/PLmutΔMΔFΔHN-GFP or SeV/ΔF-GFP were transplanted subcutaneously in the backs of C57BL/6 mice. Sera were collected at weekly intervals, and the levels of anti-SeV and SeV-neutralizing antibodies were measured. Figure 6A shows that, in comparison with the overall quantity of anti-SeV antibody produced in the case of transduction with SeV/ΔF-GFP, the production of anti-SeV antibody was greatly reduced when SeV/PLmutΔMΔFΔHN-GFP was used. Furthermore, neutralizing antibody was undetectable in mice transplanted with cells transduced by SeV/PLmutΔMΔFΔHN-GFP even at 28 days after the transplantation. The serum antibodies were characterised by Western blot analysis. Anti-HN and anti-M antibodies were not detected in the serum from the mice transplanted with SeV/PLmutΔMΔFΔHN-GFP-transduced cells (Figure 6B). However, anti-NP antibody was detected in mice transplanted with either SeV/ΔF-GFP- or SeV/PLmutΔMΔFΔHN-GFP-transduced cells. As the HN protein is known to be one of the major targets for neutralizing antibodies against SeV [29], the abolishment of neutralizing antibody in the case of SeV/PLmutΔMΔFΔHN-GFP would be caused by the absence of anti-HN antibody in the serum. Also, we previously showed that deletion of both the M and F genes from the vector genome made the SeV vector non-transmissible and caused a lack of the formation of particles that might have enhanced the immunogenicity [12]. These results clearly indicate that the deletion of all three envelope-related genes (M, F and HN) from the SeV genome significantly reduced the humoral immune reaction against the SeV vector, especially in *ex vivo* transduction.

In conclusion, we have successfully recovered and propagated a high titer of more than 10^8 CIU/ml of a new SeV vector (SeV/ΔMΔFΔHN), in which all envelope-related genes were deleted from the vector genome using a newly established packaging cell line. Amino acid substitutions in P and L proteins that induce a slower rate of transcription caused additional attenuation of this vector (SeV/PLmutΔMΔFΔHN). This new vector showed efficient transduction capability both *in vitro* and *in vivo*. Furthermore, *in vivo* as well as *ex vivo* experiments showed a significant reduction of its immunogenicity compared with that of SeV/ΔF. That is, the deletion of

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gene(s) from the SeV genome and additional introduction of amino acid substitutions into P and L is a very effective way to reduce both the immunogenic and cytopathic reactions induced by the SeV vector. These modifications are expected to improve the safety and broaden the range of clinical applications of SeV vectors such as for vaccine treatments [8,9] and cell-based treatment for hematopoietic damage including repeated dosing. The new vector, SeV/PLmutΔMΔFΔHN, is one of the most advanced genotoxicity-free cytoplasmic RNA virus vectors. This vector will also become a possible source for further improvement of SeV vector systems by introducing the additional mutations on the NP gene to modify the immune response against the vector and for alternative vector candidates such as virus-like particles (VLPs) and self-replicating ribonucleoprotein (RNP) complexes [3].

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ORIGINAL ARTICLE

Effect of tolerance induction to immunodominant T-cell epitopes of Sendai virus on gene expression following repeat administration to lung

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Sendai virus (SeV) is able to transfect airway epithelial cells efficiently in vivo. *However, as with other viral vectors, repeated administration leads to reduced gene expression. We have investigated the impact of inducing immunological tolerance to immunodominant T-cell epitopes on gene expression following repeated administration. Immunodominant CD4 and CD8 T-cell peptide epitopes of SeV were administered to C57BL/6 mice intranasally 10 days before the first virus administration with transmission-incompetent F-protein-deleted ΔF/SeV-GFP. At 21 days after the first virus administration, mice were again transfected with ΔF/SeV. To avoid interference of anti-GFP antibodies, the second transfection was carried out with ΔF/SeV-lacZ. At 2 days after the final transfection lung β-galactosidase expression, T-cell proliferation and antibody responses were measured. A state of 'split tolerance' was achieved with reduced T-cell proliferation, but no impact on antiviral antibody production. There was no enhancement of expression on repeat administration; instead, T-cell tolerance was, paradoxically, associated with a more profound extinction of viral expression. Multiple immune mechanisms operate to eradicate viruses from the lung, and these findings indicate that impeding the adaptive T-cell response to the immunodominant viral epitope is not sufficient to prevent the process.*
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Keywords: *lung; Sendai virus; T cells; tolerization*

Introduction

Cystic fibrosis (CF) is a lethal, monogenic disease, caused by mutations in the CF conductance regulator (*CFTR*) gene.[1] CF is an important candidate for gene therapy-based treatments because the median life expectancy is only approximately 30 years and treatment could potentially be administered to the lung noninvasively. CFTR is expressed in the lung in submucosal glands and airway epithelial cells (AECs),[2] the latter likely being the more important target for CF gene therapy. Gene transfer to the airway epithelium via the apical membrane using existing viral and nonviral gene transfer agents (GTAs) is generally inefficient on topical administration. Extracellular barriers such as mucus, mucociliary clearance and glycocalyx proteins limit the access of GTAs to the apical membrane.[3] In addition, the apical membrane of differentiated AECs is relatively devoid of viral receptors, such as those for adenovirus and adeno-associated virus (AAV) type 2. It also has a low basal and stimulated rate of endocytosis, thus discouraging vector entry. Transduction efficiency via the basolateral membrane is generally more efficient, but requires opening of tight junctions, which may not be a clinically viable approach in a disease with a high bacterial load in the lung. Even if the GTA can overcome these extracellular barriers, several intracellular obstacles such as endosomal escape, cytoplasmic trafficking and passage through the nuclear pore complex remain.

We have recently shown that, in contrast to adenovirus and AAV, recombinant Sendai virus (SeV), a murine paramyxovirus, transduces AECs in a variety of animal models efficiently *in vivo*.[4,5] Importantly, SeV carrying the *CFTR* cDNA is able to correct partially the characteristic CFTR-dependent chloride transport defect in the nasal epithelium of CF knockout mice.[6] Several aspects of SeV biology may explain the high gene transfer efficiency into AECs. Importantly, SeV uses cholesterol and sialic acid as receptors. Both are present on the surface of most cell types. Further, SeV requires short contact time with the target cell for internalization, and replicates in the cytoplasm of transduced cells, circumventing the nuclear membrane barrier. Gene expression is transient, with peak expression approximately 48 h after transfection, generally returning to baseline values within 2 weeks of

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transfection.[7] The mouse is a natural host for SeV and wild-type SeV infection causes pneumonia. It is currently unclear why SeV-mediated gene expression is transient. SeV-induced pulmonary inflammation is dose- and time-dependent. At high virus doses accumulation of lymphoid tissue around blood vessels and airways can be observed (J Zhu, personal communication), but elimination of infected cells is unlikely to be the only reason for transient gene expression. If SeV is to be useful in the treatment of a chronic disease such as CF, then sustained or repeated gene expression for the life-time of the patient is required.

Any therapeutic strategy must address the fact that the lung is highly evolved to recognize and eliminate pathogens using a multitude of defense mechanisms.[8] Repeat administration of adenovirus to the lung is not possible, and a variety of strategies to overcome this have been studied.[9–11] However, the efficient adaptive and innate immune responses have thus far prevented successful readministration (reviewed in Ferrari *et al.*[12]). It has been suggested that AAV may be less immunogenic than adenovirus due to the suboptimal ability of the virus to transduce dendritic cells;[13] however, studies have generated conflicting results.[13–17] During SeV replication, viral proteins are presented on the cell surface of AEC, which can act as antigen-presenting cells.[18,19] Strategies involving nonspecific immunosuppression to enable repeated SeV administration are not an option in CF because the lungs of patients are chronically infected with pathogenic bacteria. However, we hypothesized that a strategy involving the induction of specific immunological tolerance to components of the SeV might facilitate repeat administration of SeV while leaving other defenses intact.

Since the early days of cellular immunology when it was noted that administration of antigen at very high or low doses could result in specific unresponsiveness, many parameters of tolerance induction have been characterized.[20] Route of administration is one important factor, oral and intranasal pre-administration of soluble peptide having been shown to induce tolerance in murine models of autoimmunity, transplantation and allergy.[21–24] Induction of tolerance by peptide has also been successfully applied to humans.[25,26] There has been less impetus to investigate the induction of therapeutic tolerance to eliminate antiviral responses, but a number of examples have been described.[27–29] Despite the potential complexity of antiviral T-cell responses, the recognition of virus both in mouse models and in humans is often highly focused on a small number of epitopes, making peptide-induced tolerance a realistic possibility.[30–32] Immunological tolerance may act through a number of different mechanisms, including deletion of reactive clones, anergy through incomplete or altered signalling and regulation by regulatory T cells. Here we evaluate the impact of inducing intranasal tolerance to the CD4 and CD8 immunodominant T-cell epitopes of SeV on gene expression following repeated administration to the airways. This approach is based on the premise that antibody responses (except for rare examples such as the T-cell-independent responses to bacterial polysaccharides) are absolutely dependent on CD4 T-cell help, so that tolerance of T cells should impact also on the neutralizing antibody response.

Results

Repeat administration of ΔF/SeV to the lung leads to reduced gene expression

A schematic diagram illustrating the dosing regime and viral construct used is shown in Figure 1. To assess the effect of repeat administration of ΔF/SeV to the lungs, animals were transduced with ΔF/SeV-GFP on day 0, followed by repeat administration of ΔF/SeV-lacZ on day 21. β-galactosidase (βgal) expression in lung homogenate was measured 2 days after ΔF/SeV-lacZ transduction and compared to animals receiving only one administration of ΔF/SeV-lacZ. Figure 2 shows that although βgal expression levels after repeat administration were still significantly ($P<0.005$) higher than in untreated mice ($n=22$), expression was significantly ($P<0.05$) reduced by 60% when compared to one administration ($n=22$).

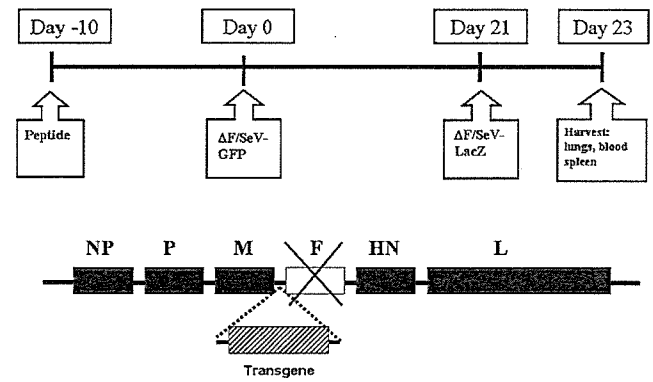

Figure 1 Schematic representation of the protocol for repeat administration of ΔF/SeV to the airways and the viral constructs used. The F protein is deleted from the viral genome and provided *in trans* during vector production. The transgene is inserted between the M and HN proteins.

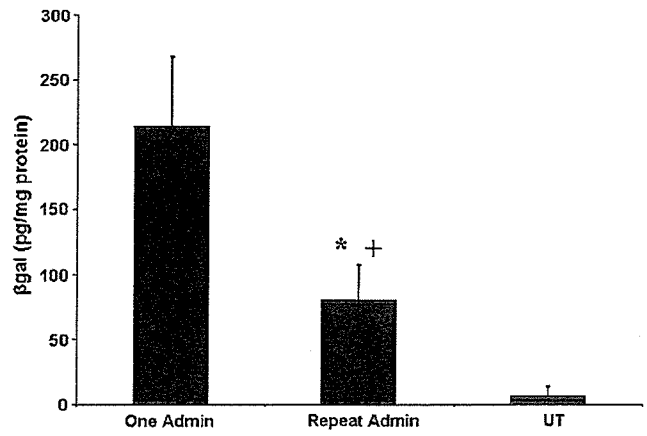

Figure 2 βgal expression after a single and repeat administration of the ΔF/SeV, to the airways. For repeated administration of ΔF/SeV lungs of mice were transduced with ΔF/SeV-GFP (day 0) and ΔF/SeV-lacZ (day 21) using the standard 'sniffing' protocol (repeat admin). The single administration (one admin) group received only the ΔF/SeV-lacZ virus. Lungs were harvested 2 days after ΔF/SeV-lacZ transfection and βgal expression was analyzed. Data are presented as mean ± s.e.m. $^{*}P<0.005$ when compared to untreated (UT), $^{+}P<0.05$ when compared to one administration, $n=22$/group.

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide epitopes impacts on T-cell proliferation

In an attempt to improve transfection efficiency after repeat administration of the ΔF/SeV, animals were pretreated intranasally with either the CD4 or CD8 immunodominant T-cell peptide epitopes to SeV at different doses 10 days before the first virus administration (Figure 1). In the C57BL/6 mouse model of lung immunity to SeV infection, the CD8 T-cell response is highly focused on the nucleoprotein (NP) 324–332 epitope and the CD4 T-cell response on hemaglutinin (HN) 419–433.[30,33] One control group of mice received an irrelevant T-cell epitope peptide, ovalbumin (OVA) 326–339, while the other control group of mice received no intranasal peptide pretreatment. The administration of SeV vectors to the mice primes T-cell proliferation to the CD4 (NP 324–332) and CD8 (HN 419–433) immunodominant epitopes. Intranasal pretreatment with a single dose of 1 μg/ml of the CD4 immunodominant peptide epitope resulted in a significant ($P<0.05$) reduction in T-cell proliferation of splenocytes *in vitro*, relative to the no peptide or the irrelevant peptide control groups. This reduced proliferation (equivalent to 2.4 ± 0.7- and 1.5 ± 0.6-fold greater proliferation than in nonantigen-exposed controls after challenge with CD4 and CD8 immunodominant epitopes, respectively) was seen when splenocytes were challenged *in vitro* with 50 μg/ml of either of the CD4 (Figure 3a) or the CD8 (Figure 3b) immunodominant epitopes.

The reduction in proliferation after challenge with the CD8 epitope in the group of mice pretreated with 1 μg/ml of the CD4 peptide epitope reflects the dependence of CD8 responses on CD4 T-cell help. Intranasal pretreatment with 1 μg/ml of the CD4, but not the CD8, immunodominant peptide epitope resulted in the induction of T-cell tolerance.

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide epitopes has no impact on anti-SeV neutralizing antibodies

To obtain a functional readout of neutralizing antibody titers, ΔF/SeV-lacZ virus was pretreated with serum from: (1) untreated mice; (2) mice that had received a single administration of ΔF/SeV-lacZ; (3) mice that had received a repeat administration of ΔF/SeV. Virus were then used to infect HeLa cells. A representative graph is shown in Figure 4a. At low serum dilutions, all the mice that had received two doses of ΔF/SeV produced anti-SeV neutralizing antibodies and showed reduced transfection of HeLa cells. The transduction efficiency was restored by subsequent serum dilutions. In contrast, serum from untreated control mice and mice receiving only one administration of ΔF/SeV (serum collected two days after transduction) did not produce any neutralizing antibodies. In Figure 4b, the serum dilution at which 50% of βgal expression of the untreated control mice was reached is plotted. There was no significant difference in neutralizing antibody measurements between the groups. In contrast to the reduced T-cell proliferation following pretreatment with intranasally-administered peptide, there was no evidence of any reduction in the neutralizing antibody titer indicating 'split tolerance'.

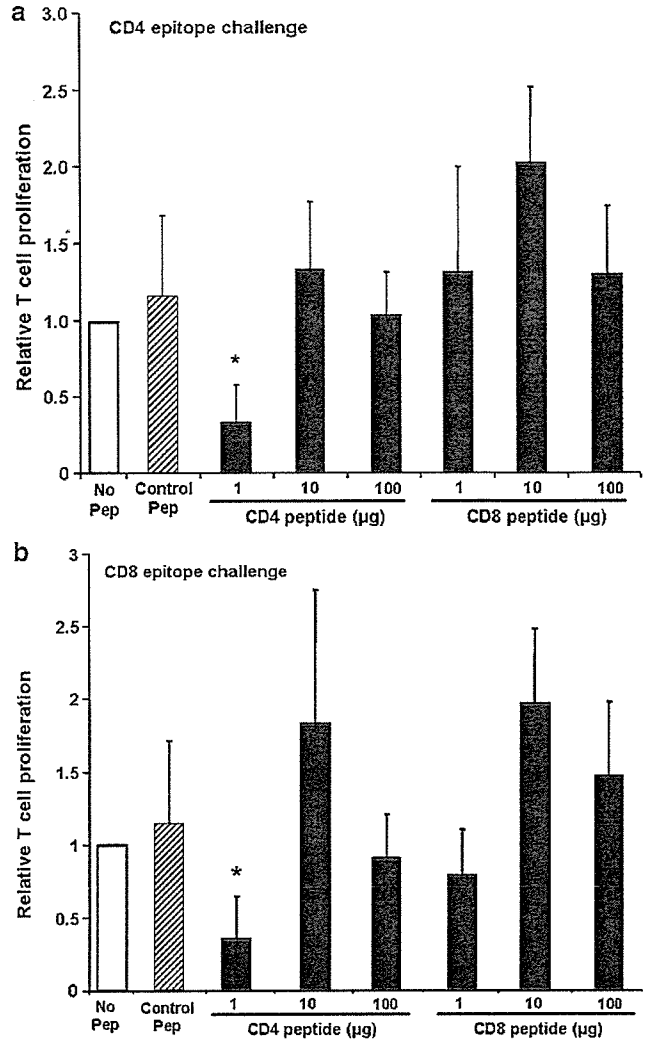

Figure 3 T-cell proliferation against CD4 (a) and CD8 (b) immunodominant T-cell epitopes of SeV following intranasal pretreatment with the CD4 and CD8 epitopes. Splenocytes were incubated in triplicate with peptide at the concentrations indicated. Cultures were harvested at 72 h for counting in a beta-scintillation counter. For the final 12 h of culture, 1 μCi of ^{3}H-thymidine was added to each well. The mean value of the triplicates was calculated and ^{3}H-thymidine incorporation data were standardized by calculating the relative ratio compared to the no peptide control group. The relative proliferation in the presence of 50 μg/ml of the CD4 (a) and CD8 (b) immunodominant epitope to SeV is shown. Groups of mice were administered the concentrations shown of the CD4 or CD8 immunodominant epitopes, control peptide or no peptide to the lungs on day −10 prior to transfection with ΔF/SeV. Data were normalized to the no peptide control group and presented as mean$\pm$s.e.m. *$P<0.05$ when compared to no peptide or irrelevant peptide control group ($n=6$–12/group).

Intranasal pretreatment with CD4 and CD8 T-cell SeV immunodominant peptide does not improve levels of gene expression after repeat administration of ΔF/SeV

To assess the effect of tolerization on repeat administration, βgal expression was measured in lung homogenate 2 days after the second ΔF/SeV transfection. Peptide preadministration did not improve gene expression. Indeed, βgal expression was reduced in mice that had a reduction in T-cell proliferation after immunization

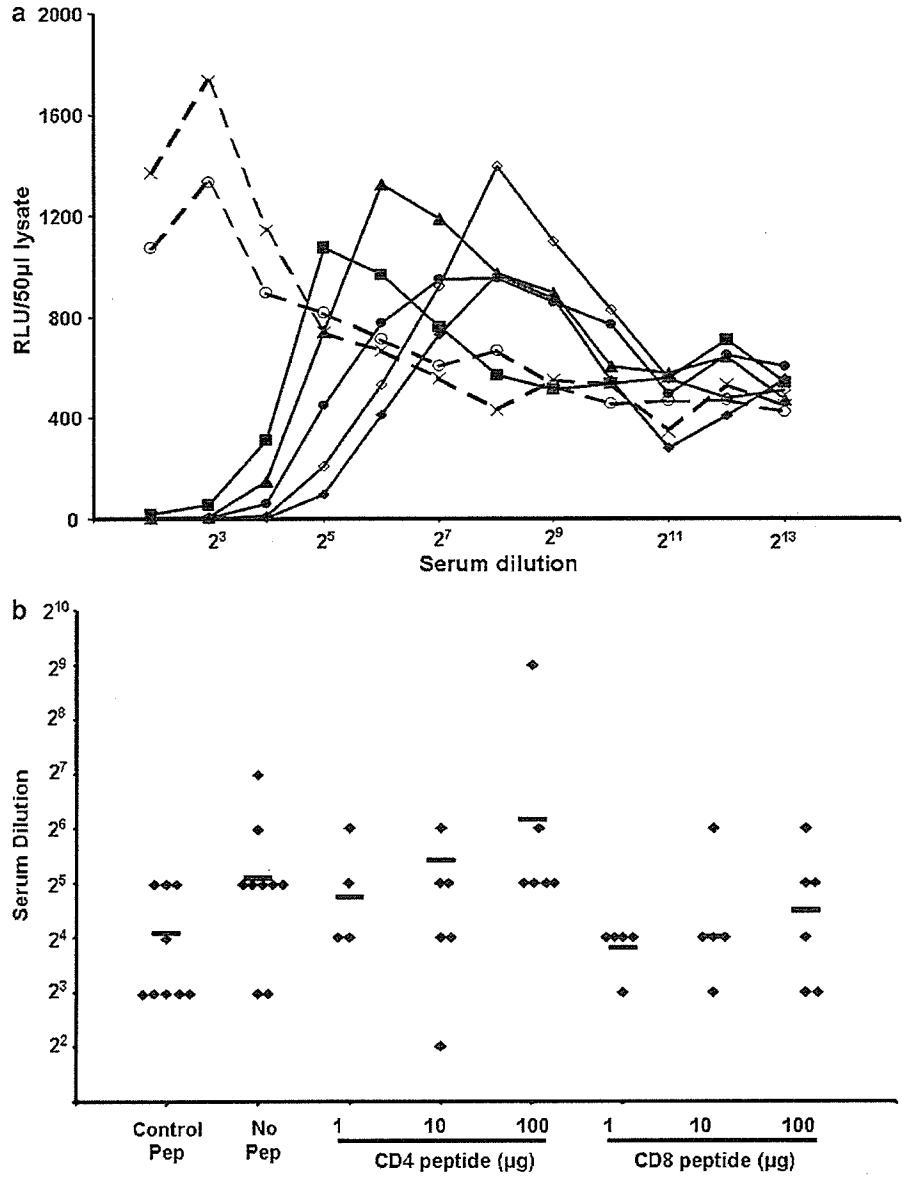

Figure 4 Anti-SeV neutralizing antibodies following pretreatment with CD4 and CD8 immunodominant T-cell epitopes to SeV. Neutralizing serum antibodies were measured by incubating ΔF/SeV-lacZ with serial dilutions of serum from all animals, with subsequent transfection of HeLa cells. A representative example for each group is shown (**a**) (cross = untreated, open circle = one administration, open diamond = no peptide animal, closed circle = control peptide, closed diamond = 1 µg CD4 peptide, closed square = 10 µg CD4 peptide, closed triangle = 100 µg CD4 peptide). The serial dilution at which 50% transfection efficiency of virus incubated with serum from untreated mice was reached was then plotted for each animal (**b**). Each diamond represent one animal and horizontal bars represent the mean.

with 1 µg/ml of CD4 immunodominant peptide compared to control groups (Figure 5).

Discussion

Administration of viral vectors to the lung must overcome the significant hurdle of highly evolved immune mechanisms that exist to recognize and eliminate pathogens.[8] As expected, we show here that βgal expression after repeat administration of recombinant ΔF/SeV-lacZ is comparatively inefficient due to potent immune responses to the vector. We, therefore, assessed the effect of tolerance induction to the immunodominant CD4 and CD8 epitopes from SeV and showed that, despite achieving a reduction in T-cell proliferation via the CD4 epitope, there was no reduction of anti-SeV neutralizing antibodies or increase in gene expression.

Nonviral GTAs are less efficient in transfecting AECs than viral vectors. However, viral GTAs are immunogenic and a significant reduction in gene expression after repeat administration has previously been reported for commonly used viral GTAs such as adenovirus and AAV, and is due to the generation of immune responses against viral proteins.[34]

First-generation recombinant SeV retains all viral genes,[35] and are transmission-competent. Second-generation, attenuated, nontransmissable SeV has been generated by deleting the F protein (ΔF/SeV) required for viral entry into the cell.[36] We have demonstrated that second generation ΔF/SeV is as efficient as first-generation viruses in transfecting AECs.[7] Following transduc-

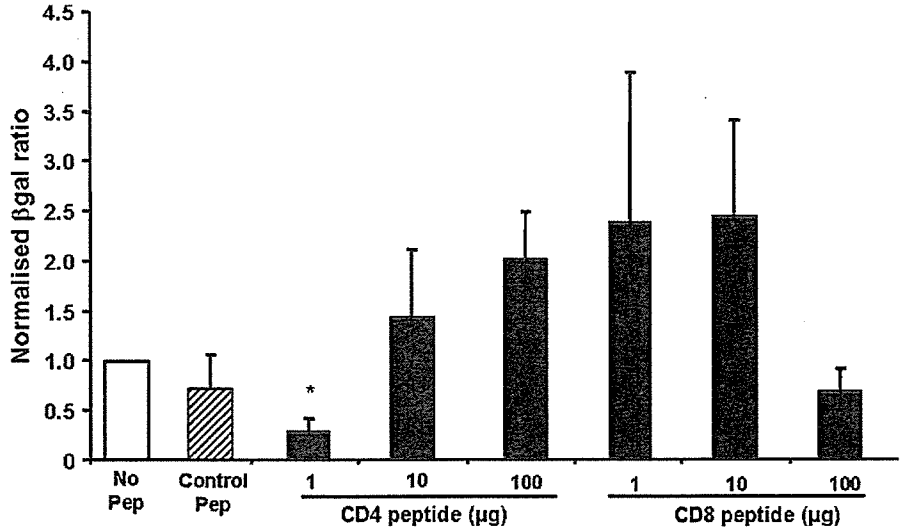

Figure 5 βgal expression after repeated administration of ΔF/SeV following pretreatment using intranasal CD4 and CD8 immunodominant T-cell epitopes to SeV. To assess the effect of peptide tolerization on gene transfer efficiency after repeat administration, peptide was administered to the lungs of mice in a single bolus of 100 μl using the standard 'sniffing' procedure on day −10. Mice were transfected with ΔF/SeV-GFP on day 0 and with ΔF/SeV-lacZ on day 21. Lungs were harvested 2 days after ΔF/SeV-lacZ transfection and βgal expression analyzed. Data were normalized to the no peptide control group and presented as mean ± s.e.m. Please refer to Figure 2 for absolute βgal expression values. The no peptide control group is identical to the repeat administration group in Figure 2. Please refer to $^*P < 0.05$ when compared to no peptide control group, $n = 12$/group.

tion, ΔF/SeV replicates in the cytoplasm and, although the F protein-deleted second-generation ΔF/SeV is transmission-incompetent, viral proteins are incorporated into the cell membrane of the infected cell and virus-like noninfective particles are released from the cells. Third-generation SeV vectors have been further depleted of the HN and M proteins, but the remaining viral proteins (NP, P, L) cannot be deleted, because they are essential for recombinant protein production.[37] Importantly, however, the complete removal of all viral proteins from adenoviral vectors has not improved their capacity for repeat administration.[38] Alternative strategies to enable repeat administration of SeV clearly need to be developed. Nonspecific immune suppression is not clinically desirable in CF, particularly in the context of SeV gene therapy, which would require prolonged immune suppression for the duration of SeV-mediated gene expression, because of the persistent virus replication. Specific tolerization of CF patients to SeV, which is not a human pathogen, however, might be a clinically feasible option. The induction of specific immunological tolerance is a manipulation that has been successfully applied in a number of other experimental and clinical settings.[26,27]

A wide variety of strategies have previously been investigated for the induction of effective immunological tolerance against viral antigens. While the goal in most systems is to promote antiviral immunity, such responses may be undesirable when they lead to loss of therapeutic gene expression as shown here, or when the response is itself associated with excessive immunopathology.[28,39] Immunological tolerance to viruses has previously been achieved by administration of tolerogenic peptides,[28,40] by oral administration of antigen,[41] by giving virus under the cover of either anti-CD4 or anti-CD40L antibody,[39] or by giving apathogenic virus intravenously.[27,42] While neonatal administration of virus might be expected to induce tolerance, this can alternatively lead in some cases to highly efficient priming of responses. More robust tolerance may be achieved through neonatal expression, which is specifically targeted to the thymus.[29] However, this approach raises concerns about the ease of translation to clinical practice. In light of these considerations, we decided to investigate peptide-induced tolerance, which has a strong track record of translation to the clinic in a range of diseases.[26,43] While there are theoretical concerns that it might not be possible to achieve immunological tolerance to a complex set of viral antigens through administration of a single epitope, analysis of viral immune responses suggests that T cells often focus on a rather small number of immunodominant epitopes.[44] Furthermore, peptides are relatively cheap to prepare, easily administered and well tolerated.

A large body of work by David Woodland and others on protective immunity against SeV indicates a vital role of T cells.[45,46] We, therefore, administered CD4 and CD8-immuno dominant SeV epitopes to the lungs of mice 10 days before ΔF/SeV. While tolerance experiments in mouse models have tended to rely on a form of 'high-dose' tolerance at 100 μg or greater,[23,47] we found, in line with recent findings from clinical studies,[25] that tolerance was actually induced after a single dose of 1 μg peptide, and not at higher doses. Interestingly, a single tolerizing dose of the CD4 epitope largely abrogated not only the response of T cells to this epitope, but also to the immunodominant CD8 class I restricted epitope, presumably because the latter response is dependent on help from CD4T cells. Despite reasonably robust T-cell tolerance, we obtained no evidence for enhanced levels of gene expression from repeat virus administration. The most likely explanation for this is that we generated 'split tolerance' that left neutralizing antibody titers unaltered, and elimination of repeat virus administration was here

achieved through antibody neutralization of input virions. 'Split tolerance', affecting one component of the immune system, but not others, is a frequent observation in viral systems, presumably because a multi-layered immune response against pathogenic viruses has been a strong evolutionary driving force.[27,48] How could an effective neutralizing antibody response develop in the face of a strong reduction in the peptide-specific CD4 helper T-cell response? One possibility is that there is sufficient, residual T-cell effector function available to supply a low level of cytokine support for the B-cell response. Another possibility is that, as a result of peptide-specific tolerance induction, the antiviral response shifts to subdominant epitopes. Analysis of these hypotheses will require further investigation. Even if one could achieve absolute tolerance with respect to the antibody and T-cell limbs of the adaptive immune response, there is still the likelihood of an efficient, or even an enhanced response using other mechanisms, for example, natural killer (NK) 1.1 T cells.[49] Thus, Scaria *et al*.[50] have demonstrated previously that inhibition of cytotoxic T cells (CTLs) through overexpression of ICP47, a protein that prevents MHC class I-mediated antigen presentation to CD8 T cells, leads to an increased activation of NK cells. Indeed, in our study, elimination of βgal viral expression appears enhanced in the presence of split tolerance. This is an important caveat for immunomodulation initiatives: compensatory mechanisms including NK cells that replace the normal, adaptive T-cell response may be even more potent.

In conclusion, induction of tolerance using an immunodominant CD4 peptide epitope does not allow more efficient gene expression following a second administration of a SeV vector.

Materials and methods

Peptide and recombinant SeV preparation

Peptides were synthesized on a 20–40 mg scale (Bio-Synthesis Incorporated, Lewisville, TX, USA) and arrived lyophilized. [(CD4 epitope – SeV HN protein 419–433): NH2-VYIYTRSSGWHSQLQIG-OH, (CD8 epitope – SeV NP protein 324–332): NH2-FAPGNYPAL-OH, control peptide epitope OVA 326–339: NH2-AVHAA-HAEINEAGR-OH]. The peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at a concentration of 75 mg/ml at −80°C. Final working concentrations of peptide for experimental use were made up on the day using PBS.

The generation and propagation of the recombinant ΔF/SeV vector carrying a green fluorescent protein (ΔF/SeV-GFP) or a lacZ reporter gene (ΔF/SeV-LacZ) was carried out as described previously.[4] The supernatant of LLC-MK2/F7 cells containing infectious particles was subsequently purified, concentrated and stored at −80°C. Virus titer was determined by infecting LLC-MK2 cells and counting the number of βgal or GFP-expressing cells. For both viruses, the titer was expressed as Cell Infectious Units (CIU) per ml.

Administration of peptide and virus to mouse lung

C57BL/6 mice (female 6–10 weeks) were anesthetized with metophane (Medical Developments Australia Pty Ltd, Springvale, Australia) and the peptide (1, 10 or 100 μg in 100 μl PBS) was placed as a single bolus into the nasal cavity and the solution rapidly 'sniffed' into the lungs. At 10 days after peptide administration, mice were transduced with ΔF/SeV-GFP (10^6 CIU/mouse in 100 μl PBS), and 21 days later with ΔF/SeV-lacZ (10^7 CIU/ mouse in 100 μl PBS) using the 'sniffing' technique. Control groups receiving no virus or one administration of ΔF/SeV-lacZ were included. At 2 days after the last virus administration, all animals were culled. Lungs from individual mice were snap-frozen in liquid nitrogen and stored at −80°C for βgal quantifiaction. Spleens from individual mice were harvested for T-cell proliferation assays. Blood was collected and stored at 37°C for a minimum of 2 h. After clotting, the blood was spun at 4000 r.p.m. for 10 min and serum was frozen at −80°C for anti-SeV neutralizing antibody measurements.

T-cell proliferation

Spleen cells were disaggregated using fine gauge needles, resuspended in HL1 serum-free medium (Hycor Biomedical, Irvine, CA, USA) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), 30 IU penicillin and 30 μg/ml streptomycin, and 4×10^5 cells/well were aliquoted in triplicate in flat-bottom microculture plates and incubated with peptide at different concentrations to measure T-cell proliferation in response to the CD4 and CD8 immunodominant peptide epitopes of SeV. For the final 12 h of culture, 1 μCi of ^{3}H-thymidine was added to each well. Cultures were harvested at 72 h for counting in a beta scintillation counter. The mean values of the triplicates were calculated and ^{3}H-thymidine incorporation data were standardized by calculating the relative ratio compared to the no peptide control group.

Neutralizing antibody assay

HeLa cells (2500 per well in a 96-well plate) were seeded 1 day before transfection. Serum was heat-inactivated for 40 min at 55°C and 1:2 serial dilutions in DMEM (+1% FCS +1% penicillin/streptomycin) prepared (1:2–1:8192). ΔF/SeV-LacZ (5×10^4 CIU) was added to each serum dilution and incubated at 37°C for 1 h in an incubator (5% CO_2). Following the incubation, the medium is removed from the HeLa cells and replaced with the 100 μl of the serum dilutions containing the virus. Cells were incubated for 5 h at 37°C, after which the serum was removed and replaced with normal growth medium (DMEM+10% FCS and 1% penicillin/streptomycin). At 24 h after transfection the medium was removed, cells are washed with PBS and lysed with 120 μl Universal lysis buffer (Roche), freeze/thawed three times, and frozen at −80°C. βgal protein expression was quantified in homogenates using the luminescent βgal Reporter System 3 (Clontech, UK) according to the manufacturer's recommendations.

Reporter gene assay

Lungs were homogenized in 300 μl Universal lysis buffer (Roche), freeze/thawed three times, spun at 10 000 r.p.m. for 10 min and supernatant was frozen. βgal protein expression was quantified in lung homogenates using the luminescent βgal Reporter System 3 (Clontech, UK) according to the manufacturer's recommendations. Total protein was quantified using the DC Protein Assay Kit