

Primary replication of a recombinant Sendai virus vector in macaques

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An efficient antigen expression system using a recombinant Sendai virus (SeV) has been established recently and its potential to induce resistance against immunodeficiency virus infections in macaques has been shown. SeV replication has been well characterized in mice, the natural host, but not in primates, including humans. Here, primary SeV replication was investigated in macaques. After intranasal immunization with a recombinant SeV expressing simian immunodeficiency virus Gag protein, SeV-Gag, robust *gag* expression was observed in the nasal mucosa and much lower but significant levels of *gag* expression were observed in the local retropharyngeal and sub-mandibular lymph nodes (LN). Expression peaked within a week and lasted at least up to 13 days after immunization. SeV-Gag was isolated from nasal swabs consistently at day 4 but not at all at day 13. *Gag* expression was undetectable in the lung as well as in remote lymphoid tissues, such as the thymus, spleen and inguinal LN, indicating that the spread of the virus was more restricted in macaques than in mice. SeV-specific T cells were detectable in SeV-immunized macaques at day 7. Finally, no naive macaques showed significant levels of anti-SeV antibodies in the plasma, even after living in a cage together with an acutely SeV-infected macaque for 5 weeks, indicating that SeV transmission from SeV-infected macaques to naive ones was inefficient. None of the SeV-immunized macaques displayed appreciable clinical manifestations. These results support the idea that this system may be used safely in primates, including humans.

Introduction

Virus-specific T cell responses have been shown to play an important role in controlling human immunodeficiency virus type 1 (HIV-1) infections (Ogg *et al.*, 1998; Rowland-Jones *et al.*, 1998; Brander & Walker, 1999; Seder & Hill, 2000). Furthermore, in macaque AIDS models, the importance of CD8⁺ T cells in the control of immunodeficiency virus infections has been shown by CD8⁺ T cell depletion using anti-CD8 antibodies *in vivo* (Matano *et al.*, 1998; Schmitz *et al.*, 1999; Jin *et al.*, 1999). Thus, induction of virus-specific T cell

responses would be of value for the protection against AIDS. Virus vector-based gene delivery is a promising AIDS vaccine strategy because of its potential for inducing cellular immune responses.

Sendai virus (SeV) is an enveloped virus with a negative-sense RNA genome of about 15.3 kb. It causes fatal pneumonia in mice, the natural host, but is thought to be nonpathogenic in humans (Nagai, 1999). We previously established a system to rescue SeV from cDNA, which enabled us to engineer the SeV genome at will and use the virus as a vector to deliver foreign genes of interest (Hasan *et al.*, 1997; Yu *et al.*, 1997; Nagai & Kato, 1999). Recent studies using animals such as mice and ferrets have shown an extremely high efficiency of SeV vector-based gene transfer *in vivo* (Yonemitsu *et al.*, 2000). Because SeV replication requires a proteinase that is localized in the

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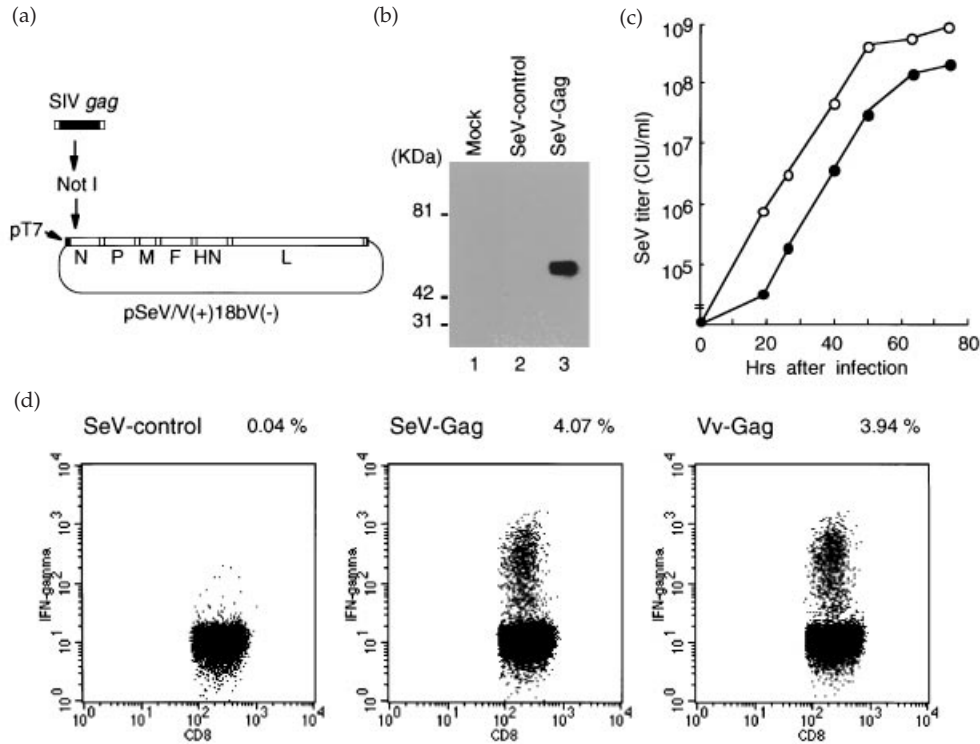


Fig. 1. SIV Gag expression in SeV-Gag-infected cells. (a) Structure of pSeV(+)/18bV(-)/SIVgag, a plasmid generating an antigenomic RNA of SeV-Gag. (b) Western blot analysis using a monoclonal mouse anti-p27 antibody. CV-1 cells were lysed 24 h after mock infection (lane 1), SeV-control infection (lane 2) or SeV-Gag infection (lane 3). (c) Replication kinetics of SeV-control (○) and SeV-Gag (●) in CV-1 cells under multiple cycle growth conditions in the presence of trypsin (7.5 µg/ml) (Sakai *et al.*, 1999). At the initial infection, cells were infected with SeV-control or SeV-Gag at an m.o.i. of 0.05. SeV titres in the supernatants harvested at several time-points were assayed on CV-1 cells by immunostaining. (d) Dot plots showing Gag-specific intracellular IFN- γ induction in PBMCs after coculture with SeV-Gag-infected cells. PBMCs from an SIV-infected macaque were cocultured with the autologous B-LCLs infected with SeV-control (left panel), SeV-Gag (middle panel) or Vv-Gag (right panel). Dot plots gated on CD3⁺CD8⁺ lymphocytes are shown. The per cent of IFN- γ ⁺ cell number to the gated cell number in each dot plot is shown.

airway epithelium for Env protein processing (Nagai, 1993), the intranasal route would be the best for efficient delivery and this may be favourable for the induction of mucosal immune responses.

We previously knocked out the SeV V gene, an accessory gene, to obtain an attenuated SeV, V⁻SeV (Kato *et al.*, 1997a, b). V⁻SeV infection is not fatal in mice and the virus retains the ability to induce efficient gene transfer. Indeed, expression levels of HIV-1 Env gp120 from recombinant V⁻SeV-infected cells reached 6 µg per 10⁶ cells, the highest among the vector systems available in mammalian cells (Yu *et al.*, 1997).

We then constructed a recombinant V⁻SeV expressing the simian immunodeficiency virus (SIV) Gag protein, SeV-Gag, to evaluate the protective efficacy of its immunization in macaque AIDS models. In two sets of macaque experiments, intranasal SeV-Gag immunization elicited resistance against a pathogenic SIV, strain mac239 (SIV_{mac239}), and a pathogenic SIV/HIV strain, SHIV89.6PD (Kano *et al.*, 2000; Matano *et al.*, 2001). The SeV-Gag-immunized macaques failed to control primary viraemia but greatly reduced the set-point plasma virus loads

after challenge. These results prompted us to determine the pattern of SeV replication in nonhuman primates for utilizing this vector as a tool for an AIDS vaccine in humans effectively and safely. In this study, we examined primary SeV replication and SeV-specific immune responses in macaques following intranasal SeV immunization.

Methods

■ **Preparation of recombinant SeV.** By using a safer V gene knock-out version of SeV, V⁻SeV (Kato *et al.*, 1997a, b), we constructed a recombinant SeV vector expressing SIV Gag, SeV-Gag. The plasmid pSeV(+)/18bV(-), which contains the full-length genome sequence of attenuated V gene-defective SeV cDNA, was described before (Kato *et al.*, 1996; Hasan *et al.*, 1997). A gene fragment encoding SIV_{mac239} Gag (nt 1306–2845, GenBank accession no. M33262) (Kestler *et al.*, 1990) was prepared by PCR and introduced into pSeV(+)/18bV(-) to obtain pSeV(+)/18bV(-)/SIVgag (Fig. 1a). Primers used for the PCR were 5' AAGCGCCGCGAGATGGGCGTGAGAACTCCG and 5' TTG-CGGCCGCGATGAACCTTTCACCTAAGTTTTTCTTACTGTGAC-TACTGGTCTCTCCAAAG. The gag gene fragment was inserted into

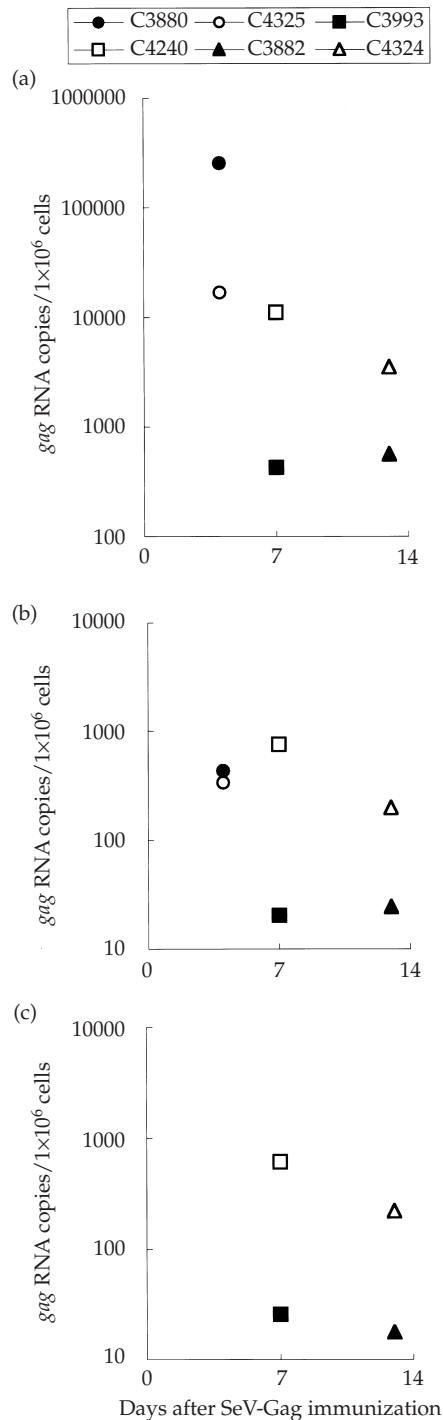


Fig. 2. Levels of SIV *gag* RNA in macaques after intranasal SeV-Gag immunization. Levels of SIV *gag* RNA in cells prepared from the nasal mucosa (a), retropharyngeal LN (b) and submandibular LN (c) are shown. RNA levels in C3880 or C4325 at day 4 were not determined because we failed to obtain the submandibular LN from these monkeys.

the *NoI* site, immediately upstream of the N-terminal coding region of pSeV(+)*18bV*(-). SeV-control and SeV-Gag were recovered from pSeV(+)*18bV*(-) and pSeV(+)*18bV*(-)/SIVgag, respectively. A

recombinant SeV expressing HIV-1 gp120 was obtained as described previously (Yu *et al.*, 1997). The titre of SeV, measured in cell infectious units (CIU)/ml was assayed on CV-1 cells by immunostaining using an anti-SeV antibody, as described before (Kiyotani *et al.*, 1990).

Western blot analysis. CV-1 cells were seeded at a density of 4×10^5 cells per well in a 6-well plate, grown overnight and then infected with SeV-control or SeV-Gag at an m.o.i. of 5. A day later, the cells were lysed with 600 μ l of triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 0.5% sodium deoxycholate, 0.1 mg/ml PMSF and 1% Triton X-100). Western blot analysis using a monoclonal mouse anti-p27 (SIV Gag CA) antibody was performed as described before (Matano *et al.*, 1993). For each lane, 10 μ l of cell lysate was loaded for analysis.

Animals. Cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) used in this study were maintained in accordance with the institutional guidelines for laboratory animals. These macaques tested negative for SeV and SIV before use. Monkey R010 received a control DNA vaccine, 800 μ g pCMVN DNA (Matano *et al.*, 2000) by intramuscular inoculation and 10 μ g pCMVN DNA by gene gun (four times), from 12 to 6 weeks before the SeV-control immunization for another experiment. Blood collections, sampling of nasal swabs and immunizations were performed under ketamine anaesthesia.

SeV-Gag RNA expression in tissues. Cells from the lymph nodes (LN), thymus and spleen were prepared by mincing the tissues. Cells from the nasal mucosa and lung were prepared after treatment with collagenase type I and dispase (Life Technologies). These cells were washed with PBS three times before RNA extraction. Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood samples using Ficoll-Paque Plus (Amersham-Pharmacia). RNA was extracted from cells using an RNA Extraction kit from Qiagen, according to the manufacturer's instructions. Nested RT-PCR was performed using SIV *gag*-specific primers (5' AGAAACTCCGTCTTGTCAGG and 5' TGAT-AATCTGCATAGCCGC for the first RT-PCR and 5' GATTAGCAGA-AAGCCTGTTGG and 5' TGCAACCTTCTGACAGTGC for the second PCR). The levels of *gag* RNA were quantified by limiting dilution of RNA samples to determine the end-point, as described before (Shibata *et al.*, 1997).

SeV recovery from nasal swabs or nasal mucosa. The nasal swab sample diluted in RPMI-1640 (Life Technologies) was injected into the allantoic cavity of chickens' eggs for recovery of SeV. In case of SeV recovery from the cells prepared from the nasal mucosa, the cells were freeze-thawed twice and 10^5 cells suspended in RPMI-1640 were injected into the allantoic cavity of chickens' eggs. After 72 h of incubation, the allantoic fluid was harvested and subjected to a haemagglutination (HA) assay to detect SeV, as described before (Kato *et al.*, 1996).

Antibody ELISA. An ELISA to measure plasma levels of anti-SeV antibodies was performed using inactivated SeV (Watanabe *et al.*, 2000).

Flow cytometry analysis of antigen-specific interferon- γ (IFN- γ) induction. Autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) (Voss *et al.*, 1992) were infected with a control vaccinia virus (VV) vector (Vv-control) (Mackett *et al.*, 1982), SeV-control, a recombinant VV vector expressing SIV Gag (Vv-Gag) or SeV-Gag at an m.o.i. of 5 and, 1 day later, used as a nonspecific control, an SeV-specific, a Gag-specific or an SeV-Gag-specific stimulator. For stimulation, 10^6 PBMCs were cocultured with 10^5 stimulator cells in the presence of GolgiStop (monensin) (Pharmingen) for 6 h. Then, intracellular IFN- γ staining was performed using Cytotfix-Cytoperm kit

(Pharminggen), according to the manufacturer's protocol, as described before (Matano *et al.*, 2001).

Results

Gag expression from SeV-Gag *in vitro*

SeV-Gag was rescued from the plasmid pSeV(+)18bV(-)/SIVgag (Fig. 1a). Expression of unprocessed SIV Gag (p55) in SeV-Gag-infected CV-1 cells was confirmed by Western blot analysis (Fig. 1b) as well as by immunostaining (data not shown) using a monoclonal anti-SIV Gag p27 antibody. The recombinant virus showed slightly slower proliferation rate compared with that of SeV-control (Fig. 1c). This retarded replication might be due to the increase in genome length (by 1.6 kb) as a result of the insertion [pSeV(+)18bV(-), 15.4 kb; pSeV(+)18bV(-)/SIVgag, 17.0 kb] of the Gag protein-coding region (Hasan *et al.*, 1997; Yu *et al.*, 1997; Sakai *et al.*, 1999).

Restimulation of Gag-specific CD8⁺ T cells by SeV-Gag-infected cells *in vitro*

Using flow cytometry analysis of antigen-specific intracellular IFN- γ induction (Lavini *et al.*, 1997; Butz & Bevan, 1998; Murali-Krishna *et al.*, 1998; Donahoe *et al.*, 2000; Gea-Banacloche *et al.*, 2000), we examined if SeV-Gag-infected cells can really restimulate Gag-specific CD8⁺ T cells *in vitro* (Fig. 1d). We used PBMCs derived from a rhesus macaque chronically infected with SIV as a source of Gag-specific CD8⁺ T cells. This animal received a proviral DNA vaccine, followed

by SIV_{mac239} challenge, as described before (Matano *et al.*, 2000).

Coculture of PBMCs with autologous B-LCLs infected with recombinant VV expressing SIV Gag (Vv-Gag) showed SIV Gag-specific intracellular IFN- γ induction in CD8⁺ T cells. Similar frequencies of CD8⁺IFN- γ ⁺ T cells were observed in PBMCs cocultured with SeV-Gag-infected B-LCLs. No significant induction of CD8⁺IFN- γ ⁺ T cells was found in the PBMCs cocultured with SeV-control-infected B-LCLs. These results indicate that the SeV-Gag-infected B-LCLs restimulated Gag-specific CD8⁺ T cells efficiently, confirming the potential of our SeV vector system for inducing specific T cell responses.

SeV-Gag expression in the nasal mucosa after its intranasal immunization into macaques

The first *in vivo* experiment was performed to examine the primary replication of SeV-Gag in macaques. Six cynomolgus macaques were inoculated intranasally with 10⁸ CIU SeV-Gag. None of the monkeys showed apparent clinical symptoms. Two of them (C3880 and C4325) were euthanized at day 4, two (C3993 and C4240) at day 7 and two (C3882 and C4324) at day 13 after the inoculation, respectively. Cells were prepared from each tissue taken at autopsy and RNA was extracted from the cells. Histological analysis showed only a slight inflammatory change in the nasal mucosa but no pathological change in other tissues, including the lung (data not shown).

In the cells prepared from the nasal mucosa, quantitative RT-PCR detected 1.7 \times 10⁴ or 2.6 \times 10⁵ copies of *gag* RNA per 10⁶ cells at day 4 (Fig. 2a). Expression levels were less

Table 1. SeV-Gag replication in macaques after intranasal inoculation

Swab samples or 10⁵ cells prepared from the nasal mucosa were injected into the allantoic cavity of chickens' eggs. After 72 h of incubation, the allantoic fluid was harvested and subjected to HA assay to detect SeV. HA-positive (+) and HA-negative (-) results are shown. If the *gag* sequence was undetectable by nested RT-PCR, then the result is tabulated as Neg; the lower limit of the detection is 1 copy/2 \times 10⁵ cells. ND, Not determined.

Macaques	Recovery of SeV from nasal swab				Nasal mucosa	Detection of <i>gag</i> RNA			
	Day 0	Day 4	Day 7	Day 13		Lung	Thymus	Spleen	Inguinal LN
Autopsy at day 4									
C3880	-	+			+	Neg	Neg	Neg	Neg
C4325	-	+			+	Neg	ND	Neg	Neg
Autopsy at day 7									
C3993	-	+	-		-	Neg	ND	Neg	Neg
C4240	-	+	+		-	Neg	Neg	Neg	Neg
Autopsy at day 13									
C3882	-	+	-	-	-	Neg	ND	Neg	Neg
C4324	-	+	+	-	-	ND	nd	ND	Neg

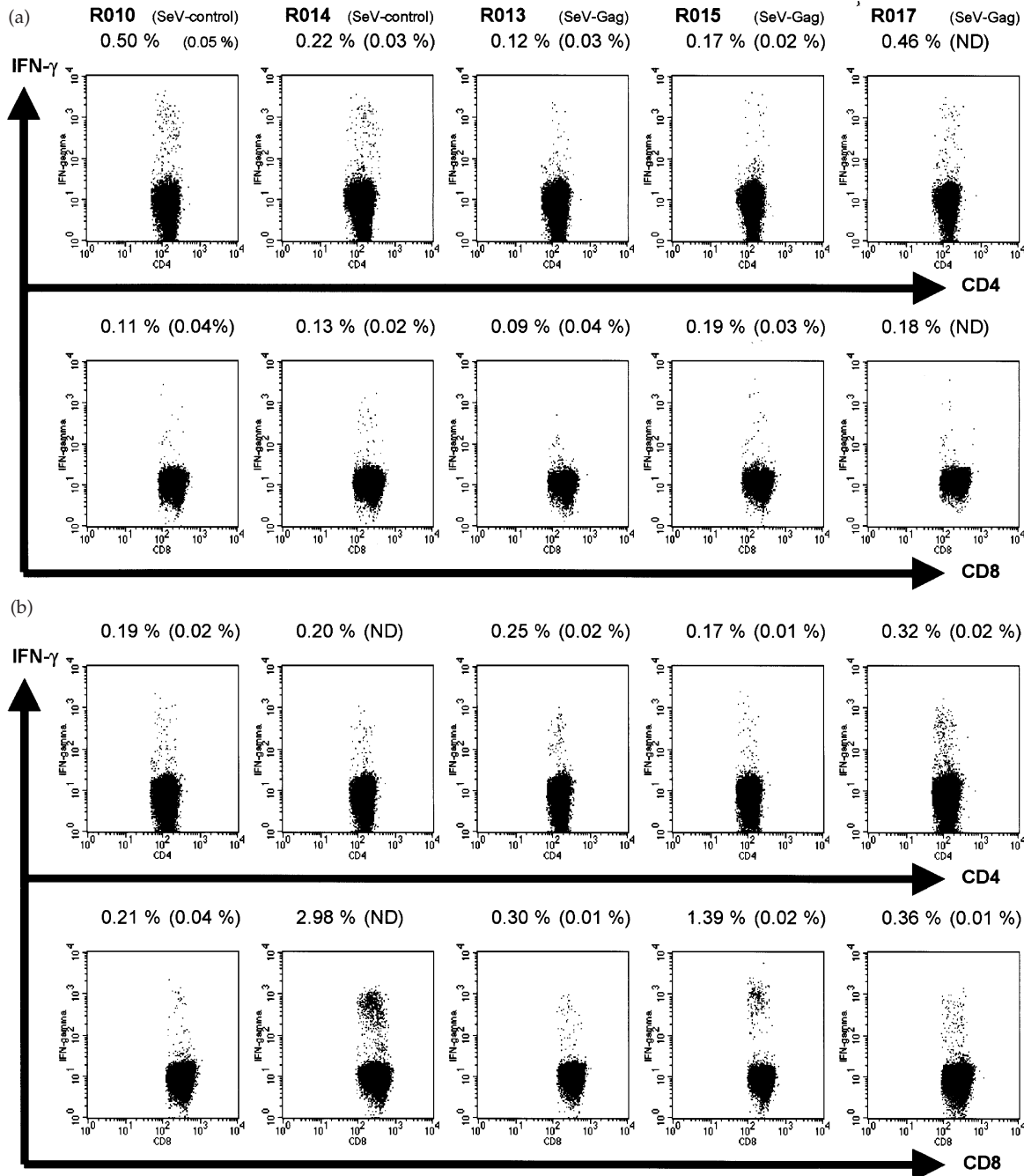


Fig. 3. Dot plots showing SeV-specific IFN- γ induction in peripheral CD4⁺ and CD8⁺ T cells in the immunized macaques. PBMCs at week 1 (a) or week 3 (b) were cocultured with B-LCLs infected with SeV-control and subjected to flow cytometry. Dot plots gated on CD3⁺CD4⁺ (upper panel in each) or CD3⁺CD8⁺ (lower panel in each) lymphocytes are shown. The per cent of IFN- γ ⁺ cell number to the gated cell number in each dot plot is shown. The per cent of IFN- γ ⁺ cell number after nonspecific stimulation (coculture with Vv-control-infected B-LCLs) is shown in parentheses. ND, Not determined.

prominent but still significant at days 7 and 13. These results indicate efficient SeV-Gag expression in the nasal mucosa after intranasal SeV-Gag inoculation.

Then, we examined if SeV was recovered from the nasal swab. As shown in Table 1, the virus was recovered from the swab at day 4 in all six animals. At day 7, virus was recovered

in two of four macaques, both of which showed higher levels of *gag* RNA in the nasal mucosa. At day 13, no virus was recovered in the animals. We also examined if the virus was recovered from 10⁵ cells prepared from the nasal mucosa (Table 1). The virus was recovered from the samples obtained at day 4 but not from those at days 7 or 13. These results

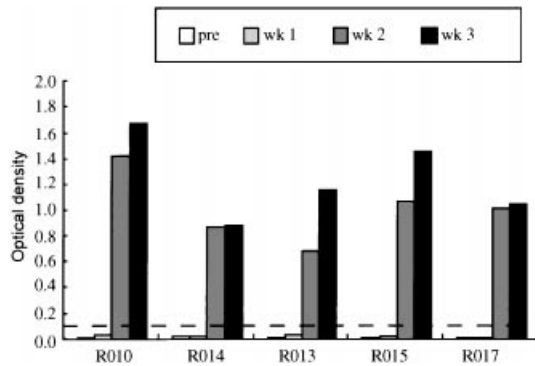


Fig. 4. Plasma anti-SeV antibody levels detected by ELISA in SeV-immunized macaques. The absorbance obtained with plasma diluted by 1:1000 is shown. The cut-off line is shown by a dotted line.

indicate that SeV-Gag replication rates reached the peak within a week after inoculation.

SeV-Gag expression in the primary LN of the nasal cavity after the immunization

The retropharyngeal LN and the submandibular LN receive the primary lymphocyte drainage from the nasal cavity (Suen & Stern, 1996) and we found *gag* RNA in both of them (Fig. 2b, c). The levels of *gag* RNA were about 1/20 of those in the nasal mucosa in each individual animal at days 7 and 13. At day 4, however, the level of *gag* RNA in the retropharyngeal LN was less than 1/50 of that in the nasal mucosa in each individual animal. Thus, no significant difference in the levels of *gag* RNA in the LN was observed between days 4 and 7, although the level of RNA in the nasal mucosa at day 4 was significantly higher than that at day 7.

SeV-Gag expression in other tissues after immunization

We also examined the expression of *gag* in the lung, thymus, spleen and inguinal LN but found no expression in any of them (Table 1).

SeV-specific immune responses in immunized macaques

The second *in vivo* experiment was performed to examine SeV-specific immune responses in macaques after intranasal SeV immunization. Two rhesus macaques (R010 and R014) received 10^8 CIU SeV-control and three (R013, R015 and R017) received 10^8 CIU SeV-Gag. The former group showed no IFN- γ induction after Gag-specific stimulation, while the latter group showed significant levels of Gag-specific T cell responses (Matano *et al.*, 2001). None of them showed apparent clinical symptoms after immunization.

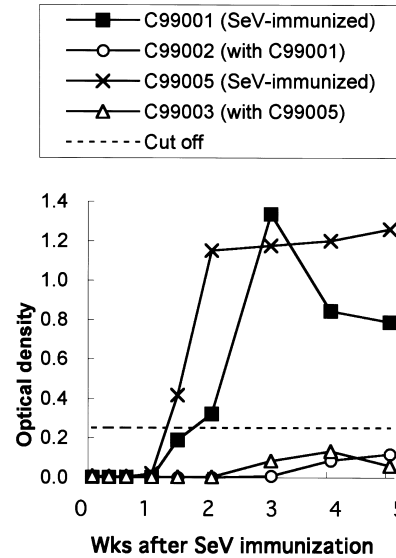


Fig. 5. Plasma anti-SeV antibody levels detected by ELISA in naive and SeV-immunized macaques being maintained together. C99002 was in a cage together with SeV-immunized C99001, and C99003 with SeV-immunized C99005. The absorbance obtained with plasma diluted by 1:1000 is shown.

Then, we examined SeV-specific T cell responses by flow cytometry analysis of antigen-specific intracellular IFN- γ induction. SeV-specific T cell responses were induced quickly in all animals after immunization (Fig. 3). Significant levels of SeV-specific T cells were detected in PBMCs at week 1. Especially, efficient induction of specific CD4 T cells was observed. At week 3, all animals showed higher frequencies of SeV-specific CD8⁺ T cells.

In all of them, plasma anti-SeV antibody was undetectable at week 1 but significant levels of the antibody appeared at week 2 after immunization (Fig. 4).

SeV transmission between macaques

The third *in vivo* experiment was performed to examine if SeV can be transmitted from SeV-immunized macaques to naive ones. In this experiment, we used recombinant SeV expressing HIV-1 gp120 (Yu *et al.*, 1997). Two pairs of cynomolgus macaques, C99001 and C99002, and C99005 and C99003, were used. In each pair, one macaque (C99001 and C99005, respectively) was immunized intranasally with 2×10^8 CIU SeV and 18 h later was put into a single cage together with its partner (C99002 and C99003, respectively). Each pair of monkeys was maintained together for 5 weeks. None of them showed apparent clinical symptoms after immunization. Due to the limitation of the cage size, small macaques that were 8 months old were chosen for this experiment.

We measured plasma anti-SeV antibody levels to examine the possibility of SeV transmission (Fig. 5). Similar to the second *in vivo* experiment, plasma anti-SeV antibodies were

clearly detectable at week 2 in both of the SeV-immunized macaques. In contrast, none of the naive macaques showed significant levels of anti-SeV antibodies. These results indicate inefficient SeV transmission from SeV-infected macaques to naive ones.

Discussion

We previously reported the potential of our SeV-Gag vaccine system for inducing cellular immune responses efficiently and its protective efficacy against SIV and SHIV infections in macaque AIDS models (Kano *et al.*, 2000; Matano *et al.*, 2001). In this study, we focused on the primary replication of the SeV vector in macaques.

In macaque experiments, SIV *gag* RNA was detected dominantly in the nasal mucosa and its local LN after intranasal SeV-Gag immunization. The detected RNA consists of the genomic RNA and the mRNA but we confirmed mRNA expression by quantitative RT-PCR using primers that discriminate SeV mRNA from SeV genomic RNA (Kato *et al.*, 2001) (data not shown). Efficient *gag* expression in the local LN as well as the nasal mucosa after immunization suggests its potential for induction of mucosal as well as systemic immune responses.

None of the monkeys in either the previous studies (Hurwitz *et al.*, 1997; Kano *et al.*, 2000) or the present study showed significant pathological symptoms after intranasal SeV immunization. SeV-Gag expression was localized in the nasal mucosa and its local LN and its expression levels reached the peak within a week after immunization. Such tissue restriction is compatible with its replication pattern in mice, which is localized in the airway epithelium. In contrast to the case in mice, we found neither pathological changes nor SeV-Gag expression in the lung in macaques, indicating that the spread of the virus was more restricted in macaques than that in mice.

In general, the proliferation of SeV-control is not slower than that of recombinant SeVs carrying inserted genes *in vitro*. However, no clinical symptoms were observed even in the SeV-control-immunized macaques. Furthermore, we found no significant differences in the efficiency of SeV recovery from the nasal swabs between SeV-control- and SeV-Gag-immunized macaques (data not shown), indicating that even SeV-control can be well controlled in macaques. Therefore, the SeV vector system could be used safely for the expression of various kinds of antigens in primates.

An intranasal SeV-Gag immunization into macaques induced anti-SeV antibodies and SeV-specific T cells quickly. Especially, all immunized macaques showed SeV-specific T cells at week 1, suggesting the potential of the specific T cells for controlling SeV replication. In spite of these responses specific to the virus vector itself, significant levels of Gag-specific T cell induction were also detected (Matano *et al.*, 2001). We found no relationship between SeV-specific and Gag-specific immune responses.

One of the characteristics of the SeV vector system is that the inserted gene is expressed more promptly and efficiently than any other SeV-specific genes derived from the vector because its accommodation is close to the 3' terminus of the SeV genome (Nagai, 1999). In addition, the inserted gene fragment has been shown to be stable in the vector both *in vitro* (Yu *et al.*, 1997) and *in vivo* (in mice) (Akaïke *et al.*, 2000). We confirmed expression of Gag as well as SeV in the cells infected with the recovered virus from the swabs in the SeV-Gag-immunized macaques used in our previous experiment (Kano *et al.*, 2000), indicating that the recovered vector still expressed the antigen, Gag. These characteristics of the SeV vector system may contribute to the efficient induction of immune responses specific to the target antigen in the presence of strong immune responses specific to the virus vector itself.

In the third *in vivo* experiment, SeV transmission from acutely SeV-infected macaques to naive ones was not detected but a little increase in anti-SeV antibody levels below the cut-off line was observed. Thus, the possibility of its transmission was not excluded completely. However, our results indicate that SeV transmission between macaques was inefficient.

In summary, we first analysed primary SeV replication and immune responses in macaques after intranasal immunization with a recombinant SeV. SeV replication was localized around the nasal mucosa and was well controlled. Furthermore, SeV transmission from SeV-immunized macaques to others was inefficient. These results may support the idea that our system can be safely used in primates, including humans. Also, a safer, replication-defective SeV vector lacking F gene is available now (Li *et al.*, 2000). In combination with our previous results, the present study indicates that the SeV vector system can induce effective cellular immune responses against pathogenic virus infections, while it is well controlled by strong immune responses against the vector itself in primates. Thus, this system may be a promising, safe and effective tool for vaccines against infections with various kinds of micro-organisms.

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