2.10. Statistical analyses

Analysis of variance (ANOVA) was first performed and then two groups were compared by Student *t*-test using Microsoft® Excel 2002. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SNV infection in EA.hy926 cells

It has been reported that HUVECs were permissive to hantavirus infection (Pensiero et al., 1992). We first tested whether immortalized human endothelial cell line, EA.hy926, would be infected with SNV. Confluent EA.hy926 cells and Vero E6 cells were inoculated with SNV at m.o.i. of 0.0025, and cell lysates and supernatants were harvested at 0, 1, 4, 8 and 12 days post-infection. To detect viral protein expression, Western blotting was performed using cell lysates (Fig. 1A). In both EA.hy926 and Vero E6 cell lysates, a weak band around 50 kDa, corresponding to the SNV N protein, was detected by rabbit anti-SNV serum (Zaki et al., 1995) at one day post-infection. The amount of the protein gradually increased until 12 days post-infection. The amount of the N protein was greater in Vero E6 cells than in EA.hy926 cells especially at later time points. Several smaller bands were also seen in the cell lysates at later time points. Similar bands were also observed when Puumala virus N protein and Seoul virus N protein were expressed in CV-1 cells and COS cells, and were suggested to be truncated forms of the N protein (Hooper et al., 1999; Terajima et al., 2002). Anti SNV N protein monoclonal antibody, HNM-6011CZ1-5 (Austral Biologicals, San Ramon, CA), also produced the same banding pattern (data not shown).

Virus titers in culture media were also measured (Fig. 1B). In culture supernatants of Vero E6 cells, the infectious virus was detected 4 days post-infection and the viral titer peaked (4.2×10^4 ffu/ml) at day 8 post-infection. In culture supernatants of EA.hy926 cells, infectious virus was first detected at 8 days post-infection, and the viral titer was about 3 logs lower than in Vero E6 cells.

These results indicate that SNV was able to infect and replicate in EA.hy926 cells, although viral protein expression and progeny virus production in EA.hy926 cells were less than in Vero E6 cells.

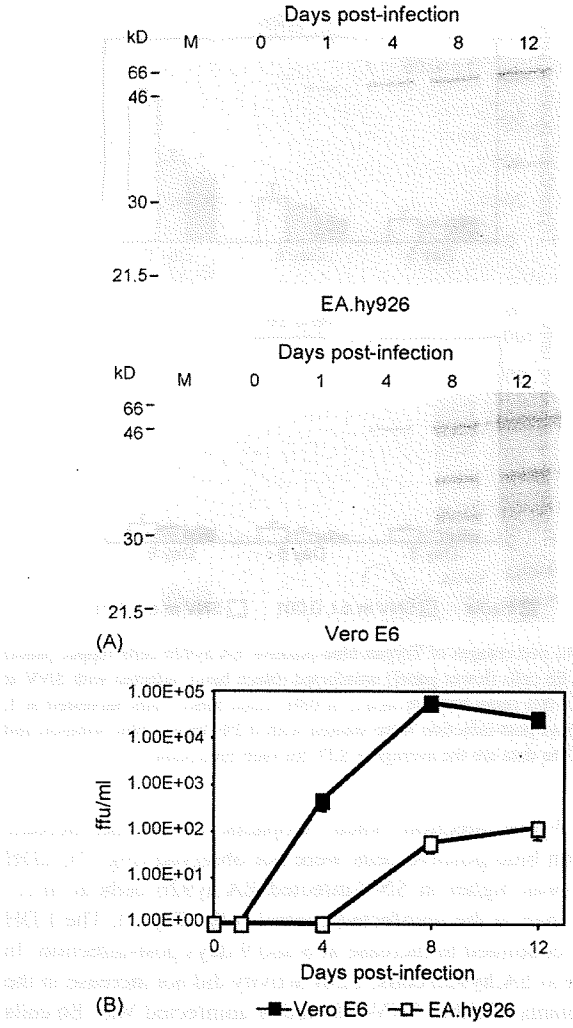

Fig. 1. SNV infection in EA.hy926 and Vero E6 cells. (A) Detection of SNV protein in EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) infected with SNV by western blotting. Cell lysates were harvested at 0, 1, 4, 8 and 12 days post-infection and reacted with rabbit anti-SNV serum. M: molecular weight markers (Rainbow™ coloured protein molecular weight markers, Amersham Pharmacia Biotech, Piscataway, NJ). (B) Infectious virus titers of SNV detected in the supernatants of EA.hy926 cells (open square) and Vero E6 cells (closed square) are shown. The titers of culture supernatants harvested at 0, 1, 4, 8 and 12 days post-infection were determined by focus-forming assay. The data are average ± S.D. for each time point.

3.2. Viabilities of EA.hy926 cells infected with SNV

Several groups reported previously that hantavirus infection did not induce visible cytopathic effects on HUVECs (Geimonen et al., 2002; Niikura et al., 2004; Pensiero et al., 1992). To examine the viabilities of EA.hy926 cells after infection with SNV, we performed a Trypan blue exclusion test. At 3 days post-infection, there was no significant difference in the percentages of SNV-infected and uninfected EA.hy926 cells stained by Trypan blue (Fig. 2). Also, no apparent cytopathic effects were observed by microscopy of SNV-infected EA.hy926 cells (data not shown). At 6 days post-infection, the percentage of Trypan blue-positive cells in EA.hy926 cells infected with SNV at m.o.i. of 0.01 was about two fold greater than uninfected cells, although the difference was not statically significant (Fig. 2). At 9 days post-infection, Trypan blue-positive cells increased to about 70%, about twice as in uninfected cells. In contrast to EA.hy926 cells, no increase in the percentage of the Trypan blue-positive cells or morphological changes was observed in Vero E6 cells infected with SNV (Fig. 2).

3.3. Cytoplasmic enzyme LDH release by SNV infection

We also assessed the viability of SNV-infected EA.hy926 cells by measuring LDH activities in supernatant released from the cytosol by increased permeability of the plasma membrane.

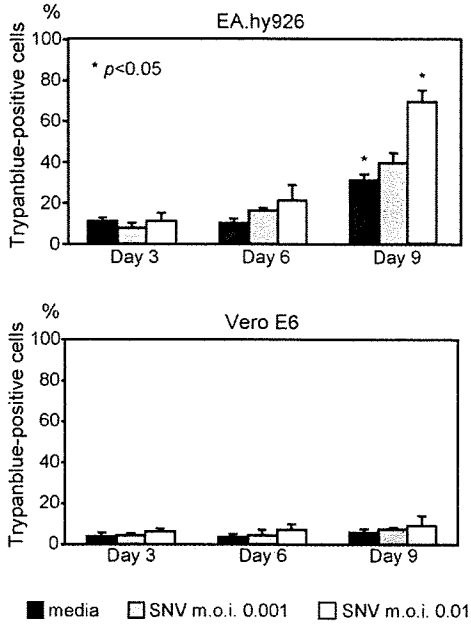

Fig. 2. The percentages of Trypan blue-positive EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) uninfected (black bars), infected with SNV at m.o.i. of 0.001 (gray bars) or m.o.i. of 0.01 (open bars). Cells harvested at 3, 6 and 9 days post-infection were stained with 0.2% Trypan blue solution and counted. The data are the average ± S.D. for each time point.

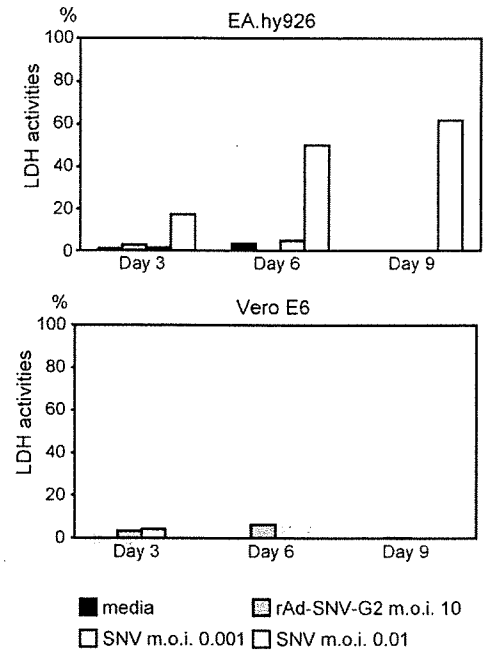

Fig. 3. LDH activity in the supernatants of EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) uninfected (black bars) or infected with rAd-SNV-G2 at m.o.i. of 10 (dark gray bars), SNV at m.o.i. of 0.001 (light gray bars) or m.o.i. of 0.01 (open bars). The LDH activities were shown as the percent values of the high control.

At day 3 post-infection, when cytopathic effects and increase in Trypan blue-positive cells were not observed (Fig. 2), LDH activity was higher in SNV-infected EA.hy926 cells at m.o.i. of 0.01 than in the uninfected control cells (Fig. 3). The LDH activity continued to increase at 6 and 9 days post-infection. In contrast to EA.hy926 cells, LDH activity did not increase in the supernatants of either SNV-infected or uninfected Vero E6 cells (Fig. 3). Infection with the recombinant replication-deficient adenovirus expressing SNV G2, rAd-SNV-G2, did not increase the LDH activity in the supernatant of infected EA.hy926 cells or Vero E6 cells even when an m.o.i. of 10 was used. These results indicate that the permeability of plasma cell membrane increased by the SNV infection in EA.hy926 cells before cytopathic effects became apparent.

3.4. CTL activity of the hantavirus-specific T cell line 1A-E2 against EA.hy926 cells

We tested whether hantavirus-specific CTLs could recognize and lyse EA.hy926 cells infected with SNV. We determined the HLA-type of EA.hy926 cell line by RT-PCR and sequencing of the PCR products. These results showed that the EA.hy926 cell line expressed HLA-A*2402, A*2501, B*1501, B*1801, Cw*0303, and Cw*1203. Then, we used a synthetic peptide containing the SNV G2 epitope restricted by HLA-A24, HAEIQNLGHWMDGTFNIKTA (minimal epitope is underlined). EA.hy926 cells were pulsed with the peptide and incubated with the hantavirus-specific CTL line 1A-E2 at various *E/T* ratios for 16 h (Fig. 4). It is not possible to estimate the actual effecter (SNV-specific CTL)/target (SNV-infected endothelial cells) ratio in vivo, but, it may not be very high. We, therefore, used relatively low *E/T* ratio (0.01, 0.1 and 1) in our experiments. CTL activity was peptide concentration and *E/T* ratio-dependent. Next, confluent EA.hy926 cells were infected with rAd-SNV-G2 at an m.o.i. of 10 and after 3 days CTL assays were performed. Similar to the results of the peptide-pulsed cells, 1A-E2 lysed rAd-SNV-G2-infected EA.hy926 cells (Fig. 4). Finally, we infected EA.hy926 cells with SNV at m.o.i. of 0.0001, 0.001 and 0.01 for 3 days and then CTL assays were performed. At 3 days post-infection apparent cytopathic effects were not observed (Section 3.2), although LDH release was detected in culture media (Section 3.3). To eliminate the accumulated LDH in the culture media the supernatants were removed before adding the effector cells (1A-E2). 1A-E2 lysed SNV-infected EA.hy926 cells at m.o.i. of 0.01 and *E/T* ratio of 1 (Fig. 4). These results indicate that hantavirus-specific human CTL line, 1A-E2, recognized and lysed the human endothelial cell line infected with SNV in vitro.

3.5. Increased permeability of EA.hy926 cell monolayer caused by SNV infection and the virus-specific CTLs

To examine whether SNV infection alone or the combination of SNV infection and the virus-specific CTLs could increase permeability of EA.hy926 cell monolayer, we performed transwell permeability assays (Fig. 5). SNV infection alone caused an increase of permeability at 4 days after infection, compared to media control. The difference, however, was not always statistically significant in every experiment due to the variance

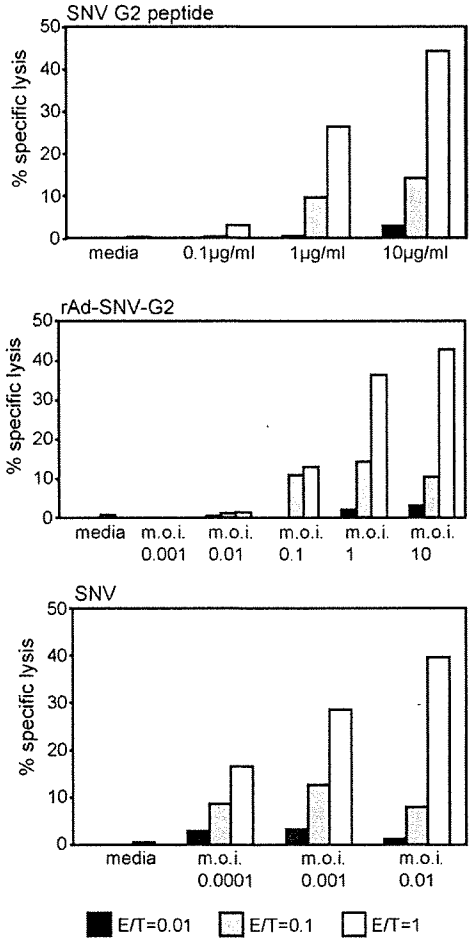

Fig. 4. CTL activity by the 1A-E2 CTL line against EA.hy926 cells pulsed with the SNV G2 peptides, HAEIQNLGHWMDGTFNIKTA (upper panel), infected with rAd-SNV-G2 (middle panel), or SNV (bottom panel). Effector 1A-E2 cells were added to target EA.hy926 cells at *E/T* ratio of 0.01 (black bars), 0.1 (gray bars) or 1 (open bars).

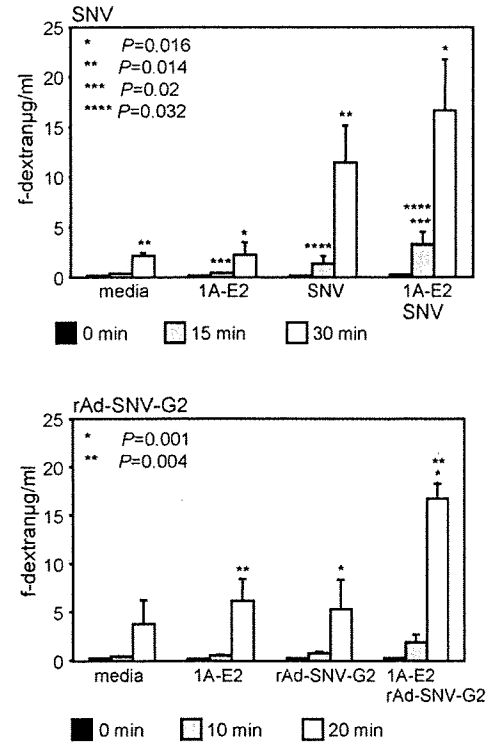

Fig. 5. Permeability changes in EA.hy926 cells infected with SNV (upper panel) or rAd-SNV-G2 (lower panel), with or without the addition of the 1A-E2 CTL line. 500 μg/ml of f-dextran was added to the upper chamber and medium harvested from lower wells. Concentrations of the f-dextran at 0 min (black bars), 15 min (gray bars) and 30 min (open bars) after addition are shown. The data are the average ± S.D. for each time point. Data shown in the top panel are the representative of four independent experiments.

of the assay. The CTL line, 1A-E2, which was added 3 days post-infection, further increased permeability of SNV-infected EA.hy926 cell monolayer, but not the permeability of uninfected cell monolayer (Fig. 5). To test whether the CTL alone could increase permeability, we performed the same assay using EA.hy926 cell monolayer infected with rAd-SNV-G2, which did not increase the LDH activity in the supernatant of infected EA.hy926 cells (Fig. 3). In contrast to SNV, rAd-SNV-G2 infection alone did not increase permeability (Fig. 5). Addition of 1A-E2 cells resulted in a significant increase of permeability of the rAd-SNV-G2-infected EA.hy926 cell monolayer (Fig. 5). These results suggest that the virus-specific CTLs recognizing infected EA.hy926 cell monolayer alone were able to increase permeability.

4. Discussion

We demonstrated that hantavirus-specific CTLs increased the permeability of SNV-infected EA.hy926 cell monolayer after recognition of the antigen presented on cell surface. By using the recombinant replication-deficient adenovirus, which did not increase permeability of infected EA.hy926 cells, we show that CTLs alone were able to increase permeability after recognition of the antigen presented on infected cell.

We did not perform experiments analyzing the mechanisms of permeability change caused by CTLs. Since EA.hy926 cells pulsed with peptides containing the SNV G2 epitope, infected with the recombinant adenovirus expressing the SNV G2 protein, or infected with SNV were lysed efficiently by the CTLs (Fig. 4.), it is likely that the permeability change was at least in part caused by the lysis of the EA.hy926 cell monolayer. We were not able to determine what percent of cells in EA.hy926 cell monolayer was infected because of the detection limit of our immunohistochemical staining against SNV antigen in EA.hy926 cell monolayer. Cytokines released by CTLs, such as TNF-α, were also likely to be involved in the increase of permeability.

In previous reports by other groups, in vitro hantavirus infection alone did not increase permeability of infected human endothelial cell monolayers (Khaiboullina et al., 2000; Niikura et al., 2004; Sundstrom et al., 2001), although Khaiboullina et al. (2000) observed a tendency for increased permeability in SNV-infected HUVECs, compared to the uninfected control. Our data of the direct effect of SNV infection on EA.hy926 cell monolayer permeability is not conclusive. As for the

mechanisms of the permeability change induced by the virus infection alone, there are reports showing that the replication of many hantaviruses could induce characteristic features of apoptosis and/or cytopathic effects in some human and primate cell lines (Kang et al., 1999; Li et al., 2002, 2004; Markotic et al., 2003). We, however, observed very few detached cells after incubation of EA.hy926 cell monolayer with SNV. Another likely mechanism is the alteration of tight junction or membrane association, which was observed with rotavirus (Obert et al., 2000).

Khaiboullina et al. (2000) showed that SNV-infected human alveolar macrophages produced TNF-α. The culture supernatant from SNV-infected human alveolar macrophages, however, did not increase the permeability of the HUVEC monolayer (Khaiboullina et al., 2000). Niikura et al. (2004) observed prolonged hyper-permeability induced by TNF-α in Hantaan virus-infected HUVECs and suggested the involvement of infected monocytic cells producing the low level of TNF-α in the development of the capillary leakage in vivo. Our results showed that hantavirus-specific CTL were able to further increase permeability after recognition of the antigen presented on cell surface. Gavrilovskaya et al. (2002) found that pathogenic hantaviruses inhibited β_3 integrin-directed endothelial cell migration. In vivo these three mechanisms: [1] the attack of infected endothelial cells by virus-specific CTLs, [2] TNF-α production by infected monocyte/macrophages, (Khaiboullina et al., 2000; Niikura et al., 2004) and [3] the direct effect of viral infection on endothelial cell functions (Gavrilovskaya et al., 2002; Niikura et al., 2004) may contribute to the severe capillary leakage. Autopsies performed on patients with fatal HPS demonstrated that infected lung endothelial cells were not necrotic and the lung architecture appeared to be grossly intact (Nolte et al., 1995; Zaki et al., 1995). These observations are obviously not consistent with the possibility of the endothelial cell lysis by the virus-specific CTLs. In vivo CTLs may induce capillary leakage mainly by the release of cytokines, such as TNF-α, and/or only a small percentage of the infected endothelial cells are lysed by CTLs, which may not be detectable in autopsy tissues. To examine whether CTLs can cause capillary leakage in vivo, it is important to attempt to establish an appropriate animal model. Laboratory mice and the natural rodent reservoirs do not develop any symptoms similar to the human diseases, HPS and HFRS. Syrian hamsters do develop a disease similar to human HPS when infected with Andes virus, but not SNV (Hooper et al., 2001; Milazzo et al., 2002). It would be interesting to know whether excess expansion of virus-specific CTLs, which is observed in human disease (Kilpatrick et al., 2004), develops in infected Syrian hamsters. There are, however, few reagents available to study the immune responses of hamsters to the virus (Enserink, 2001).

In conclusion, our results suggest that $CD8^+$ T cells contribute to capillary leakage observed in the patients with HPS or HFRS in addition to the direct effects of viral infection on endothelial cell functions. $CD8^+$ T cells-mediated capillary leakage might also be involved in the pathogenesis of other viral hemorrhagic fevers, where immunopathological mechanisms are speculated (e.g. dengue virus and arenavirus infections) (Peters and Zaki, 2002).

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Letter to the Editor

Immunopathogenesis of hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome: Do CD8$^+$ T cells trigger capillary leakage in viral hemorrhagic fevers?

Abstract

There are many viruses known to cause viral hemorrhagic fevers in humans. The mechanisms causing hemorrhage are likely to vary among viruses. Some viruses, such as Marburg virus, are directly cytopathic to infected endothelial cells, suggesting infection of endothelial cells alone can cause hemorrhage. On the other hand, there are viruses which infect endothelial cells without causing any cytopathic effects, suggesting the involvement of host immune responses in developing hemorrhage. Typical examples of these include viruses of the hantavirus species. We hypothesize that impairment of endothelial cell's defense mechanisms against cytotoxic CD8$^+$ T cells is the mechanism of capillary leakage in hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome, which may be common to other viral hemorrhagic fevers. CD8$^+$ T cells may be a potential target for therapy of some viral hemorrhagic fevers.
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Keywords: Hantavirus; Endothelial cells; Cytotoxic T lymphocytes; PD-1; PD-L1; PD-L2; Hantavirus pulmonary syndrome; Hemorrhagic fever with renal syndrome

There are many viruses known to cause viral hemorrhagic fevers in humans [1]. The mechanisms causing hemorrhage are likely to vary among viruses [2]. Some viruses, such as Marburg virus, are directly cytopathic to infected endothelial cells [3], suggesting infection of endothelial cells alone can cause hemorrhage. On the other hand, there are viruses which infect endothelial cells without causing any cytopathic effects, suggesting the involvement of host immune responses in developing hemorrhage. Typical examples of these include hantaviruses [4–7], which belong to genus *Hantavirus*, family *Bunyaviridae*.

Hantaviruses are RNA viruses possessing a segmented negative-stranded RNA genome [8,9]. Hantaviruses are conventionally divided into the Old World and the New World hantaviruses based on the geographic regions where they occur, although phylogenetic tree based on the genomic RNA sequences forms three main groups, Hantaan virus-like viruses, Puumala virus-like viruses and Sin Nombre virus (SNV)-like viruses [10]. The Old World hantaviruses, including Hantaan, Seoul, Dobrava and Puumala viruses which are seen throughout Europe and Asia, cause a human disease known as hemorrhagic fever with renal syndrome (HFRS) with more than 100,000 cases diagnosed annually. Clinically, HFRS is initially characterized by non-specific flu-like symptoms followed by thrombocytopenia, and a capillary leak syndrome with hemoconcentration. In severe cases renal failure and shock can develop. Mortality rates vary from approximately 1 to 15%, depending on the individual virus [11,12]. The New World hantaviruses include SNV and Andes virus, and are seen in North, Central, and South America [11,12]. While these hantaviruses have likely existed in the Americas for many years, they were recognized as a cause of disease when the first outbreak of hantavirus pulmonary syndrome (HPS) occurred in the southwestern United States in 1993 [13,14]. HPS shares many characteristics with HFRS, including thrombocytopenia and a capillary leak syndrome. However, there are some differences. The pathology seen with Old World hantaviruses focuses on the kidneys, but the major target organ for the New World hantaviruses is the lung. HPS cases progress to a severe degree more frequently than HFRS cases. After a prodromal phase similar to that of HFRS, patients very rapidly develop pulmonary edema, and shock, which often requires mechanical ventilation and/or extracorporeal membrane oxygenation [15], and the case fatality rate is approximately 35% (http://www.cdc.gov/ncidod/diseases/hanta/hps/noframes/caseinfo.htm). Thus, the New World hantaviruses cause some of the most lethal acute viral infections known, and antiviral therapy or vaccines are not yet available. In autopsied cases, most SNV antigens are found in endothelial cells, especially alveolar endothelial cells; but some other cells are also positive for viral antigen, such as monocyte/macrophages [16].

There have been three hypotheses to explain the mechanisms of increased capillary permeability. (1) The attack of infected endothelial cells by virus-specific cytotoxic T lymphocytes (CTLs); (2) tumor necrosis factor (TNF)-α production by infected monocyte/macrophages; and (3) the direct effect of viral infection on endothelial cell functions.

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Both in vivo and in vitro observations suggest that SNV is in general not directly cytopathic to infected cells including endothelial cells [6,7,16,17], although there are papers reporting apoptosis in some human and primate cell lines, such as human embryonic kidney 293 cells and African green monkey kidney Vero E6 cells, infected with hantaviruses [18–21]. β_3-Integrin is a cellular receptor for human-pathogenic hantaviruses [22,23]. Since the ligation of endothelial $\alpha_V\beta_3$-integrin increased transcapillary liquid flux [24], it was speculated that the virus–β_3-integrin interaction might be the mechanism of increased capillary permeability [22,23]. Infection of endothelial cells alone, however, failed to increase their permeability [6,7,25]. Hantavirus infection did inhibit β_3-integrin-directed migration of endothelial cells, which might contribute to hantavirus pathogenesis [26].

Mechanisms involving CTLs have been suggested by us [27–29] and others [30–32]. Lung tissues obtained at necropsy from HPS patients contain abundant large immunoblasts consisting of $CD4^+$ and $CD8^+$ T cells [16] [17], and high numbers of cytokine-producing cells including TNF-α, interleukine-2, and interferon (IFN)-γ [33], which could mediate capillary leakage. In addition, preliminary evidence suggests that, in SNV infection, the HLA-B*3501 allele is associated with increased risk for developing severe HPS, implying involvement of $CD8^+$ T cells [34]. We demonstrated very high frequencies of SNV-specific $CD8^+$ T cells detected by MHC class I/peptide tetramer staining in HPS patients' blood during acute illness, and the magnitude of virus-specific T cell responses was significantly higher in the patients with clinically severe HPS (patients who met clinical criteria requiring mechanical ventilation) than in patients with moderate disease (hospitalized but not requiring mechanical ventilation) [29]. We also showed that specific CTL recognized and increased the permeability of an immortalized HLA-matched human endothelial cell monolayer infected with SNV in transwell permeability assays [35]. These data suggest that SNV-specific $CD8^+$ T cells contribute to the observed capillary leakage during HPS. Since infected lungs at autopsy had no obvious damage in endothelial cells [16,17], capillary leakage is more likely to be caused by cytokine release than by endothelial cell lysis. Lysis of a small percentage of endothelial cells, which is difficult to detect in tissue sections, may be enough to cause capillary leakage, although bleeding is very rare in HPS.

A linkage between disease severity and MHC haplotype was also observed in patients with nephropathia epidemica (NE), a milder form of HFRS caused by Puumala virus infection. HLA-B8-DR3 extended haplotype was associated with severe outcome of the disease [36,37], and HLA-B27 was associated with milder disease [38], implying involvement of $CD8^+$ T cells in NE pathogenesis. In NE patients the kidney biopsies showed interstitial infiltration of lymphocytes, plasma cells, monocytes/macrophages, and polymorphonuclear leukocytes. An increased expression of cytokines including TNF-α and endothelial adhesion molecules was observed [39]. Plasma TNF-α levels were also elevated [40], and urinary excretion of interleukin-6 correlates with proteinuria [41].

In laboratory mice infected with Hantaan virus, virus-specific $CD8^+$ T cells, not neutralizing antibodies, were important for clearance of the virus [42–44]. These laboratory mice, as well as natural rodent reservoirs of hantaviruses, do not develop any disease similar to HPS or HFRS, suggesting in mice these virus-specific T cell are protective, not immunopathogenic. It is not understood why these rodents do not develop the disease. We should remember that there are many differences in the immune systems of humans and mice [45].

In transgenic mouse model of influenza infection, in which lung alveolar epithelial cells expressed influenza A virus hemagglutinin (HA), adoptive transfer of HA-specific $CD8^+$ T cells into the HA-transgenic mice induced lung injury, which was mediated by TNF-α produced by the HA-specific $CD8^+$ T cells and chemokines produced by alveolar epithelial cells attacked by the HA-specific $CD8^+$ T cells [46,47]. A similar mechanism may occur as a result of endothelial cell–$CD8^+$ T cell interactions.

A series of experiments performed by transplantation immunologists, however, showed that, contrary to lung alveolar epithelial cells, endothelial cells were protected from humoral and cellular immune responses in laboratory mice [48,49]. Johnson et al. analyzed humoral and cellular immune responses against β-galactosidase (BG) protein expressed in the endothelial cells of transgenic mice. In theory immune responses against BG protein would not be induced in BG-transgenic mice in which BG should be tolerated. Infection with recombinant vaccinia virus encoding BG, however, induced humoral and cellular immune responses in the BG-transgenic mice at the same level as responses in wild type FVB mice (from which the BG-transgenic were generated), and, more surprisingly, these infected mice remained healthy. No damage was observed in endothelial cells. The BG-transgenic mice also remained healthy after primed spleen cells or lymph node cells from immunized, wild type FVB mice, which contained $CD8^+$ (and $CD4^+$) T cells reacting to BG protein, were adoptively transferred into them. These results are surprising, but, nevertheless consistent with the down-regulation of $CD8^+$ T cell activation and cytolysis by PD-L (PD-1 ligand) 1 and PD-L2 molecules expressed on IFN-γ-activated endothelial cells both in humans (in vitro study) and mice [50–52] ($CD8^+$ T cells express programmed death-1 (PD-1) molecule).

To reconcile these two findings, the immunopathological mechanisms we would like to propose are:

(1) In HPS and HFRS patients the mechanisms to down-regulate $CD8^+$ T cell activation and cytolysis for protection of endothelial cells may be overwhelmed by the excess amount of activated $CD8^+$ T cells, which appears to occur in HPS [29].
(2) The protecting mechanisms may not be functioning properly because of the infection of endothelial cells. Glycoproteins of human-pathogenic hantaviruses have been found to have immunomodulatory functions [53,54], although the effects of hantavirus infection on PD-1, PD-L1 or PD-L2 are not known. It is also not known whether T cells are infected with hantavirus in vivo.

These two mechanisms can work synergistically, and infected monocyte/macrophages also can contribute to the

immunopathogenesis by TNF-α production [6,25]. Involvement of virus-specific $CD4^+$ T cells is also likely, although there are no data suggesting the direct role of $CD4^+$ T cells in HPS or HFRS. Recently Salazar et al. reported that HLA-B35 molecule, a risk factor for severe HPS, increased susceptibility of cells to apoptosis [55].

In conclusion, we hypothesize that impairment of endothelial cell's defense mechanisms against cytotoxic $CD8^+$ T cells is the mechanism of capillary leakage in HPS and HFRS, which may be common to other viral hemorrhagic fevers. Immunopathological mechanisms are speculated to underlie diseases caused by dengue virus and arenavirus infections [2,56]. $CD8^+$ T cells may be a potential target for therapy of some viral hemorrhagic fevers.

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Construction and application of chimeric virus-like particles of tick-borne encephalitis virus and mosquito-borne Japanese encephalitis virus

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We have previously reported a system for packaging tick-borne encephalitis (TBE) virus subgenomic replicon RNAs into single-round infectious virus-like particles (VLPs) by using *in trans* expression of viral C/prM/E structural proteins. In this study, the *trans*-packaging system was applied to the generation of chimeric VLPs with mosquito-borne Japanese encephalitis (JE) virus. Although *trans*-expression of TBE virus C and JE virus prM/E proteins resulted in the secretion of VLPs, the expression of JE virus C/prM/E proteins did not lead to the secretion of VLPs, suggesting that homologous interaction between C and non-structural proteins or the genomic RNA is important for efficient assembly of infectious particles. Neutralization testing showed that the antigenic characteristics of the VLPs were similar to those of the native virus. Furthermore, the infectivities of the TBE virus- and JE virus-enveloped VLPs for the ISE6 tick cell line and C6/36 mosquito cell line were investigated. The VLPs were able to enter only those cells that were derived from the natural vectors for the respective viruses. TBE virus replicon RNA packaged in VLPs produced TBE virus non-structural proteins in tick cells, but could neither replicate nor produce viral proteins in mosquito cells. These findings indicate the importance of specific cellular factors for virus entry and replication during flavivirus infection of arthropods. These results demonstrate that chimeric VLPs are useful tools for the study of viral genome packaging and cellular factors involved in vector specificity, with the additional safety aspect that these chimeric VLPs can be used instead of full-length chimeric viruses.

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INTRODUCTION

The genus *Flavivirus* (family *Flaviviridae*) contains important human pathogens, including tick-borne encephalitis (TBE) virus, Japanese encephalitis (JE) virus, yellow fever virus, dengue virus and West Nile (WN) virus. Flaviviruses can be divided into three phylogenetic and ecological groups: the tick-borne group, the mosquito-borne group and the no-known-vector group (Gaunt *et al.*, 2001; Gould *et al.*, 2003; Kuno *et al.*, 1998). The extent of transmission of arthropod-borne viruses depends on both ecological and physiological parameters, of which vector competence is the most important factor. Vector competence is determined by extrinsic and intrinsic factors, such as the physiological ability of vector tissue to become infected and to maintain a particular infectious agent (Hardy *et al.*, 1983; Kramer & Ebel, 2003; Nuttall & Labuda, 2003). The involvement of these factors in flavivirus infection is not well understood.

A supplementary table showing primers used in this study is available with the online version of this paper.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e. the core (C), premembrane (prM) and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), within a single long open reading frame (Chambers *et al.*, 1990). The 5′- and 3′-untranslated regions (UTRs) have predicted secondary structures that are implicated in viral replication, translation and packaging of the genomes (Gritsun *et al.*, 1997; Proutski *et al.*, 1997; Rauscher *et al.*, 1997). In the process of assembly of virus particles, viral structural proteins are inserted co-translationally into the endoplasmic reticulum (ER) and processed by the NS2B–NS3 protease complex and signal peptidase, and the C proteins and genomic RNA, which form the icosahedral nucleocapsid, are encapsidated by budding into the luminal side of the ER (Mackenzie & Westaway, 2001). However, little is known about the details of the molecular mechanism of packaging.

The development of stable, infectious cDNA clones of flaviviruses has enabled the construction of chimeras of

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different flaviviruses (Caufour *et al.*, 2001; Guirakhoo *et al.*, 2001; Mathenge *et al.*, 2004; Pletnev & Men, 1998; Pletnev *et al.*, 1992, 2002). These chimeric viruses are useful tools for the study of viral replication cycles and for vaccine development. Moreover, they can be applied to the study of host factors that are involved in vector competence for flavivirus transmission in cases where chimeric viruses are generated from flaviviruses borne by different arthropods.

For several flaviviruses, such as TBE virus (Gehrke *et al.*, 2003; Hayasaka *et al.*, 2004), Kunjin (KUN) virus (Khromykh & Westaway, 1997), WN virus (Scholle *et al.*, 2004; Shi *et al.*, 2002), dengue virus (Pang *et al.*, 2001) and yellow fever virus (Molenkamp *et al.*, 2003), subgenomic replicons have recently been constructed by deleting genes for viral structural proteins. These replicons can replicate in cultured cells by virtue of functioning NS proteins, but they cannot produce progeny infectious viruses owing to the lack of viral structural proteins. In recent studies, the expression of viral structural proteins in cells harbouring replicon RNA has resulted in the secretion of particles, which have been designated virus-like particles (VLPs). VLPs are infectious and the replicon RNAs packaged in VLPs replicate in infected cells. However, as viral structural proteins are not encoded by the replicon, progeny viruses cannot be produced. Thus, this single-round infectivity feature of VLPs enables safe handling under biosafety level 2 (BSL-2) conditions. VLP systems were developed for TBE virus (Gehrke *et al.*, 2003; Yoshii *et al.*, 2005), KUN virus (Harvey *et al.*, 2004; Khromykh *et al.*, 1998) and WN virus (Scholle *et al.*, 2004). The VLPs of flaviviruses are similar to the native virus in terms of their physical features and functional characteristics for infection. Therefore, VLPs can be substituted for native virions in investigations into the biological properties of flaviviruses.

Previously, we constructed a subgenomic replicon of Far-Eastern subtype TBE virus and developed the *trans*-packaging system for VLPs further (Hayasaka *et al.*, 2004; Yoshii *et al.*, 2005). In this study, the *trans*-packaging system was applied to the generation of chimeric VLPs between TBE virus and JE virus (tick- and mosquito-borne flaviviruses, respectively), to investigate the molecular mechanism of flavivirus packaging and the specificity of the competence of the natural host vectors for flavivirus infection.

METHODS

Cells and viruses. The baby hamster kidney (BHK)-21 cell line was grown at 37 °C in Eagle's minimal essential medium (MEM) that was supplemented with 8 % fetal calf serum (FCS) and L-glutamine. C6/36 cells, which are derived from the mosquito *Aedes albopictus*, were grown at 28 °C in MEM with 10 % FCS (Igarashi, 1978). The ISE6 cell line from the tick *Ixodes scapularis* (kindly donated by Dr U. G. Munderloh, University of Minnesota, MN, USA) was grown at 34 °C in L-15B medium with 10 % FCS, as described by Munderloh *et al.* (1994).

TBE virus strain Oshima 5-10 and JE virus strain Nakayama were used in this study (Mason *et al.*, 1991; Takashima *et al.*, 1997).

Antibodies. Rabbit polyclonal anti-prM, anti-E and anti-NS3 antibodies were generated by immunization with the recombinant prM, E and NS3 proteins, as described in our previous study (Yoshii *et al.*, 2004). For the neutralization tests, we used the mouse anti-E monoclonal antibodies (mAbs) 1H4 and 4H8, which were prepared in our laboratory (Komoro *et al.*, 2000). Mouse mAbs 10B4 (anti-JE virus E) and 13E7 (anti-JE virus prM), which were provided by Dr E. Konishi (Department of Health Sciences, Kobe University School of Medicine, Kobe, Japan), were used in Western blotting. The anti-TBE virus, anti-Langat virus and anti-JE virus mouse polyclonal antibodies were prepared from the sera of mice infected with TBE virus strain Oshima 5-10, Langat virus strain TP-21 and JE virus strain Nakayama, respectively.

Plasmid construction. Derivation of the recombinant plasmid pcTBECME, which expresses all of the viral structural proteins derived from the Oshima 5-10 strain of TBE virus, has been described previously (Yoshii *et al.*, 2005). pcJEME, which is a pcDNA3-based plasmid that encodes the JE virus (Nakayama strain) genes for the signal sequence of prM and the prM and E proteins, was provided by Dr E. Konishi and Dr P. W. Mason (Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA) (Konishi *et al.*, 1998). For the construction of pcJECME, which encodes all of the viral structural proteins of the JE virus, viral RNA was extracted from a JE virus-inoculated suckling mouse brain, and RT-PCR was performed by using the primers *Bam*HIJE5′f (forward) and pCJEME1200r (reverse) (see Supplementary Table S1, available in JGV Online) as described previously (Takashima *et al.*, 1997). The PCR products were digested with *Bam*HI and *Bsi*WI and inserted into the pcJEME plasmid.

For the construction of pcTBEC-JEME, which encodes TBE virus C and the JE virus signal sequence of prM and the prM and E genes, these fragments were amplified by fusion PCR. First, the DNA fragment that encodes the region of the TBE virus gene from the 5′-UTR to the NS2B–NS3 cleavage site of protein C was amplified by using pcTBECME as template with the *Bam*HITBE5′f and CrGGN primers, and the JE virus signal sequence of prM and the prM and E genes were amplified by using pcJEME as the template with primers CfGGN and pCJEME1200r. The CfGGN and CrGGN primers contain a complementary sequence that encodes the 3′ end of the TBE virus C gene and the signal sequence of the JE virus prM. These two PCR fragments were subsequently used as templates in a second round of PCR with the *Bam*HITBE5′f and pCJEME1200r primers. The PCR products were digested with *Bam*HI and *Bsi*WI and inserted into the pcJEME plasmid.

For the construction of pcTBENS2B/3, which expresses the TBE virus NS2B–NS3 polyprotein, PCR was carried out using the *Xho*INS2Bf and *Eco*RINS3r primers. The PCR products were digested with *Xho*I and *Eco*RI and inserted into the pCAGGS plasmid (Niwa *et al.*, 1991).

The TBE replicon RNA transcripts were prepared from the Oshima REPpt plasmid, as described previously (Hayasaka *et al.*, 2004).

Preparation of VLPs. TBE replicon RNA was transcribed from the Oshima REPpt plasmid and electroporated into BHK-21 cells, as described previously (Hayasaka *et al.*, 2004). After 24 h culture, the cells were transfected with the plasmid that expressed the flavivirus structural proteins, which was complexed with the *Trans*IT-LT1 reagent (PanVera Corporation), as described previously (Yoshii *et al.*, 2004). At 36 h post-transfection, the supernatant was harvested and cleared by centrifugation at 1000 *g* for 10 min. The particles in the supernatant were precipitated with 10 % PEG (M_r=8000) and 1.9 % NaCl for 2 h at 4 °C, and pelleted at 10 000 *g* for 20 min. The pellets were resuspended in PBS supplemented with RNase A (20 μg ml^{-1}).

Infectivity assays. Titration of the VLPs was carried out as described previously (Yoshii *et al.*, 2005). Briefly, BHK-21 cells grown on

chamber slides were infected with serially diluted VLP solutions and incubated for 24 h at 37 °C. The cells were fixed with 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100. After blocking with 2 % BSA, the cells were incubated with anti-TBE virus NS3 rabbit IgG antibodies for 1 h and then treated with fluorescein isothiocyanate-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch). The images were viewed and recorded by using confocal microscopy.

In the particle neutralization test, 100 IU (infectious units) of VLPs were incubated with a serial dilution of the antibodies (described above) prior to infection of BHK-21 cells for 1.5 h. Particle-infected cells were visualized as described above.

In the case of experimental infections of ISE6 or C6/36 cells, cells grown on 16-well chamber slides were infected with VLPs at an m.o.i. of 1 and incubated for 48 h. Infected cells were visualized as described above.

SDS-PAGE and Western blotting. Transfected cells and supernatants were electrophoresed in SDS/polyacrylamide gels. The protein bands on the gels after SDS-PAGE were transferred onto PVDF membranes and incubated with 1 % gelatin in 25 mM TBS that contained 0.01 % Tween 20 (TBST). After washing with TBST, the membranes were reacted with the anti-E and/or anti-prM antibodies and then treated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (Promega).

Infectivity to arthropod cells. ISE6 or C6/36 cells were infected with infectious VLPs at an m.o.i. of 1. Following VLP absorption for 1 h, the supernatants were harvested and remaining VLPs in the supernatant were titrated. The cells were treated with 0.1 mg Pronase ml^{-1} (Roche) for 40 min to prevent non-specific binding of VLPs to the cell surface, as described previously (Mizutani *et al.*, 2003). The cell samples were harvested at 1, 2, 4 and 8 h post-infection. RNA was extracted from the cell and reverse transcription was carried out as described previously (Takashima *et al.*, 1997). PCR was carried out by using the following primers: for the region between the 5′-UTR and NS1 of TBE virus, TBE5′f and TBENS1r; for the *I. scapularis* β-actin gene, I-actin-F and I-actin-R; and for the *A. albopictus* β-actin gene, A-actin-F and A-actin-R (see Supplementary Table S1, available in JGV Online). The actin gene expression levels were used in control RT-PCR experiments to normalize the amount of cDNA used in each reaction.

RESULTS

Packaging of TBE virus replicon RNA into single-round infectious particles that contain the JE virus envelope

For expression of the flavivirus structural proteins used to package the TBE virus replicon RNA, the following four plasmid vectors, in which TBE virus and/or JE virus structural protein genes were cloned, were prepared (Fig. 1): pcTBECME, which encodes the TBE virus C/prM/E; pcJEME, which encodes the JE virus signal sequence of prM and the prM and E regions; pcJECME, which encodes the JE virus C/prM/E; and pcTBEC-JEME, which encodes the TBE virus C gene and the JE virus signal sequence of the prM–prM–E region.

For the RNA-packaging experiment, TBE virus replicon RNA was prepared from the Oshima REPpt plasmid, which

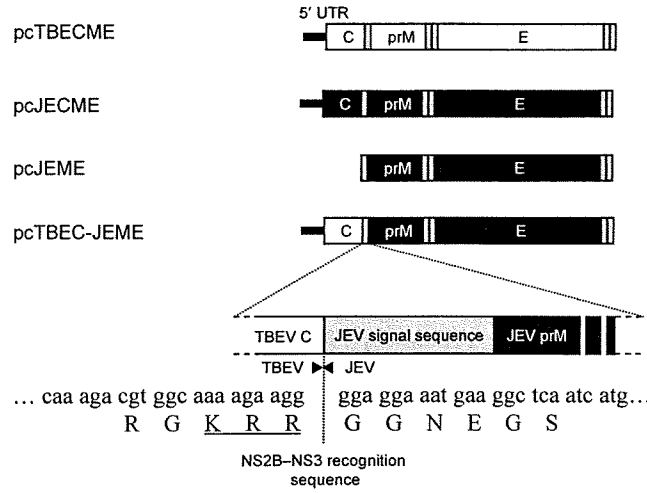

Fig. 1. Schematic representation of the plasmids used for expression of the flavivirus structural proteins. White regions are derived from TBE virus and black regions from JE virus. The signal sequence of prM of pcTBEC-JEME is derived from JE virus.

was constructed from the TBE virus infectious cDNA O-IC plasmid (Hayasaka *et al.*, 2004). In the BHK-21 cells that were infected with PEG-precipitated supernatant from pcTBEC-JEME- or pcTBECME- and replicon-transfected cells, the production of TBE virus NS3 proteins was detected by immunofluorescence assay (IFA) (Fig. 2a, d), which contrasted with the lack of viral protein production observed for cells that were infected with the supernatant of pcJEME- and replicon-transfected cells or that were transfected with replicon RNA alone (Fig. 2b, e). In the case of the supernatant from pcJECME- and replicon-transfected cells, IFA-positive cells were rarely seen (Fig. 2c; representative field). A second passage, in which the supernatants of the infected BHK-21 cell cultures were transferred to fresh BHK-21 cells, resulted in infection in the case of the RNA transfection from full-length infectious cDNA O-IC (authentic virus particles; Fig. 2i), but not in the case of transfection with replicon Oshima REPpt and pcTBEC-JEME or pcTBECME (Fig. 2g, h). These data demonstrate that the particles (VLPs) secreted by pcTBEC-JEME- or pcTBECME- and TBE virus replicon Oshima REPpt-transfected cells have only single-round infectivity potential.

C RNA homology requirement for the secretion of VLPs

To analyse the secretion of VLPs from cells transfected with plasmids that express flavivirus structural proteins and the replicon, transfected cells and supernatant were subjected to Western blot analysis (Fig. 3a). In cells that were transfected with pcTBECME and Oshima REPpt, TBE virus prM and E protein bands were detected by the TBE virus prM- and E-specific antibodies. Moreover, similar intensities were detected by the JE virus prM- and

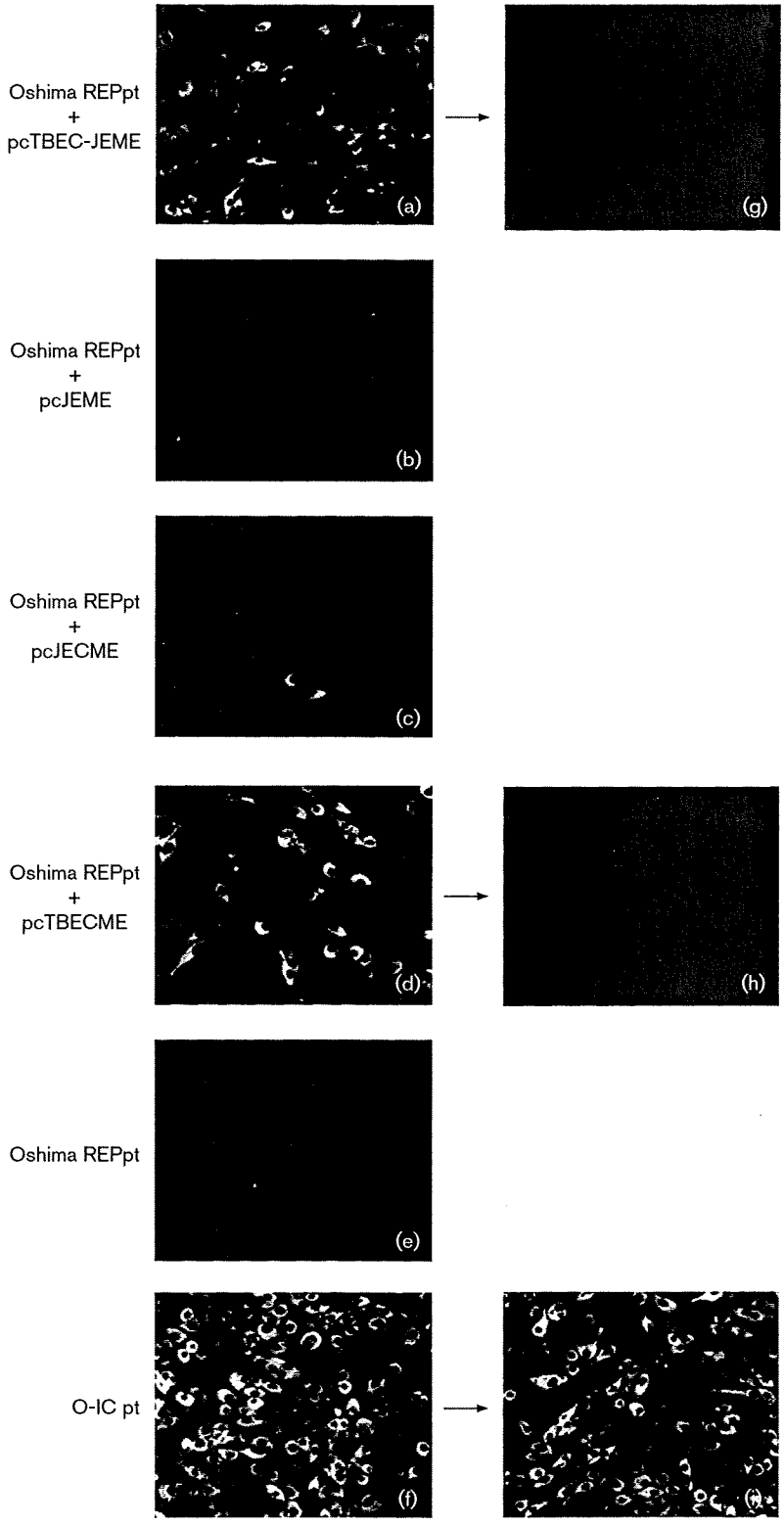

Fig. 2. Secretion of chimeric particles with single-round infectivity. (a–f) Fresh BHK-21 cells were infected with the culture supernatants of cells that were transfected with *in vitro*-synthesized Oshima REPpt RNA and (a) pcTBEC-JEME, (b) pcJEME, (c) pcJECME or (d) pcTBECME, or (e) Oshima REPpt alone. As a control (f), RNA from the full-length TBE virus infectious cDNA O-IC pt was used. (g–i) Reinfection of fresh BHK-21 cells with the culture supernatants: (a)→(g), (d)→(h) and (f)→(i). Expression of viral proteins was visualized by immunofluorescence using anti-NS3 antibodies.

E-specific antibodies for the JE virus prM and E protein bands in Oshima REPpt replicon-electroporated cells that were transfected sequentially with the pcTBEC-JEME, pcJECME and pcJEME plasmids. It has been reported previously that the C–prM junction is cleaved first by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER-luminal side (Amberg *et al.*, 1994; Lobigs, 1993; Sato *et al.*, 1993).

In this case, prM protein bands were detected as independent forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by viral NS3 protease derived from the expression of the TBE virus replicon RNA. On the other hand, viral protein secretion was observed in the culture supernatants of replicon-electroporated cells that were transfected sequentially with the pcTBECME, pcTBEC-JEME and pcJEME plasmids. However, low levels of E protein were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Furthermore, the harvested supernatant was used to infect BHK-21 cells for titration of infectious VLPs (Fig. 3b). A relatively high titre of infectious VLPs ($>10^6$ IU ml^{-1}) was secreted from cells that were transfected with both the pcTBECME and pcTBEC-JEME plasmids, whereas few VLPs (250 IU ml^{-1}) were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Semiquantitative RT-PCR showed that the secretion levels of replicon RNAs also corresponded to the infectious titre of VLPs from cells transfected with each plasmid (Fig. 3c). On the other hand, pcJEME plasmid transfection into replicon-electroporated cells resulted in no secretion of infectious VLPs, despite the secretion of viral structural proteins. This viral protein secretion was due to the secretion of subviral particles (SPs), which consist of a viral envelope without nucleocapsid or genomic RNA, as reported in our previous study (Konishi *et al.*, 2001). These data indicate that the efficient packaging and incorporation of nucleocapsid require homologous interactions between flavivirus C proteins and non-structural proteins or genomic RNA, but not between C proteins and viral envelope proteins.

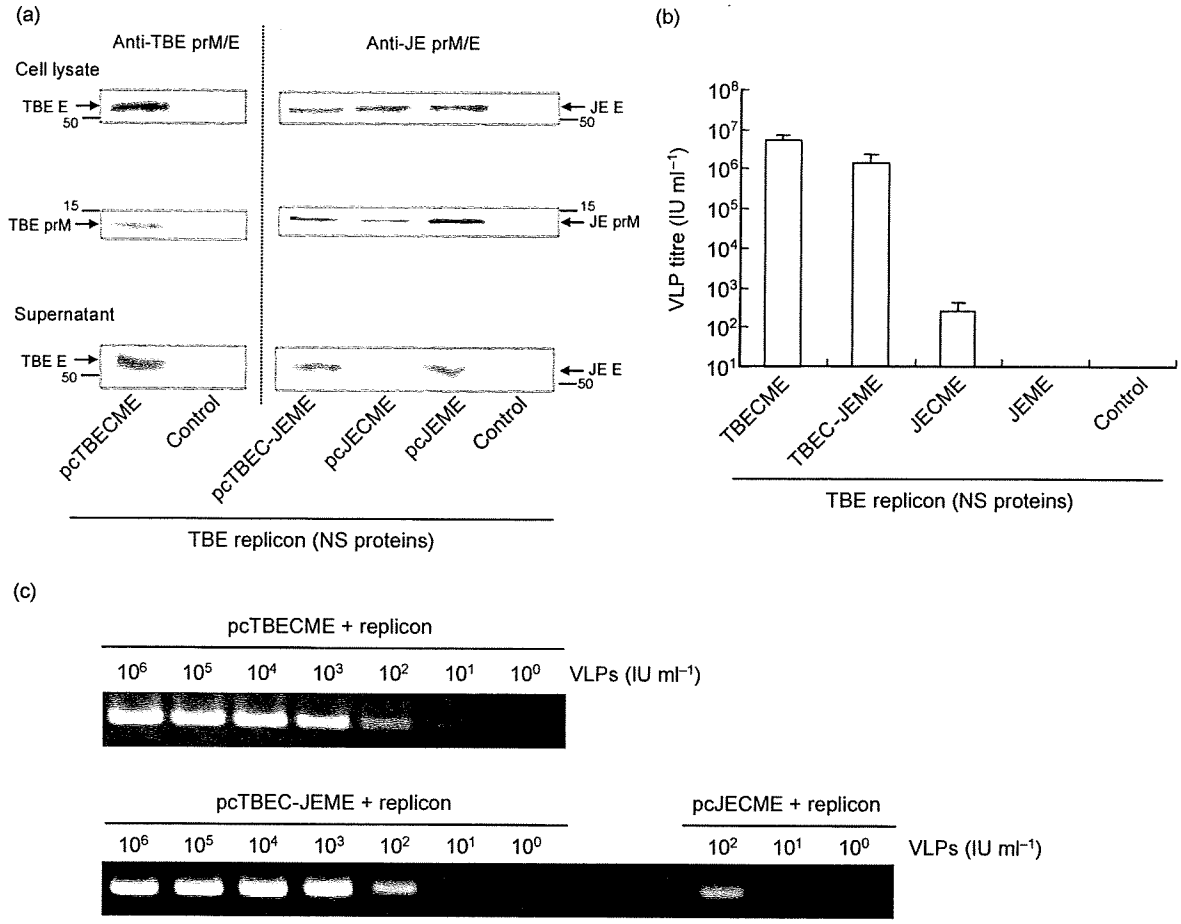

Fig. 3. Kinetics of VLP secretion. (a) Detection of flavivirus structural proteins. TBE virus replicon-electroporated BHK-21 cells were transfected sequentially with pcTBECME, pcTBEC-JEME, pcJECME, pcJEME or control plasmid. At 36 h post-transfection of the plasmid, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated. (b) Titres (IU) contained in the culture fluids were determined by infectivity assay to BHK-21 cells. The data are means from four independent experiments (error bars indicate SEM). (c) Detection of replicon RNAs packaged in VLPs. Secreted VLPs were diluted serially and subjected to RT-PCR for the detection of TBE virus replicon RNA.

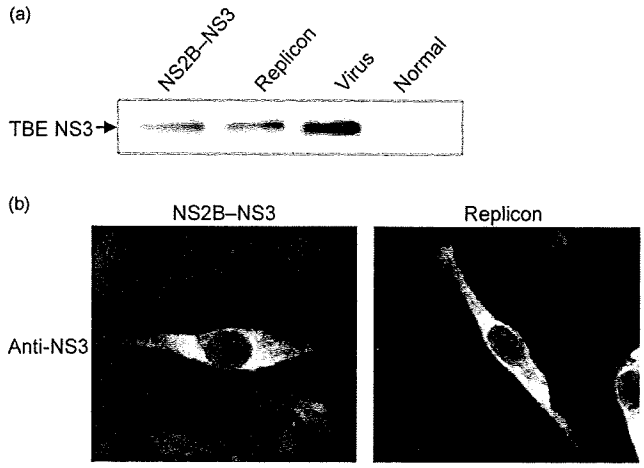

Fig. 4. (a) Expression of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid and replicon RNAs. TBE virus-infected cells and untreated cells were used as a control. (b) Localization of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid (left panel) and replicon RNAs (right panel). Expression of NS3 proteins was visualized by anti-NS3 antibodies at 36 h post-transfection.

Secretion of subviral particles from cells that express chimeric structural proteins and NS2B–NS3 proteins

It is known that expression of the flavivirus envelope proteins prM and E leads to budding and secretion of SPs. To examine the mechanism involved in the differential secretion of infectious VLPs observed for pcTBEC-JEME and pcJECME transfection of replicon-electroporated cells, we investigated SP secretion from cells that expressed C–prM–E polyproteins with TBE virus NS2B–NS3 protease. The pcTBENS2B/3 plasmid, which expresses TBE virus NS2B–NS3 polyproteins, was prepared. The same amount of NS3 was expressed and similar cytoplasmic localization was observed in cells transfected with pcTBENS2B/3 compared with those transfected with the TBE virus replicon RNAs (Fig. 4).

Individual plasmids that express flavivirus C/prM/E structural proteins (pcTBECME, pcTBEC-JEME or pcJECME) were transfected alone or together with pcTBENS2B/3 into BHK-21 cells. When the C/prM/E viral structural proteins were expressed without NS2B–NS3 proteins, C–prM polyprotein bands larger than those seen for prM were detected by the prM-specific antibodies (Fig. 5; lower panel with cell lysate samples). When the

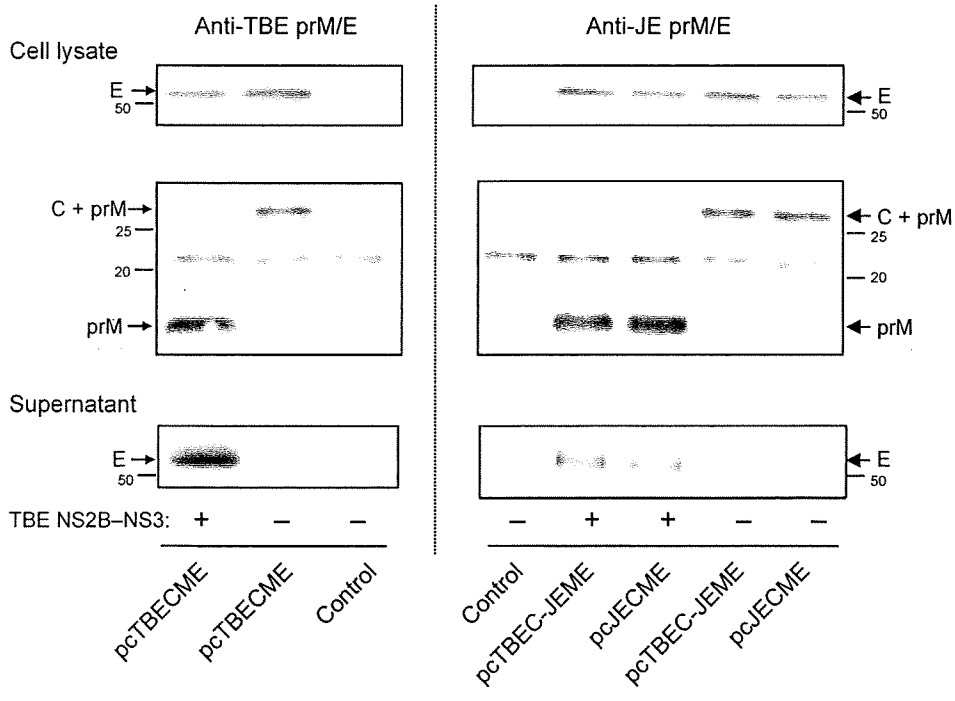

Fig. 5. Detection of flavivirus structural proteins in transfected BHK-21 cells. BHK-21 cells that were transfected with (+) or without (–) the pcTBENS2B/3 plasmid were co-transfected with pcTBECME, pcTBEC-JEME, pcJECME or control plasmid. At 36 h post-transfection, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by using specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated.

viral structural proteins were expressed with TBE virus NS2B–NS3 proteins, the prM protein bands were detected as separate forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by TBE virus NS3 protease, as observed for the co-expression of the TBE virus replicon RNA (Fig. 3). On the other hand, whilst no viral protein was secreted without NS2B–NS3 expression, viral protein secretion was observed in all supernatant samples of cells that expressed the flavivirus C/prM/E structural proteins together with TBE virus NS2B–NS3 proteins (Fig. 5; panel with supernatant samples), indicating that SPs are secreted by cleavage of the C–prM junction. This suggests that the low level of VLP secretion from cells that were transfected with pcJECME and TBE virus replicon RNA (Fig. 3) is not due to the budding property of the viral envelope prM/E proteins after processing by NS2B–NS3 protease, and that other factors, such as the mechanism for genome replication and packaging, are involved in the efficient secretion of infectious chimeric VLPs.

Neutralizing test for VLP infection of BHK-21 cells

To confirm the antigenic characteristics of the secreted VLPs, the reactivities of VLPs with anti-flavivirus antibodies were examined. The neutralization test for VLP infection of BHK-21 cells was performed by using the mouse mAbs and polyclonal antibodies against TBE virus, Langat virus (tick-borne flavivirus) and JE virus. As shown in Table 1, mAb 1H4, anti-TBE virus polyclonal antibodies and anti-Langat virus polyclonal antibodies neutralized TBE-envelope VLP infectivity at almost the same concentrations as they did native TBE virus infectivity. In addition, the anti-JE virus polyclonal antibodies neutralized JE-envelope VLP infectivity at almost the same concentration as they did JE virus infectivity. The cross-reactive mAb 4H8 showed similar reactivities for the VLPs and naïve viruses. These data show that the envelope glycoproteins of VLPs have the same antigenicity as those of authentic virus particles.

Table 1. Neutralizing titres of antibodies against infectious particles of flaviviruses

Data are reciprocal numbers of the highest serum dilution that reduced the virus focal count by 50 %.

Antibody	VLPs*			
	TBE-envelope	JE-envelope	TBE virus	JE virus
mAbs†				
1H4	>640	<20	>640	<20
4H8	40	80	40	80
Polyclonal antibodies				
Anti-TBE virus	160	<20	320	<20
Anti-Langat virus	>640	<20	>640	<20
Anti-JE virus	<20	320	<20	640

*VLPs were prepared from BHK-21 cells transfected with Oshima REPpt replicon and pcTBEC-JEME (TBE-envelope VLPs) or pcJECME (JE-envelope VLPs).
†1H4, Specific for tick-borne flavivirus; 4H8, cross-reactive against tick-borne and mosquito-borne flavivirus.

Infectivities of TBE-envelope and JE-envelope VLPs for arthropod cells

The JE-envelope VLPs contain envelope proteins that are derived from the mosquito-borne JE virus and carry nucleocapsid and replicon RNA derived from the tick-borne TBE virus. Therefore, we examined the susceptibilities of cell lines derived from arthropods to infection with chimeric JE-envelope VLPs. ISE6 cells, derived from *I. scapularis*, and C6/36 cells, derived from *A. albopictus*, were infected with the TBE-envelope VLPs, JE-envelope VLPs, TBE virus or JE virus, and viral protein production was detected by IFA.

As shown in Fig. 6, the tick cell line ISE6 was susceptible to infection by the TBE virus and TBE-envelope VLPs and the viral proteins showed a cytoplasmic distribution. However, the ISE6 cells were not susceptible to infection by the JE virus or JE-envelope VLPs, although the JE-envelope VLPs contained replicon RNA derived from the TBE virus. On the other hand, the mosquito cell line C6/36 showed no signs of infection by the TBE virus or TBE-envelope VLPs. The C6/36 cells were positive for viral antigen after infection with the mosquito-borne JE virus. Interestingly, the C6/36 cells that were infected with JE-envelope VLPs were not positive by IFA.

In order to reveal the mechanism involved in the lack of susceptibility of tick and mosquito cells to JE-envelope VLPs, the internalization of VLPs was examined (Fig. 7). Following 1 h VLP absorption, replicon RNA was detected in ISE6 cells that were infected with TBE-envelope VLPs, whereas most of the JE-envelope VLPs remained in the supernatant and no replicon RNA was detected intracellularly. This indicates that the entry of JE-envelope VLPs into ISE6 cells is inefficient and, thus, no viral proteins are detected by IFA (Fig. 6). In contrast, the JE-envelope VLPs entered the C6/36 cells after 1 h VLP absorption, whereas the TBE-envelope VLPs remained in the supernatant (Fig. 7a). However, intracellular replicon RNAs disappeared at 8 h post-infection (Fig. 7b). Taken together with the IFA results (Fig. 6), this suggests that JE-envelope VLPs can enter C6/36 mosquito cells, but cannot replicate therein, due to fact that their genomic replicon RNA is derived from the tick-borne TBE virus. Direct transfection of C6/36 cells with TBE virus full-length RNA or replicon RNA resulted in neither viral protein production nor virus particle secretion, as reported previously (Mandl *et al.*, 1991).

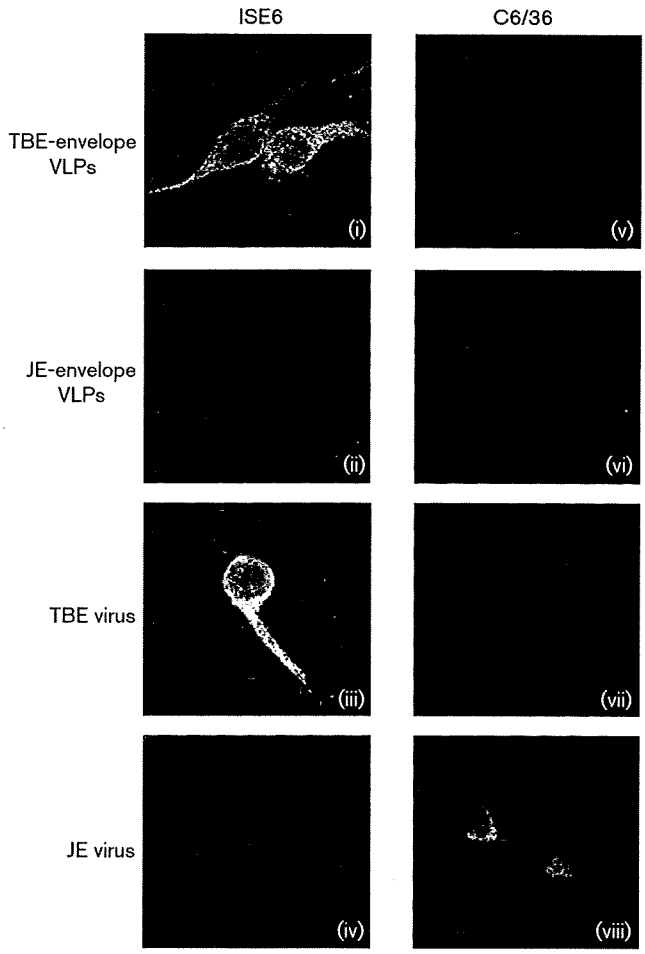

Fig. 6. Susceptibility of ISE6 (tick) and C6/36 (mosquito) cell lines to infectious flavivirus particles. ISE6 (i–iv) and C6/36 (v–viii) cells were infected with TBE-envelope VLPs (i, v), JE-envelope VLPs (ii, vi), TBE virus (iii, vii) or JE virus (iv, viii), prepared from the culture supernatants of BHK-21 cell cultures. Viral protein production was visualized by immunofluorescence using specific antibodies, as described in Methods.

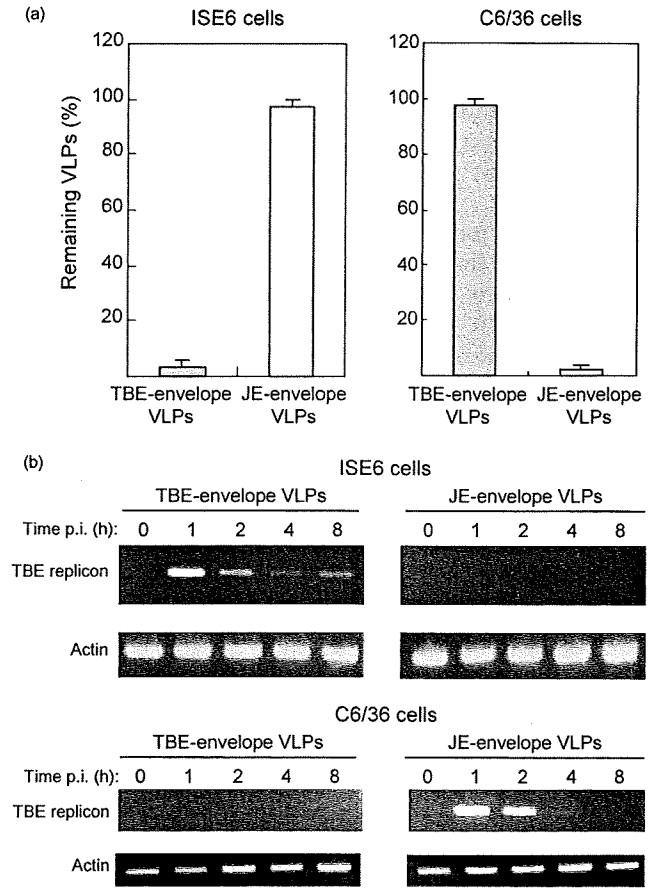

Fig. 7. (a) Internalization of TBE- or JE-envelope VLPs into arthropod cells. ISE6 and C6/36 cells were infected with VLPs. After 1 h absorption, the supernatants were harvested and remaining VLPs were titrated. Results are expressed as percentages of remaining VLPs compared with untreated controls. The data are means from three independent experiments (error bars indicate SEM). (b) Detection of replicon RNA from arthropod cells infected with TBE- or JE-envelope VLPs. ISE6 and C6/36 cells were infected with the VLPs. At each time point post-infection (p.i.), RNA samples were extracted and subjected to RT-PCR for the detection of TBE virus replicon RNA. Actin mRNA was used as an internal control.

DISCUSSION

In this study, we developed, for the first time, chimeric VLPs between mosquito-borne and tick-borne flaviviruses, using the *trans*-packaging system for TBE virus subgenomic replicons. In recent years, the development of reverse-genetic technologies has enabled the construction of flavivirus chimeras in various combinations (Caufour *et al.*, 2001; Guirakhoo *et al.*, 2000, 2001; Huang *et al.*, 2000; Mathenge *et al.*, 2004; Monath *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992, 2002). These chimeras may be useful tools for studying the biological features of flaviviruses and for vaccine development. However, several aspects of flavivirus chimeras require further study. The family *Flaviviridae* contains many important human pathogens that cause severe symptoms and high mortality (approx. 30 %). Thus, recent studies of flavivirus chimeras have tended to use less pathogenic or vaccine strains of flavivirus for the development of live-attenuated vaccines. On the other hand, flavivirus VLPs have the same antigenicity and virus-entry characteristics as the wild-type virus, and the single-round infectivity of VLPs allows infection experiments to be conducted under BSL-2 conditions (Gehrke *et al.*, 2003; Khromykh *et al.*, 1998; Scholle *et al.*, 2004; Yoshii *et al.*, 2005). Therefore, our strategy is that chimeric VLPs can substitute for chimeric viruses in investigations of the biological properties of flaviviruses.

Chimeric VLPs that packaged the TBE replicon were recovered only following *trans*-expression of the TBE virus

C and JE virus prM/E proteins. In many studies of flavivirus chimeras, chimeric viruses have been recovered successfully by replacement of the viral envelope protein prM and E genes by those from other flaviviruses. These data indicate that the C proteins (or nucleocapsid) interact non-specifically with viral envelope proteins and that this interaction is not important for the assembly of virus particles. On the other hand, in experiments using *trans*-expression of the JE virus C/prM/E proteins, we failed to recover chimeric VLPs. Similar results have been shown previously in several studies of chimeric viruses, in which substitution of the C/prM/E protein genes of a tick-borne flavivirus with those of mosquito-borne flaviviruses resulted in inefficient recovery of chimeric virus (Chambers *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992) and, in a study by Harvey *et al.* (2004), packaging efficiency of dengue type 2 virus replicon RNAs by *trans*-expression of the KUN virus C/prM/E proteins was lower than that of KUN virus replicon RNA; the detailed mechanism underlying this finding has not been analysed.

It is known that the C terminus of the C protein is processed by the activity of the viral NS2B–NS3 protease, and that the N terminus of prM is subsequently cleaved by the cellular signal peptidase (Amberg & Rice, 1999; Amberg *et al.*, 1994; Stocks & Lobigs, 1998; Yamshchikov & Compans, 1995; Yamshchikov *et al.*, 1997). The processed prM protein then forms a heterodimer with the E protein, which is essential for the maturation and assembly of infectious particles (Allison *et al.*, 1995; Konishi & Mason, 1993; Lorenz *et al.*, 2002). In cells that expressed the JE virus C/prM/E structural proteins and TBE virus replicon or NS2B–NS3, prM proteins were detected in the form cleaved from the C protein, which indicates that the JE virus C–prM junctions are cleaved by the TBE virus NS2B–NS3 protease (Figs 3 and 5). However, in spite of the processing of the C–prM junctions, the expression of JE virus C/prM/E proteins in cells that harboured the TBE virus replicon resulted in a low level of secretion of viral proteins compared with cells that expressed the TBE virus C and JE virus prM/E proteins or the TBE virus C/prM/E proteins, which secreted VLPs that packaged the replicon RNA. The difference between the *trans*-expressed polyproteins is a reflection of whether the respective C proteins are homologous to the genomic replicon RNA. Thus, in addition to the processing of C–prM junctions, other factors related to the homology between the C protein and genomic RNA regulate the assembly of VLPs that package replicon RNA.

Efficient secretion of E, in the form of non-infectious SPs, was observed by the expression of JE virus C/prM/E proteins and TBE virus NS2B–NS3 proteins, but few infectious VLPs and fewer E proteins were secreted by the expression of JE virus C/prM/E proteins in TBE replicon-transfected cells. This indicated that some factor involved in the replication of replicon RNA regulated the process of viral particle assembly and secretion and retained viral structural proteins intracellularly. Recent studies of flavivirus non-structural proteins have revealed the involvement of NS2A and NS3 in the assembly and/or release of infectious virus particles (Khromykh *et al.*, 2000; Kummerer & Rice, 2002; Liu *et al.*, 2002) and it has been reported that the packaging of flavivirus genomic RNA into infectious particles is coupled to genome replication (Khromykh *et al.*, 2001). From these studies, it has been concluded that homologous interaction between viral C and non-structural proteins or genomic RNA is important for the efficient assembly and secretion of infectious particles packaging genomic RNA. No signals or motifs in flavivirus RNA or C protein that determine the specificity of packaging have yet been defined. However, these findings can contribute to future studies of packaging signals and increase understanding of how flavivirus virions are assembled and secreted.

It has been shown that, in general, arthropod-borne flaviviruses infect either mosquito or tick cells (Lawrie *et al.*, 2004). However, it is not known whether the ability of certain flaviviruses to infect certain cells but not others is due to viral entry into the cells or to replication and subsequent release from infected cells. The chimeric VLPs developed in our study have viral envelopes that are derived from mosquito-borne JE virus, and nucleocapsid (C protein plus subgenomic replicon RNA) derived from tick-borne TBE virus. Virus particles of flaviviruses enter cells by receptor-mediated endocytosis, the viral envelope fuses with the endosomal membrane and then the genomic RNAs are uncoated from the nucleocapsid and replicate (Heinz *et al.*, 2004). Thus, our chimeric VLP system has the advantage that it can separate particle-internalization events from sequential viral-replication events. As shown in Figs 6 and 7, TBE virus and JE virus infected either ISE6 or C6/36 cells, and TBE-envelope VLPs infected only ISE6 cells. However, no TBE virus NS proteins were produced in either tick or mosquito cells that were infected with JE-envelope VLPs. RT-PCR revealed that the TBE- and JE-envelope VLPs could enter ISE6 or C6/36 cells, and that the TBE virus replicon RNAs could not replicate in C6/36 cells that were infected with JE-envelope VLPs. Similar results were reported in a study by Pletnev *et al.* (1992), in which a chimeric virus of TBE virus/dengue type 4 virus that contained prM/E protein genes from the TBE virus was restricted in its ability to enter C6/36 cells. It is not known whether JE virus genomic RNA can replicate in tick cells, but these data indicate that vector-specific factors in arthropod cells may be required for each step of virus entry and replication during flavivirus infection. Although the susceptibility of a tick- or mosquito-derived cell to a particular arbovirus does not always reflect vector association, it is a useful indicator.

In addition to their use in investigations of the biological properties of flaviviruses, chimeric VLPs can be applied to serological diagnosis as a substitute for neutralization testing, which uses infectious viruses. As shown in Table 1, infections with TBE- and JE-envelope VLPs were neutralized by mAbs and immune sera with titres similar to those

used to neutralize the TBE and JE viruses, indicating that the chimeric VLP system is an effective alternative to the use of native flaviviruses in neutralization tests. This chimeric VLP-based neutralization system does not require a high-level containment laboratory, as the subgenomic replicon RNA packaged in the VLPs does not have the genes that encode the viral structural proteins and thus is unable to produce infectious progeny viruses. Furthermore, by replacement of the prM/E genes with those from other flaviviruses in plasmids used for the *trans*-expression of viral structural proteins, the chimeric VLP system can be adjusted easily to develop neutralization tests for a variety of flaviviruses, as substitutes for tests involving the native viruses.

In summary, we have generated chimeric VLPs that comprise the JE virus envelope and TBE virus nucleocapsid, which contains the subgenomic replicon of the TBE virus. JE-envelope VLPs were secreted when the TBE virus C protein and JE virus prM/E proteins were expressed in TBE virus replicon-electroporated cells, but not when all of the structural proteins of JE virus were expressed. The TBE- and JE-envelope VLPs were neutralized by antibodies against flaviviruses with efficacies similar to those for the native TBE and JE viruses. The infectivities of the TBE- and JE-envelope VLPs for tick and mosquito cells suggest that vector host cell-specific factors are involved in each step of flavivirus entry and replication during arthropod infection.

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