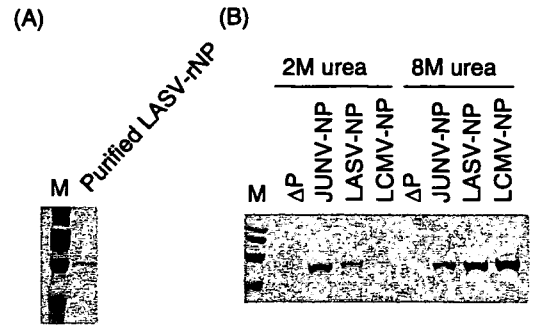


FIG. 2. SDS-PAGE analyses of the purification of His-LASV-rNP using the Ni^{2+} column purification method (A) and of the semipurification strategy based on the hydrophobic property of arenavirus nucleoproteins (B). The supernatant fractions of the Ac-ΔP-, Ac-His-LASV-NP-, Ac-LCMV-NP-, or Ac-JUNV-NP-infected Tn5 cells treated with PBS–2 M urea (B, left part) are shown. The pellet fractions of these cells treated with PBS–2 M urea were further solubilized with PBS–8 M urea (B, right part).

were shifted by 1 amino acid (aa), with a consecutive overlap of 9 aa to cover the entire LASV-NP1 (aa 1 to 100) and LASV-NP5 (aa 361 to 460) fragments. Linear epitopes on the NP were determined by using Pepscan (Chiron Technologies, Clayton, Australia) according to the manufacturer's instructions. Ninety-six peptides were prepared as 14-aa biotinylated peptides, including a 4-aa spacer sequence (SGSG) at the amino-terminal end, according to each of the amino acid sequences of the LASV-rNP1 and LASV-rNP5 of the LASV Josiah strain. The methods were previously described in detail (33).

IgG-ELISA. Immunoglobulin G (IgG)-ELISA was performed as previously described except for the antigen preparation (38, 39). Briefly, ELISA plates (96-well type plate, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified His-LASV-rNP, LCMV-rNP, or JUNV-rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (M-T-PBS), followed by incubation for 1 h for blocking. The plates were washed three times with T-PBS and then inoculated with the test samples (100 μl/well), which were diluted fourfold from 1:100 to 1:6,400 with M-T-PBS. After a 1-h incubation period, the plates were washed three times with T-PBS, and then the plates were inoculated with goat anti-human IgG antibody labeled with HRPO (1:1,000 dilution; Zymed Laboratory). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2'azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and optical density was at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} was calculated by subtracting the OD of the negative antigen-coated wells from that of the corresponding wells. The means and standard deviations were calculated from the 96 control sera. The cutoff value for the assay was defined as the mean plus 3 standard deviations.

Immunofluorescence. The pQE32-LASV-NP was digested with BamHI, and the insert was subcloned into the BamHI site of the pKS336 vector (40). The LASV-NP gene that was inserted into the pKS336 vector, pKS336-LASV-NP, was confirmed to be in the correct orientation to the promoter, tested for nucleotide sequencing as described above, and the nucleotide sequence of the gene was confirmed to be identical to the original sequence. HeLa cells were then transfected with pKS336-LASV-NP by using a FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. The cells transfected with the plasmid were selected with 3 μg of blasticidin *S*-hydrochloride/ml in MEM-10FBS. The HeLa cell clones were analyzed for the expression of LASV-rNP by IIFA using the rabbit serum raised against His-LASV-rNP. The cells expressing LASV-rNP were subcloned and used as IIFA antigens.

Ag-capture ELISA. Ag-capture ELISA was performed as previously described (32, 41). The purified MAb to His-LASV-rNP, MAb 4A5, produced in the present study was diluted in PBS solution, and 100 μl was adsorbed overnight at 4°C onto the immunoplates (96-well type plate, Pro-Bind, Falcon; Becton Dickinson Labware). Purified MAb 4A5 was coated onto the immunoplates at a concentration of approximately 100 ng/well in 100 μl of PBS. The difference in the procedures between the Ag-capture ELISA in the present study and those in previous studies (32, 37, 41) is that the MAb, MAb 4A5, and rabbit serum raised to His-LASV-rNP were used as capture and detector antibodies, respectively. The procedure for the Ag-capture ELISA was performed as follows. The ELISA plate was coated with capture MAb, followed by blocking of the plate with M-T-PBS, addition of the samples to the ELISA plate, detection of the captured LASV-NP with rabbit serum raised to His-LASV-rNP, detection of rabbit IgG antibody that reacted with the captured antigen with goat anti-rabbit IgG antibodies conjugated with HRPO (Zymed Laboratories), and substrate reaction. In each run of the Ag-capture ELISA, the negative control antigen (M-T-PBS) was also tested. Serially diluted samples were added to the MAb-coated wells. The OD_{405} values of each well were adjusted by subtracting the OD_{405} value of the negative control antigen from the corresponding well. The adjusted OD_{405} was taken as a measure of the amount of antigen specifically bound. All samples were treated with 1% Nonidet-P40 (NP-40) in PBS to destroy the LASV virion and expose the nucleoprotein in the LASV virion.

RT-PCR. RT-PCR was performed as previously described (10). The primers used in the RT-PCR were 36E2 (5'-ACCGGGGATCCTAGGCATT-3') and 80F2 (5'-ATATAATGATGACTGTTGTTCTTTGTGCA-3'). The RT-PCR was carried out with a Ready-to-Go RT-PCR tube (Pharmacia). The amplified PCR products were visualized with ethidium bromide in 2% agarose gel after electrophoresis.

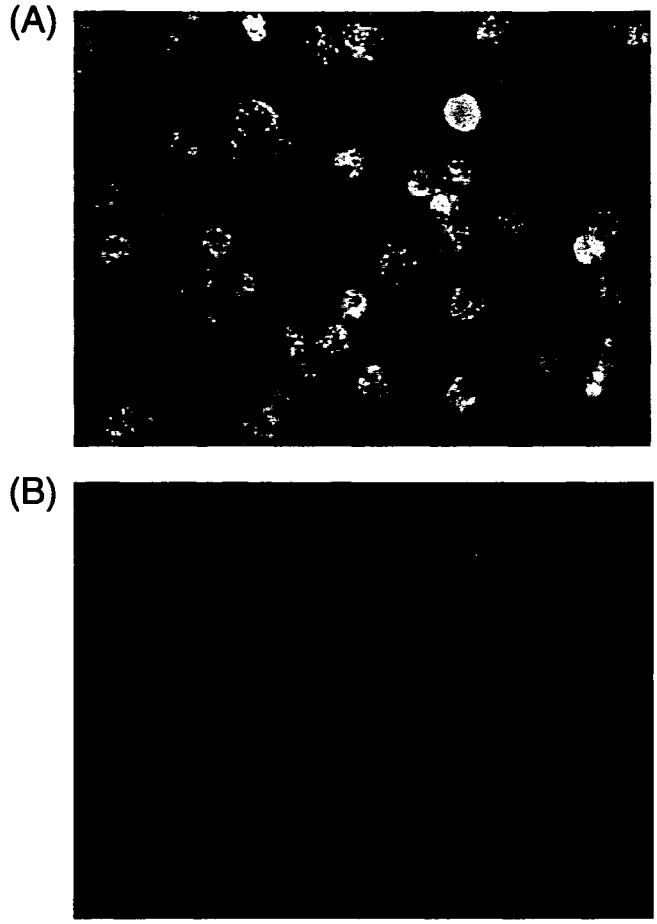


FIG. 3. Staining patterns of LASV-rNP-expressing HeLa cells by sera from an LF patient (A) and a healthy control (B) in an IIFA.

RESULTS

Expression of His-LASV-rNP. Tn5 cells infected with each of the recombinant baculoviruses—Ac-His-LASV-NP, Ac-LCMV-rNP, and Ac-JUNV-rNP—were suspended in PBS–2 M urea. Most of the cell proteins were solubilized by this

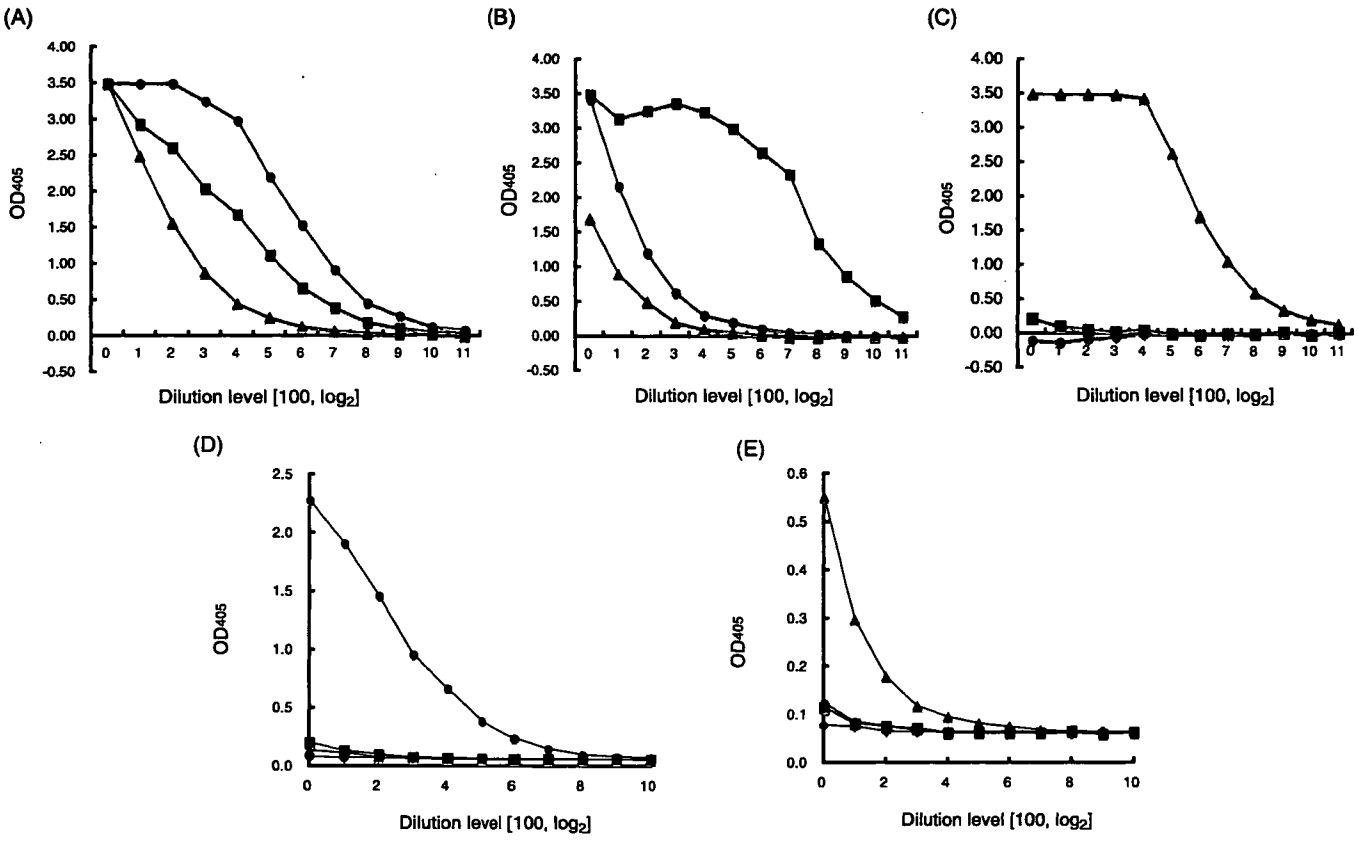


FIG. 4. Reactivity of antibodies to arenaviruses (LASV, LCMV, and JUNV) to the rNPs of these viruses. The reactivities of rabbit sera raised to LASV-rNP (●), LCMV-rNP (■), or JUNV-rNP (▲) with the antigens His-LASV-rNP (A), LCMV-rNP (B), and JUNV-rNP (C) in an IgG-ELISA are shown. The reactivities of the sera collected from patients with LF (D) and AHF (E) with the antigens LASV-rNP (●), LCMV-rNP (■), and JUNV-rNP (▲) and negative control antigen (◆) in an IgG-ELISA are also shown.

treatment, whereas the rNPs of these viruses remained insoluble. After centrifugation at 15,000 × *g* for 10 min, pellet fractions were collected. The rNPs, which were still present in the pellet fractions, were completely solubilized in PBS–8 M urea. The samples were then centrifuged at 15,000 × *g* for 10 min, and the supernatant fractions of the PBS–8 M urea were confirmed to contain highly purified recombinant rNPs of arenaviruses (Fig. 2).

Development of indirect immunofluorescence. The LASV-rNP was expressed in HeLa cells by transfection with the expression vector, pKS336-LASV-NP. The transfected cells were stained by anti-His-LASV-rNP rabbit serum and human serum samples from LF patients (Fig. 3). All 4 serum samples collected from two LF patients showed a positive staining, but 96 control serum samples did not. The LASV-rNP-based IIFA was also evaluated using serum samples collected from monkeys experimentally infected with LASV. All of the sera collected from five LASV-infected monkeys showed a positive staining, but those from four mock-infected monkeys did not.

Development of His-LASV-rNP-based IgG-ELISA. Four serum samples collected from LF patients were determined to be positive by His-LASV-rNP-based IgG-ELISA, whereas 94 of the 96 control serum samples were determined to be negative. Thus, the sensitivity and specificity of the ELISA were 100 and 96%, respectively. All serum samples collected from five LASV-infected monkeys were determined to be positive, whereas those from four mock-infected monkeys were negative.

In order to examine cross-reactivity among arenaviruses in the LASV-rNP-based IgG-ELISA, antisera against LASV-rNP, LCMV-rNP, or JUNV-rNP were examined (Fig. 4). The anti-LASV-rNP serum showed a strongly positive reaction, and anti-LCMV rNP and anti-JUNV-rNP sera showed strongly positive reactions in the IgG ELISA using the respective antigens (Fig. 4A, B, and C). Anti-LCMV-rNP and anti-JUNV-rNP sera showed a less strongly positive reaction in the His-LASV-rNP-based IgG-ELISA than anti-LASV-rNP serum (Fig. 4A). Anti-LASV-rNP and anti-JUNV-rNP also showed a less strongly positive reaction in the His-LCMV-rNP-based IgG-ELISA than anti-LCMV-rNP serum (Fig. 4B). However, anti-LASV-rNP and anti-LCMV-rNP sera showed a negative reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4C). Human sera from LF patients showed a highly positive reaction in the LASV-rNP-based IgG-ELISA, but sera from patients with Argentine hemorrhagic fever (AHF), which is caused by JUNV, did not (Fig. 4D). Serum from an AHF patient showed a highly positive reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4E). These results suggest that cross-reactive antibody among arenaviruses may be detected by the newly developed LASV-rNP-based IgG-ELISA.

Development of LASV Ag-capture ELISA. Three clones of a hybridoma that excreted an MAb to His-LASV-rNP were es-

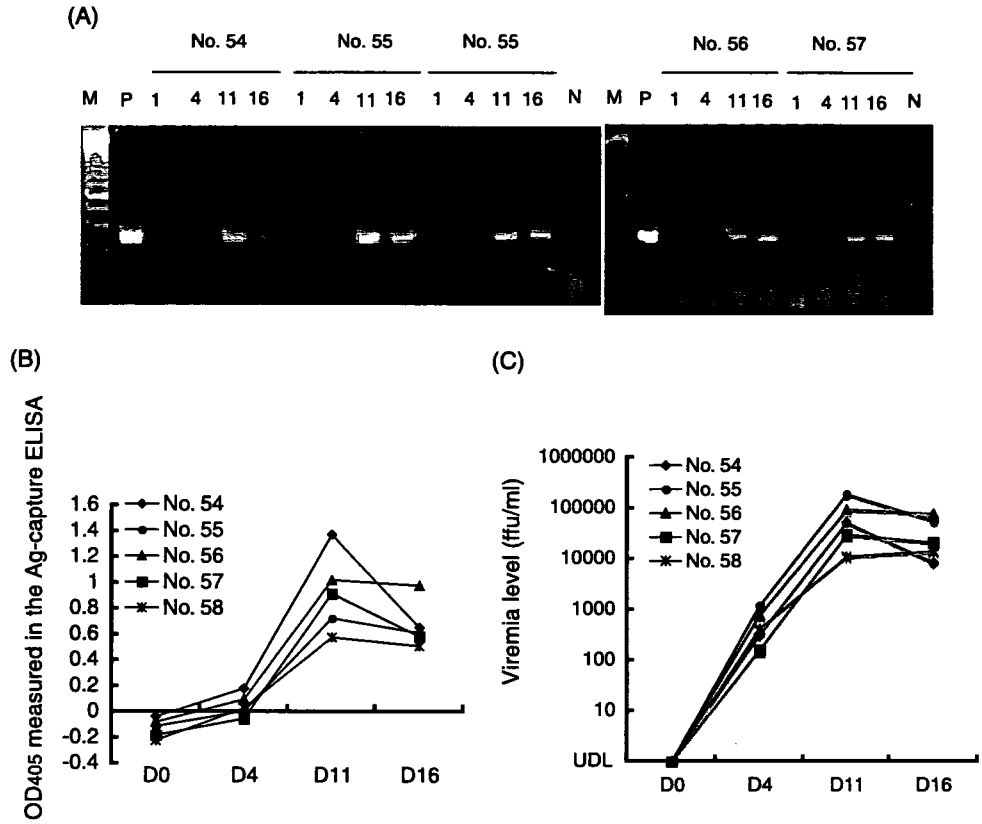


FIG. 5. Detection of the LASV genome by the RT-PCR (A), LASV-NP by the LASV-NP-Ag-capture ELISA (B), and the infectious dose of LASV (C) in serially collected sera of hamsters experimentally infected with LASV. The OD_{405} values in panel B were obtained at a dilution of 1:40.

tablished. The isotype of the three MAbs were identified as IgG1. These MAbs were designated MAb 4A5, MAb 6C11, and MAb 2-11. Of these MAbs, MAb 4A5 was the most efficient in capturing His-LASV-rNP in the Ag-capture ELISA format. The Ag-capture ELISA with MAb 4A5 detected His-LASV-rNP concentrations as low as 800 pg/ml (data not shown). Furthermore, the Ag-capture ELISA detected the MOPV-NP but not the rNPs of LCMV and JUNV (data not shown).

All of the sera collected from five LASV-infected hamsters on days 11 and 16 postinfection were antigen positive in the Ag-capture ELISA using MAb 4A5 as a capture antibody, whereas the sera collected on days 0 and 4 were antigen negative. The OD_{405} values in the ELISA were highest on day 11. The reactivity patterns in each hamster in the ELISA were similar to the viremia levels (Fig. 5). The sera collected on days 11 and 16 were found to be LASV genome positive by RT-PCR (10). Thus, the sensitivity of the Ag-capture ELISA was similar to that of the RT-PCR.

Determination of the epitope recognized by the monoclonal antibodies. The epitope recognized by MAbs was determined. MAb-4A5 reacted in Western blots with GST-LASV-rNP1-6 (full-length LASV-rNP), GST-LASV-rNP1-5, and GST-LASV-rNP1-4 but not with the other truncated LASV-rNPs shown in Table 1, suggesting that MAb 4A5 reacted with a conformational epitope located on the amino-terminal portion of LASV-rNP. The epitope was maintained when the extreme amino-terminal portion, LASV-rNP1, was present but was lost when LASV-rNP1 was removed. These results suggest that the extreme amino-terminal portion, LASV-rNP1, is essential for the maintenance of the conformational epitope. MAbs 6C11 and 2-11 reacted in Western blots with GST-LASV-rNP1 and GST-LASV-rNP5, respectively (Table 1).

The Pepscan analyses indicated that MAbs 6C11 and 2-11

TABLE 1. Reactivities of the MAbs developed in the present study with the GST-tagged truncated LASV-rNP in Western blot analyses

Truncated LASV-rNP	Reactivity with MAb[a]:		
	6C11	4A5	2-11
LASV-rNP1	+	–	–
LASV-rNP2	–	–	–
LASV-rNP3	–	–	–
LASV-rNP4	–	–	–
LASV-rNP5	–	–	+
LASV-rNP6	–	–	–
LASV-rNP1-2	ND	–	ND
LASV-rNP1-3	ND	–	ND
LASV-rNP1-4	ND	+	ND
LASV-rNP1-5	ND	+	ND
LASV-rNP1-6[b]	ND	+	ND
LASV-rNP2-7	ND	–	ND
LASV-rNP3-6	ND	–	ND
LASV-rNP4-6	ND	–	ND
LASV-rNP5-6	ND	–	ND

[a] "+" and "–" indicate positive and negative reactions, respectively. ND, not determined.
[b] LASV-rNP1-6 indicates LASV-rNP.

TABLE 2. Reactivities of the MAbs developed in the present study with the NPs of LASV, MOPV, LCMV, and JUNV in Western blot analyses

MAb	Reactivity of MAb[a] with NP of:			
	LASV	MOPV	LCMV	JUNV
4A5	+	+	-	-
6C11	+	ND	+	-
2-11	+	ND	-	-

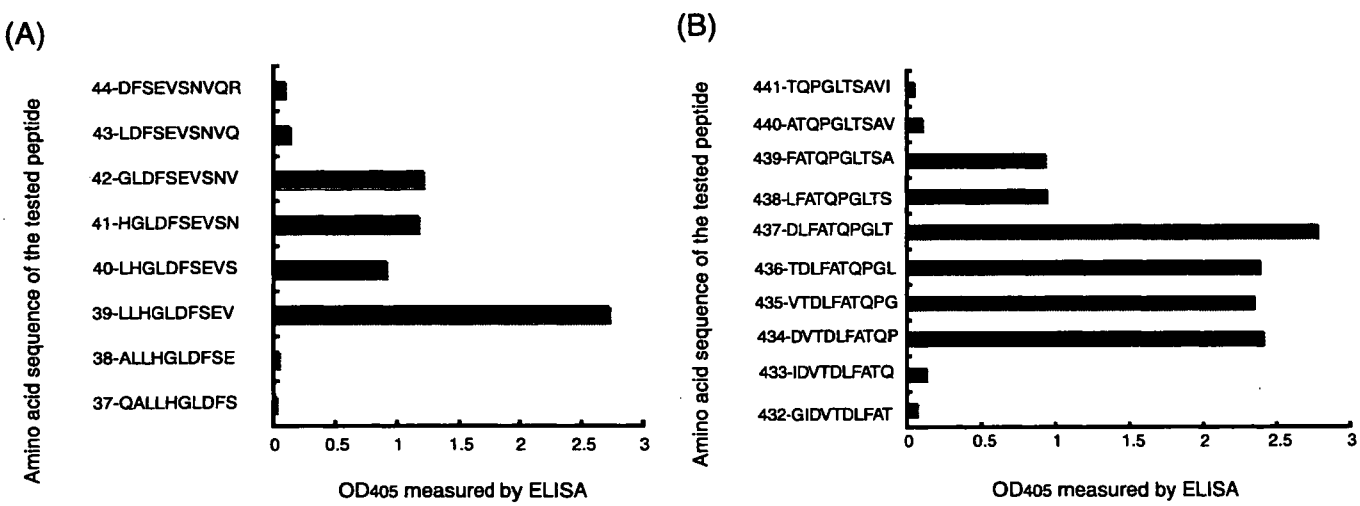
[a] "+" and "-" indicate positive and negative reactions, respectively. ND, not determined. The reactivities of MAb 6C11 and MAb 2-11 were not evaluated with MOPV-NP. However, theoretically, MAb 6C11 should be reactive with MOPV-NP due to the presence of the amino acid residues that can react with MAb 6C11, but MAb 2-11 should not react with MOPV-NP due to the absence of the amino acid residues that can react with MAb 2-11.

recognized linear epitopes. MAbs 6C-11 and 2-11 recognized GLDFSEV (aa 41 to 47) within LASV-rNP1 and FATQP (aa 439 to 443) within LASV-rNP5, respectively (Fig. 6). The reactivity patterns of these MAbs with NPs of LASV, MOPV, LCMV, and JUNV are summarized in Table 2.

DISCUSSION

We report here the development of diagnostic systems (antibody and antigen detection systems) for LF using LASV-rNP. The LASV-rNP-based IgG-ELISA was sensitive and specific in detecting anti-LASV-IgG. Although the data were not shown, an IgM-capture ELISA using purified LASV-rNP as an antigen was developed in the same way as that shown in previous reports and detected LASV-IgM antibody (42, 43). All sera collected from LF patients and monkeys infected with LASV showed positive reactions in the LASV-rNP-based IIFA. The staining patterns of the rNP with these sera were granular in the IIFA (Fig. 3), making it easy to distinguish positives from negatives. IIFA using LASV-rNP-expressing HeLa cells was also highly sensitive and specific in detecting LASV-IgG. In the preliminary study, ca. 15% of the sera collected from 334 Ghanaians and only less than 1% of 280 Zambians showed positive reactions in the LASV-rNP-based IgG ELISA (our data). The results are considered to be compatible with the fact that LF is endemic to the western African region, including Ghana, but not to the eastern African region. The LASV-rNP-based antibody detection systems such as ELISA and IIFA were suggested to be useful not only in the diagnosis of but also in the seroepidemiological study of LF.

The LASV-rNPs were expressed by a transformation system in *E. coli* or by recombinant baculovirus systems and have already been applied as antigens in ELISA, Western blotting, and IIFA for the detection of antibodies to LASV (4, 14, 16, 22, 23, 44). In the present study, an Ag-capture ELISA using



(C)

Arenavirus-NP	Amino acid sequence of the corresponding region: MoAb-6C11	MoAb-2-11
LASV-NP	31-VVKDAQALLHGLDFSEVNVQRLMRKERRD	431-HGIDVTDLFATQPGLTSAVI
MOPV-NP	31-VIKDAQALLHGLDFSEVANVQRLMRKEKRD	432-HGIDIQDLFSVQPGLTSAVI
LCMV-NP	31-VIKDATNLLNGLDFSEVSNVQRIMRKEKRD	424-HGMDLADLFNAQPGLTSSVI
JUNV-NP	31-VLKDAKLIADSIDFNQVAQVQRALRKTKRG	422-HGILMKDIEDAMPGVLSYVI

FIG. 6. Pepscan analyses to determine the epitopes of MAb 6C11 (A) and MAb 2-11 (B). The vertical bar indicates the amino acid residues with an amino acid position within the LASV-NP. MAb 6C11 was confirmed to react with 7 aa residues positioned from aa 42 to 48 (GLDFSEV) within LASV-NP1. MoAb-2-11 was confirmed to react with 5 aa residues positioned from aa 439 to 443 (FATQP) within LASV-NP5. (C) The corresponding amino acid residues to the epitope of the MAb 6C11 and MAb 2-11 among MOPV, LCMV, and JUNV are shown. The GenBank accession numbers for the S genes of LASV, MOPV, LCMV, and JUNV are NC_004296, AY772170, AY847350, and DQ272266, respectively. The epitope of the MAb 6C11 is present not only in the nucleoprotein of LASV but also in those of MOPV and LCMV—but not in that of JUNV.

MAbs to LASV-rNP was also developed. Furthermore, detection of the cross-reactive antibody by LASV-rNP-based IgG-ELISA was examined. The results for cross-reactivity indicate that the LASV-rNP-based IgG-ELISA detects not only antibodies to LASV but also those to LCMV.

The Ag-capture ELISA using MAb 4A5 was confirmed to be useful in the detection of authentic LASV antigen in sera serially collected from hamsters infected with LASV. The sensitivity of the MAb 4A5-based Ag-ELISA was similar to that of conventional RT-PCR, the efficiency of which in the diagnosis of LF was previously reported (10). Therefore, the MAb 4A5-based Ag-capture ELISA is regarded as useful in the diagnosis of LF. Unfortunately, the efficacy of the MAb 4A5-based Ag-capture ELISA in the diagnosis of LF was not evaluated using serum samples from patients. Thus, further study is still required. The three MAbs, including MAb 4A5, were characterized, and the corresponding amino acid residues within the nucleoproteins of LASV, MOPV, LCMV, and JUNV to the epitopes of MAb 6C11 and MAb 2-11 are summarized in Fig. 6C. It was of interest that LASV, MOPV, LCMV, and JUNV might be identified by analyses of the reactivity patterns of MAbs 4A5, 6C11, and 2-11 to the nucleoproteins of each virus. The nucleoproteins of all LASV strains circulating in the western and central parts of Africa would be detected by the MAb 4A5-based Ag-capture ELISA, since this ELISA was able to detect MOPV-NP that was different from LASV in terms of genetic and evolutional characteristics.

We have thus far reported the development of antibody and antigen detection systems using the recombinant nucleoproteins of the viruses for Ebola hemorrhagic fever, Marburg hemorrhagic fever, and Crimean-Congo hemorrhagic fever (32, 33, 36–42). Recently, a number of highly pathogenic emerging virus infections in humans appeared, such as Nipah virus encephalitis (8), SARS-coronavirus infections (21, 35), and highly pathogenic avian influenza virus infections (9, 45, 46). The strategy shown here might be applicable to the development of diagnostic systems for severe viral infections whose etiologic agents are highly pathogenic to humans as an alternative method to methods using infectious viruses.

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REFERENCES

1. **Anonymous.** 2000. Lassa fever imported to England. Commun. Dis. Rep. CDR Wkly. **10**:99.
2. **Anonymous.** 2000. Lassa fever, imported case, The Netherlands. Wkly. Epidemiol. Rec. **75**:265.
3. **Baize, S., D. Pannetier, C. Faure, P. Marianneau, I. Marendat, M. C. Georges-Courbot, and V. Deubel.** 2006. Role of interferons in the control of Lassa virus replication in human dendritic cells and macrophages. Microbes Infect. **8**:1194–1202.
4. **Barber, G. N., J. C. Clegg, and G. Lloyd.** 1990. Expression of the Lassa virus nucleocapsid protein in insect cells infected with a recombinant baculovirus: application to diagnostic assays for Lassa virus infection. J. Gen. Virol. **71**(Pt. 1):19–28.
5. **Borio, L., T. Inglesby, C. J. Peters, A. L. Schmaljohn, J. M. Hughes, P. B. Jahrling, T. Ksiazek, K. M. Johnson, A. Meyerhoff, T. O'Toole, M. S. Ascher, J. Bartlett, J. G. Breman, E. M. Eitzen, Jr., M. Hamburg, J. Hauer, D. A. Henderson, R. T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G. J. Nabel, M. T. Osterholm, T. M. Perl, P. Russell, and K. Tonat.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA **287**:2391–2405.
6. **Bossi, P., A. Tegnell, A. Baka, F. Van Loock, J. Hendriks, A. Werner, H. Maidhof, and G. Gouvras.** 2004. Bichat guidelines for the clinical management of haemorrhagic fever viruses and bioterrorism-related haemorrhagic fever viruses. Eur. Surveill. **9**:E11–E12.
7. **Carey, D. E., G. E. Kemp, H. A. White, L. Pinneo, R. F. Addy, A. L. Fom, G. Stroh, J. Casals, and B. E. Henderson.** 1972. Lassa fever. Epidemiological aspects of the 1970 epidemic, Jos, Nigeria. Trans. R. Soc. Trop. Med. Hyg. **66**:402–408.
8. **Chua, K. B., K. J. Goh, K. T. Wong, A. Kamarulzaman, P. S. Tan, T. G. Ksiazek, S. R. Zaki, G. Paul, S. K. Lam, and C. T. Tan.** 1999. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. Lancet **354**:1257–1259.
9. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351**:472–477.
10. **Demby, A. H., J. Chamberlain, D. W. Brown, and C. S. Clegg.** 1994. Early diagnosis of Lassa fever by reverse transcription-PCR. J. Clin. Microbiol. **32**:2898–2903.
11. **Ghiringhelli, P. D., R. V. Rivera Pomar, N. I. Baro, M. F. Rosas, O. Grau, and V. Romanowski.** 1989. Nucleocapsid protein gene of Junin arenavirus (cDNA sequence). Nucleic Acids Res. **17**:8001.
12. **Ghiringhelli, P. D., R. V. Rivera-Pomar, M. E. Lozano, O. Grau, and V. Romanowski.** 1991. Molecular organization of Junin virus S RNA: complete nucleotide sequence, relationship with other members of the *Arenaviridae* and unusual secondary structures. J. Gen. Virol. **72**(Pt. 9):2129–2141.
13. **Gunther, S., P. Emmerich, T. Laue, O. Kuhle, M. Asper, A. Jung, T. Grewing, J. ter Meulen, and H. Schmitz.** 2000. Imported Lassa fever in Germany: molecular characterization of a new Lassa virus strain. Emerg. Infect. Dis. **6**:466–476.
14. **Gunther, S., O. Kuhle, D. Rehder, G. N. Odaibo, D. O. Olaleye, P. Emmerich, J. ter Meulen, and H. Schmitz.** 2001. Antibodies to Lassa virus Z protein and nucleoprotein co-occur in human sera from Lassa fever endemic regions. Med. Microbiol. Immunol. **189**:225–229.
15. **Hirabayashi, Y., S. Oka, H. Goto, K. Shimada, T. Kurata, S. P. Fisher-Hoch, and J. B. McCormick.** 1988. An imported case of Lassa fever with late appearance of polyserositis. J. Infect. Dis. **158**:872–875.
16. **Hummel, K. B., M. L. Martin, and D. D. Auperin.** 1992. Baculovirus expression of the glycoprotein gene of Lassa virus and characterization of the recombinant protein. Virus Res. **25**:79–90.
17. **Ikegami, T., M. Niikura, M. Saijo, M. E. Miranda, A. B. Calaor, M. Hernandez, L. P. Acosta, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2003. Antigen capture enzyme-linked immunosorbent assay for specific detection of Reston Ebola virus nucleoprotein. Clin. Diagn. Lab. Immunol. **10**:552–557.
18. **Jeffs, B.** 2006. A clinical guide to viral haemorrhagic fevers: Ebola, Marburg, and Lassa. Trop. Doct. **36**:1–4.
19. **King, L., and R. Possee.** 1992. The baculovirus expression system: a laboratory guide. Chapman and Hall, New York, NY.
20. **Kitts, P. A., M. D. Ayres, and R. D. Possee.** 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. **18**:5667–5672.
21. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348**:1953–1966.
22. **Lloyd, G., G. N. Barber, J. C. Clegg, and P. Kelly.** 1989. Identification of Lassa fever virus infection with recombinant nucleocapsid protein antigen. Lancet **ii**:1222.
23. **Lukashevich, L. S., J. C. Clegg, and K. Sidibe.** 1993. Lassa virus activity in Guinea: distribution of human antiviral antibody defined using enzyme-linked immunosorbent assay with recombinant antigen. J. Med. Virol. **40**: 210–217.
24. **Macher, A. M., and M. S. Wolfe.** 2006. Historical Lassa fever reports and 30-year clinical update. Emerg. Infect. Dis. **12**:835–837.
25. **Mahdy, M. S., W. Chiang, B. McLaughlin, K. Derksen, B. H. Truxton, and K. Neg.** 1989. Lassa fever: the first confirmed case imported into Canada. Can. Dis. Wkly. Rep. **15**:193–198.
26. **Matsuura, Y., R. D. Possee, and D. H. Bishop.** 1986. Expression of the S-coded genes of lymphocytic choriomeningitis arenavirus using a baculovirus vector. J. Gen. Virol. **67**(Pt. 8):1515–1529.
27. **McCormick, J. B., P. A. Webb, J. W. Krebs, K. M. Johnson, and E. S. Smith.** 1987. A prospective study of the epidemiology and ecology of Lassa fever. J. Infect. Dis. **155**:437–444.
28. **Merryweather, A. T., U. Weyer, M. P. Harris, M. Hirst, T. Booth, and R. D. Possee.** 1990. Construction of genetically engineered baculovirus insecticides

containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. J. Gen. Virol. 71(Pt. 7):1535–1544.
29. **Monath, T. P.** 1975. Lassa fever: review of epidemiology and epizootiology. Bull. W. H. O. **52**:577–592.
30. **Monath, T. P., P. E. Mertens, R. Patton, C. R. Moser, J. J. Baum, L. Pinneo, G. W. Gary, and R. E. Kissling.** 1973. A hospital epidemic of Lassa fever in Zorzor, Liberia, March-April 1972. Am. J. Trop. Med. Hyg. **22**:773–779.
31. **Monson, M. H., J. D. Frame, P. B. Jahrling, and K. Alexander.** 1984. Endemic Lassa fever in Liberia. I. Clinical and epidemiological aspects at Curran Lutheran Hospital, Zorzor, Liberia. Trans. R. Soc. Trop. Med. Hyg. **78**:549–553.
32. **Niikura, M., T. Ikegami, M. Saijo, I. Kurane, M. E. Miranda, and S. Morikawa.** 2001. Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J. Clin. Microbiol. **39**:3267–3271.
33. **Niikura, M., T. Ikegami, M. Saijo, T. Kurata, I. Kurane, and S. Morikawa.** 2003. Analysis of linear B-cell epitopes of the nucleoprotein of Ebola virus that distinguish Ebola virus subtypes. Clin. Diagn. Lab. Immunol. **10**:83–87.
34. **Peters, C. J.** 2002. Clinical virology, p. 949–969. *In* D. D. Richman, R. J. Whitley, and F. G. Hayden (ed.), Arenaviruses, 2nd ed. ASM Press, Washington, DC.
35. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300**:1394–1399.
36. **Saijo, M., M. Niikura, T. Ikegami, I. Kurane, T. Kurata, and S. Morikawa.** 2006. Laboratory diagnostic systems for Ebola and Marburg hemorrhagic fevers developed with recombinant proteins. Clin. Vaccine Immunol. **13**: 444–451.
37. **Saijo, M., M. Niikura, A. Maeda, T. Sata, T. Kurata, I. Kurane, and S. Morikawa.** 2005. Characterization of monoclonal antibodies to Marburg virus nucleoprotein (NP) that can be used for NP-capture enzyme-linked immunosorbent assay. J. Med. Virol. **76**:111–118.
38. **Saijo, M., M. Niikura, S. Morikawa, T. G. Ksiazek, R. F. Meyer, C. J. Peters, and I. Kurane.** 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J. Clin. Microbiol. **39**:1–7.
39. **Saijo, M., T. Qing, M. Niikura, A. Maeda, T. Ikegami, C. Prehaud, I. Kurane, and S. Morikawa.** 2002. Recombinant nucleoprotein-based enzyme-linked immunosorbent assay for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J. Clin. Microbiol. **40**:1587–1591.
40. **Saijo, M., T. Qing, M. Niikura, A. Maeda, T. Ikegami, K. Sakai, C. Prehaud, I. Kurane, and S. Morikawa.** 2002. Immunofluorescence technique using HeLa cells expressing recombinant nucleoprotein for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J. Clin. Microbiol. **40**:372–375.
41. **Saijo, M., Q. Tang, B. Shimayi, L. Han, Y. Zhang, M. Asiguma, D. Tianshu, A. Maeda, I. Kurane, and S. Morikawa.** 2005. Antigen-capture enzyme-linked immunosorbent assay for the diagnosis of Crimean-Congo hemorrhagic fever using a novel monoclonal antibody. J. Med. Virol. **77**:83–88.
42. **Saijo, M., Q. Tang, B. Shimayi, L. Han, Y. Zhang, M. Asiguma, D. Tianshu, A. Maeda, I. Kurane, and S. Morikawa.** 2005. Recombinant nucleoprotein-based serological diagnosis of Crimean-Congo hemorrhagic fever virus infections. J. Med. Virol. **75**:295–299.
43. **Tang, Q., M. Saijo, Y. Zhang, M. Asiguma, D. Tianshu, L. Han, B. Shimayi, A. Maeda, I. Kurane, and S. Morikawa.** 2003. A patient with Crimean-Congo hemorrhagic fever serologically diagnosed by recombinant nucleoprotein-based antibody detection systems. Clin. Diagn. Lab. Immunol. **10**:489–491.
44. **Ter Meulen, J., K. Koulemou, T. Wittekindt, K. Windisch, S. Strigl, S. Conde, and H. Schmitz.** 1998. Detection of Lassa virus antinucleoprotein immunoglobulin G (IgG) and IgM antibodies by a simple recombinant immunoblot assay for field use. J. Clin. Microbiol. **36**:3143–3148.
45. **Tran, T. H., T. L. Nguyen, T. D. Nguyen, T. S. Luong, P. M. Pham, V. C. Nguyen, T. S. Pham, C. D. Vo, T. Q. Le, T. T. Ngo, B. K. Dao, P. P. Le, T. T. Nguyen, T. L. Hoang, V. T. Cao, T. G. Le, D. T. Nguyen, H. N. Le, K. T. Nguyen, H. S. Le, V. T. Le, D. Christiane, T. T. Tran, J. Menno de, C. Schultsz, P. Cheng, W. Lim, P. Horby, and J. Farrar.** 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. N. Engl. J. Med. **350**:1179–1188.
46. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351**:467–471.

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Increased permeability of human endothelial cell line EA.hy926 induced by hantavirus-specific cytotoxic T lymphocytes

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Abstract

Hantavirus infection causes two human diseases, hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. The typical feature of these diseases is increased permeability in microvascular beds in the kidneys and the lungs, respectively. The mechanism of capillary leakage, however, is not understood. Some evidence suggests that hantavirus disease pathogenesis is immunologically mediated by cytotoxic T lymphocytes and other immune cells in target organs producing inflammatory cytokines. In this study we examined the roles of virus-specific cytotoxic T lymphocytes in increased permeability of human endothelial cells infected with hantavirus. We used a human $CD8^+$ hantavirus-specific cytotoxic T lymphocyte line, 1A-E2, specific for the HLA-A24-restricted epitope in Sin Nombre and Puumala virus G2 protein, and the human endothelial cell line, EA.hy926 that expresses HLA-A24 molecule. The cytotoxic T lymphocyte line recognized and lysed target cells infected with Sin Nombre virus, and in transwell permeability assays increased permeability of EA.hy926 cell monolayer infected with Sin Nombre virus or recombinant adenovirus expressing the Sin Nombre virus G2 protein. These results suggest that cytotoxic T lymphocyte activity contribute to capillary leakage observed in patients with hantavirus pulmonary syndrome or hemorrhagic fever with renal syndrome.
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Keywords: Sin Nombre virus; Hantavirus pulmonary syndrome; Hemorrhagic fever with renal syndrome; Cytotoxic T lymphocyte; Endothelial cell; Transwell permeability assay

1. Introduction

Hantaviruses, belonging to the family Bunyaviridae, are distributed worldwide. Two forms of zoonotic human diseases are caused by hantavirus species (Enria et al., 2001; Linderholm and Elgh, 2001; Schmaljohn and Hjelle, 1997). Hemorrhagic fever with renal syndrome (HFRS) and its mild form nephropathia epidemica (NE) are caused by Old World (Europe and Asia) hantaviruses, such as Hantaan, Seoul, Dobrava and Puumala viruses. Hantavirus pulmonary syndrome (HPS) is caused by New World (North and South America) hantaviruses, such as Sin Nombre and Andes viruses (Khaiboullina and St Jeor, 2002; Zeier et al., 2005). The hantavirus genome consists of three RNA segments, large, medium and small segments, encoding RNA-dependent RNA polymerase, envelope glycoproteins (G1 and G2), and nucleocapsid (N) protein, respectively (Jonsson and Schmaljohn, 2001). Hantaviruses persistently infect their natural rodent reservoirs without apparent diseases (Meyer and Schmaljohn, 2000). Humans are infected with hantaviruses by direct contact with infected rodents or through the inhalation of excreted viral aerosols (Linderholm and Elgh, 2001).

Human hantavirus diseases are characterized by an increased permeability in microvascular beds of the kidneys in HFRS and the lungs in HPS, and endothelial cells are considered to be the primary targets of hantavirus infection (Hautala et al., 2002; Khaiboullina and St Jeor, 2002; Mustonen et al., 1994; Temonen et al., 1996; Zaki et al., 1995). In vitro hantavirus infection alone, however, did not induce visible cytopathic effects in cultured human endothelial cells (Geimonen et al., 2002; Niikura et al., 2004; Pensiero et al., 1992) nor did it increase capillary permeability of an infected endothelial cell monolayer (Khaiboullina et al., 2000; Niikura et al., 2004; Sundstrom et al., 2001). Increased levels of cytokines including

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tumor necrosis factor (TNF) -α, interleukin (IL) -2, IL-6 and interferon (IFN) -γ have been detected in sera of HFRS and HPS patients (Khaiboullina and St Jeor, 2002; Vapalahti et al., 2001). An expanded leukocyte population including monocytes, T and B cells and an increase of $CD8^+/CD4^+$ ratio have been observed in HFRS patients (Chen and Yang, 1990; Huang et al., 1994; Lewis et al., 1991; Markotic et al., 1999). We found significantly higher frequencies of Sin Nombre virus (SNV)-specific T cells in patients with severe HPS requiring mechanical ventilation (up to 44.2% of $CD8^+$ T cells) than in moderately ill HPS patients hospitalized but not requiring mechanical ventilation (up to 9.8% of $CD8^+$ T cells). These results suggest that virus-specific $CD8^+$ T cells contribute to HPS disease outcome (Kilpatrick et al., 2004). Increased numbers of $CD8^+$ T cells are found in the kidneys of HFRS (Temonen et al., 1996) and in the lungs of HPS patients (Zaki et al., 1995). There is an abundance of immune cells expressing a variety of cytokines in the lungs of HPS cases (Mori et al., 1999; Zaki et al., 1995) and in the kidneys of NE cases (Temonen et al., 1996). In addition, preliminary evidence suggests that HLA-B*3501 is associated with severe HPS in SNV infection, implying involvement of $CD8^+$ cytotoxic T lymphocytes (CTLs) (Kilpatrick et al., 2004) (Koster et al., 2001). A similar linkage between disease severity and MHC haplotype was observed between Puumala virus infection and the HLA-B8-DR3 extended haplotype (severe outcome) or the HLA-B27 (milder disease) (Makela et al., 2002; Mustonen et al., 1998). These reports suggest that host immune responses against hantavirus, especially virus-specific CTLs and inflammatory cytokines produced by virus-specific T cells may contribute to disease pathogenesis of HPS and HFRS (Terajima et al., 2004).

We have established panels of CTL lines from the PBMC of NE and HPS patients (Ennis et al., 1997; Kilpatrick et al., 2004; Terajima et al., 2002, 2004; Van Epps et al., 1999, 2002). These cell lines were able to recognize and lyse autologous B lymphoblastoid cell lines pulsed with the epitope peptide or infected with recombinant vaccinia viruses expressing the hantaviral protein containing the epitope. In this report, we tested one of these CTL lines against human endothelial cell line, EA.hy926, infected with SNV in vitro. Primary human umbilical vein endothelial cells (HUVECs) or human lung microvascular endothelial cells (HMVEC-Ls) have been used to examine the effect of hantavirus infection on endothelial cell function (Gavrilovskaya et al., 2002; Khaiboullina et al., 2000; Niikura et al., 2004; Sundstrom et al., 2001). Although ideally the interaction between hantavirus-specific CTL and hantavirus-infected endothelial cells should also be analyzed using HUVECs or HMVEC-Ls, it is difficult to obtain endothelial cells that express the MHC class I molecules by which our CTL lines were restricted. Therefore, we used the immortalized human endothelial cell line EA.hy926 (Edgell et al., 1983), which we found to express the HLA-A24 allele (in this report), and one of our hantavirus-specific CTL line, 1A-E2, which was established from convalescent PBMC from the patient infected with Puumala virus and was restricted by HLA-A24 (Terajima et al., 2002). 1A-E2 recognized the epitope, HWMDATFNL, encoded by Puumala virus G2 protein, and was cross-reactive to the corresponding peptide, HWMDGTFNI, in SNV G2 protein (Terajima et al., 2002). EA.hy926 cells are the most similar to HUVEC among the available immortalized human endothelial cell lines and have been used to study the endothelial cell/leukocyte interactions (Lidington et al., 1999).

In this study, we first tested the infectivity of SNV to EA.hy926 cells. Next, we showed that the hantavirus-specific $CD8^+$ T cell line 1A-E2 could recognize and lyse EA.hy926 cells infected with SNV or presenting SNV antigen. Finally, we demonstrated that 1A-E2 enhanced the permeability of EA.hy926 cells infected with SNV. These results suggest that virus-specific CTLs contribute to the capillary leakage.

2. Materials and methods

2.1. Virus and cell lines

SNV stock virus (strain CC107, kindly provided by Connie S. Schmaljohn) (Schmaljohn et al., 1995) was propagated in Vero E6 cells and aliquots were stored in −80 °C. All experiments using cultured live SNV were performed in biosafety level 3 laboratory of University of Massachusetts Medical School according to standard BSL3 guidelines. The EA.hy926 cell line, which had been derived by fusing HUVECs with the permanent human cell line A549, were kindly provided by Cora-Jean S. Edgell, University of North Carolina (Edgell et al., 1983). Vero E6 cells (ATCC CRL-1586) and EA.hy926 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) containing 2% fetal bovine serum (FBS). CTL line, 1A-E2, was established from convalescent PBMC of the patient infected with Puumala virus (Terajima et al., 2002). 1A-E2 was maintained in RPMI (Invitrogen Corporation) containing 10% FCS.

2.2. HLA-typing of EA.hy926 cells

HLA-type of EA.hy926 cell line was determined by RT-PCR and nucleotide sequencing as described previously (Co et al., 2002). Total RNA was extracted from EA.hy926 cells. cDNA was synthesized. HLA-A, B and C genes were amplified using common 5′ primer, HLA-5P2 containing *SalI* restriction enzyme site, and HLA-A, B and C-specific 3′ primers, HLA-3PA, HLA-3PB, and HLA-3PC containing *HindIII* restriction enzyme site, respectively (Zemmour et al., 1992). These PCR products were cloned into the pBluescript II SK(+) vector. Six plasmid clones from each of the HLA alleles were purified, and the nucleotide sequences were determined. The PCR products were also directly sequenced (Ennis et al., 1990). The sequencing reactions were performed by the Nucleic Acid Facility of the University of Massachusetts Medical School. These sequencing results showed that the EA.hy926 cell line expressed HLA-A*2402, A*2501, B*1501, B*1801, Cw*0303, and Cw*1203.

2.3. Virus titration

Titers of SNV were determined in a focus-forming assay with some modifications (Takashima et al., 1997). Briefly, confluent Vero E6 cells in 96-well plates were inoculated with serially

diluted culture supernatants in 2% FCS DMEM. After 90 min of incubation, virus inocula were removed and cells were washed with PBS. Infected cells were incubated in 2% FCS DMEM containing 1.5% carboxymethylcellulose (Sigma, St. Louis, MO) for 7 days. Virus foci were detected using a rabbit anti-SNV serum (kindly provided by Patrick C. Stockton and Thomas G. Ksiazek) (Zaki et al., 1995) and using a peroxidase-anti-peroxidase (PAP) staining technique (Polysciences, Warrington, PA) combined with DAB Substrate Kit for peroxidase (Vector Laboratories, Burlingame, CA). Foci were counted under a dissecting microscope. Virus titers were calculated as average number of foci in triplicate wells.

2.4. Virus growth curve and Western blotting

Confluent Vero E6 cells and EA.hy926 cells were infected with SNV at multiplicity of infection (m.o.i) of 0.0025 in 6-well plates. At 0, 1, 4, 8 and 12 days post-infection, supernatants and cells were harvested separately, and stored at −80 °C until use. Virus titers in supernatants were determined by the focus-forming assay described above. Cell lysates were separated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes as previously described (Maeda et al., 2005). SNV proteins were detected by rabbit anti-SNV serum (Zaki et al., 1995) and visualized by Opti-4CN Detection kit (Bio-Rad Laboratories, Hercules, CA).

2.5. Trypan blue exclusion test

Confluent Vero E6 cells and EA.hy926 cells in 96-well plates were infected with SNV at an m.o.i of 0.01 or 0.001 in triplicates. At 1, 3, 6 and 9 days post-infection, both adherent and detached cells were harvested, and resuspended in 0.2% Trypan blue solution. Stained and unstained cells were counted under microscopy.

2.6. Measurement of lactate dehydrogenase (LDH) activity in the supernatants of SNV infected cells

Confluent Vero E6 cells and EA.hy926 cells in 96-well plates were infected with SNV or recombinant adenovirus containing the cDNA encoding the SNV G2 protein. At 3, 6 and 9 days post-infection, supernatants were collected and LDH activity released from the cytosol of damaged cells into the culture supernatant was measured in triplicates by Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol. Relative LDH activity in the supernatants was calculated as follows.

$$\text{relative LDH activity (\%)} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

High control in the assay was uninfected cells treated with 2% Triton X-100 solution for 10 min. Low control was uninfected cells incubated with culture medium alone.

2.7. CTL assay

Confluent EA.hy926 cells were infected with SNV at m.o.i of 0.01 in 96-well flat-bottom plates. At 3 days post-infection, medium was removed and cells were washed with PBS, then 1A-E2 cells were added as effector/target (*E/T*) ratio of 1 and incubated for 4 or 16 h. Supernatants were harvested and CTL activity was measured by Cytotoxicity Detection Kit (LDH) (Roche Applied Science) described in the previous subsection (2.6). In the CTL assays low control was infected cells incubated without effector cells (1A-E2). 10% specific lysis above background was considered positive.

2.8. Transwell permeability assay

The assay was performed following the previously published method with some modification (Shaw et al., 2001). EA.hy926 cells were cultured in 24-well transwell inserts (Polycarbonate membrane Transwell® Inserts, Corning Incorporated, Acton, MA). Confluent EA.hy926 cell monolayers were infected with SNV at m.o.i. of 0.01 in the upper chambers. After 90 min of incubation, virus inocula were removed and washed with PBS. Infected monolayers were incubated in 2% FCS DMEM. Three days after infection, 1A-E2 cells were added at *E/T* ratio of 1 (for wells without CTL, only media were changed), and cells were incubated for 16 h. Then, media in the upper chambers were replaced with fresh media containing 500 μg/ml of f-dextran (dextran, fluorescein, 70,000 MW, anionic, Molecular Probes, Eugene, OR). The media in the lower wells were taken at 0, 15 and 30 min and the absorbances at 485 nm wavelength were measured. Since we observed an increase in the permeability in negative control wells ("media" in Fig. 5) after 30 min of incubation in the first set of the assays, we used earlier time points (0, 10 and 20 min) in the next set of the assays. The concentrations of f-dextran were calculated from a standard curve.

2.9. Generation of recombinant adenoviruses expressing the SNV G2 protein

A recombinant adenovirus which expresses the SNV G2 protein was constructed as we reported previously (Maeda et al., 2005). An Adenovirus Expression Vector Kit (Takara Shuzo, Kyoto, Japan) was used. The full-length SNV G2 protein cDNA was excised from the plasmid, pGEM-G2 (kind gift from Christina Spiropoulou of the CDC), with restriction enzyme *BamHI* and *EcoRI*, blunt-ended with Klenow fragment, and inserted into the cosmid vector, pAxCAwt, at its *SwaI* site. The cosmid carrying the SNV G2 cDNA under the control of the strong CAG promoter, pAxCAwt-SNV-N, was packaged using GigapackR III XL packaging extract (Stratagene, La Jolla, CA). 293 cells (ATCC number CRL-1573) were transfected with pAxCAwt-SNV-G2 and restriction enzyme-digested DNA-TPC using Calcium Phosphate Transfection Kit (Invitrogen). Recombinant adenovirus, rAd-SNV-G2, was cloned by limiting dilution and grown in 293 cells for preparation of high-titer virus stock solutions. Viral titers were determined by the 50% tissue culture infectious dose ($TCID_{50}$) method in 293 cells.

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.

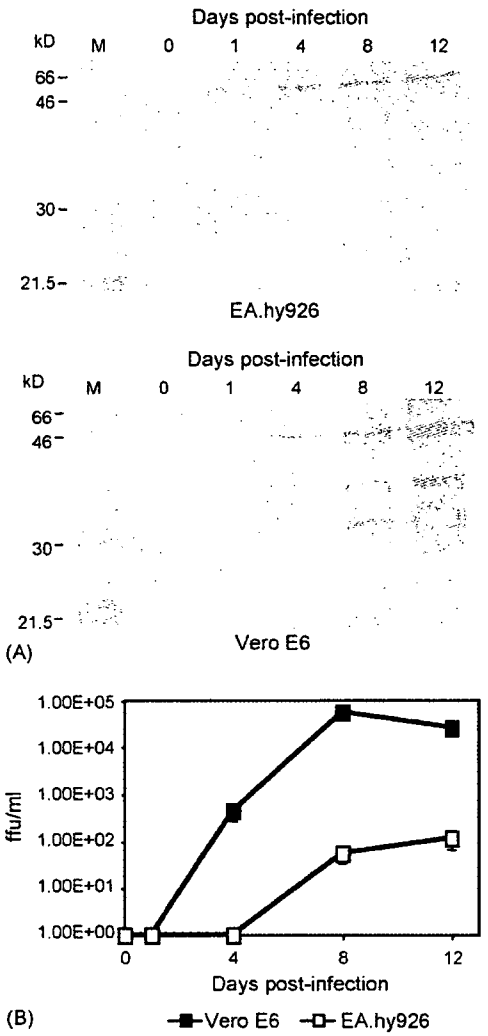

Fig. 1. SNV infection in EA.hy926 and Vero E6 cells. (A) Detection of SNV protein in EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) infected with SNV by western blotting. Cell lysates were harvested at 0, 1, 4, 8 and 12 days post-infection and reacted with rabbit anti-SNV serum. M: molecular 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.

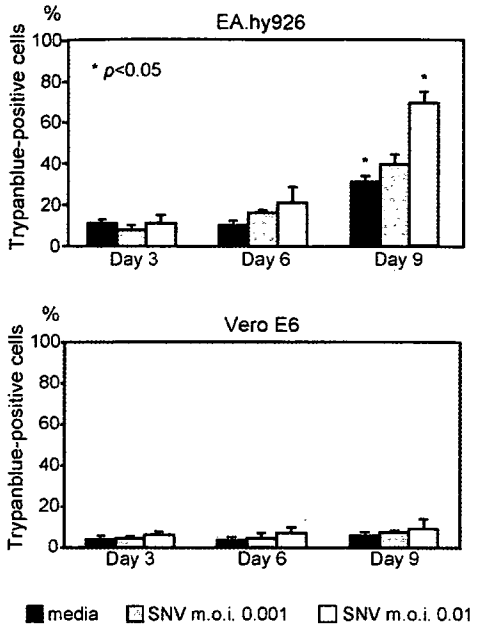

Fig. 2. The percentages of Trypan blue-positive EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) uninfected (black bars), infected with SNV at m.o.i. of 0.001 (gray bars) or m.o.i. of 0.01 (open bars). Cells harvested at 3, 6 and 9 days post-infection were stained with 0.2% Trypan blue solution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

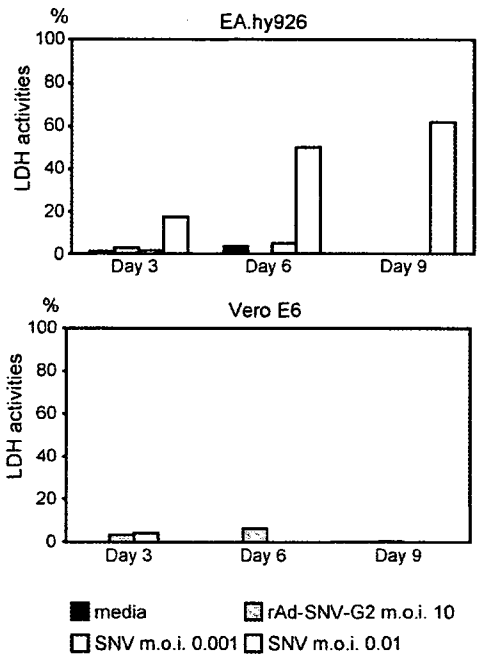

Fig. 3. LDH activity in the supernatants of EA.hy926 cells (upper panel) and Vero E6 cells (lower panel) uninfected (black bars) or infected with rAd-SNV-G2 at m.o.i. of 10 (dark gray bars), SNV at m.o.i. of 0.001 (light gray bars) or m.o.i. of 0.01 (open bars). The LDH activities were shown as the percent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

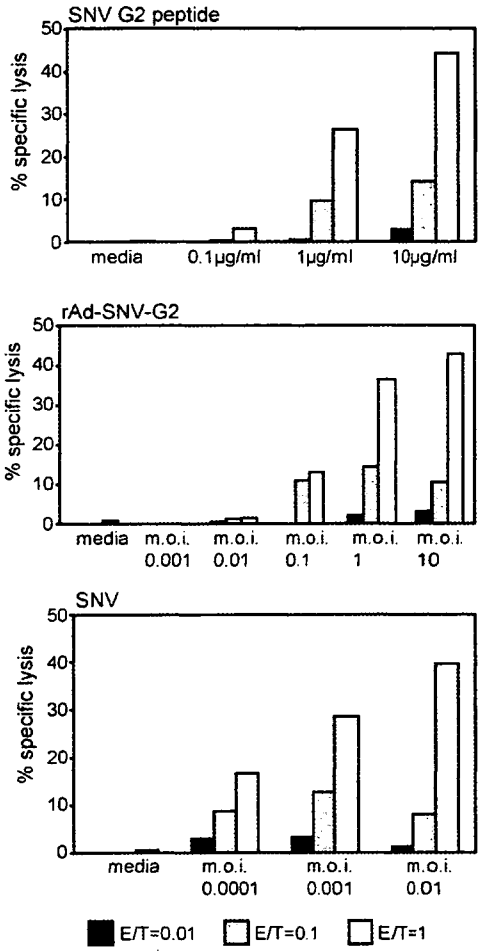

Fig. 4. CTL activity by the 1A-E2 CTL line against EA.hy926 cells pulsed with the SNV G2 peptides, HAEIQNLGHWMDGTFNIKTA (upper panel), infected with rAd-SNV-G2 (middle panel), or SNV (bottom panel). Effector 1A-E2 cells were added to target EA.hy926 cells at *E/T* ratio of 0.01 (black bars), 0.1 (gray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

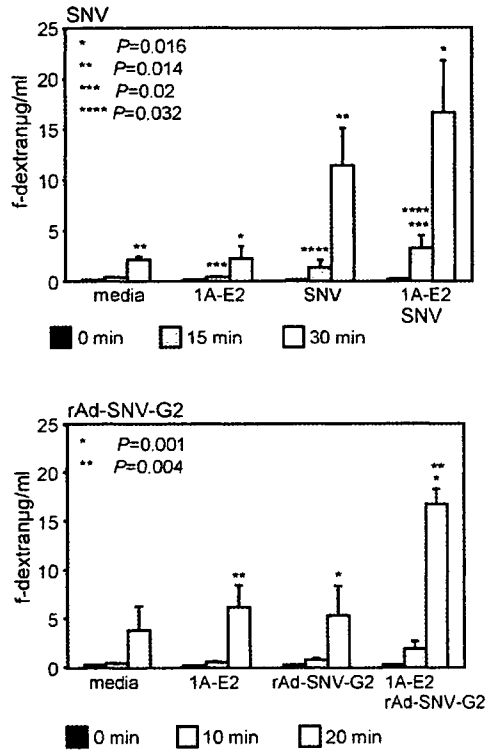

Fig. 5. Permeability changes in EA.hy926 cells infected with SNV (upper panel) or rAd-SNV-G2 (lower panel), with or without the addition of the 1A-E2 CTL line. 500 μg/ml of f-dextran was added to the upper chamber and medium harvested from lower wells. Concentrations of the f-dextran at 0 min (black 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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References

Chen, L.B., Yang, W.S., 1990. Abnormalities of T cell immunoregulation in hemorrhagic fever with renal syndrome. J. Infect. Dis. 161 (5), 1016–1019.

Co, M.D., Terajima, M., Cruz, J., Ennis, F.A., Rothman, A.L., 2002. Human cytotoxic T lymphocyte responses to live attenuated 17D yellow fever vaccine: identification of HLA-B35-restricted CTL epitopes on nonstructural proteins NS1, NS2b, NS3, and the structural protein E. Virology 293 (1), 151–163.

Edgell, C.J., McDonald, C.C., Graham, J.B., 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridisation. Proc. Natl. Acad. Sci. U.S.A. 80 (12), 3734–3737.

Ennis, F.A., Cruz, J., Spiropoulou, C.F., Waite, D., Peters, C.J., Nichol, S.T., Kariwa, H., Koster, F.T., 1997. Hantavirus pulmonary syndrome: CD8+ and CD4+ cytotoxic T lymphocytes to epitopes on Sin Nombre virus nucleocapsid protein isolated during acute illness. Virology 238 (2), 380–390.

Ennis, P.D., Zemmour, J., Salter, R.D., Parham, P., 1990. Rapid cloning of HLA-A, B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. Proc. Natl. Acad. Sci. U.S.A. 87 (7), 2833–2837.

Enria, D.A., Briggiler, A.M., Pini, N., Levis, S., 2001. Clinical manifestations of New World hantaviruses. Curr. Top. Microbiol. Immunol. 256, 117–134.

Enserink, M., 2001. Virology. Finally, a handle on the hantaviruses. Science 293 (5534), 1414–1415.

Gavrilovskaya, I.N., Peresleni, T., Geimonen, E., Mackow, E.R., 2002. Pathogenic hantaviruses selectively inhibit beta3 integrin directed endothelial cell migration. Arch. Virol. 147 (10), 1913–1931.

Geimonen, E., Neff, S., Raymond, T., Kocer, S.S., Gavrilovskaya, I.N., Mackow, E.R., 2002. Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses. Proc. Natl. Acad. Sci. U.S.A. 99 (21), 13837–13842.

Hautala, T., Sironen, T., Vapalahti, O., Paakko, E., Sarkioja, T., Salmela, P.I., Vaheri, A., Plyusnin, A., Kauma, H., 2002. Hypophyseal hemorrhage and panhypopituitarism during Puumala Virus Infection: Magnetic resonance imaging and detection of viral antigen in the hypophysis. Clin. Infect. Dis. 35 (1), 96–101.

Hooper, J.W., Kamrud, K.I., Elgh, F., Custer, D., Schmaljohn, C.S., 1999. DNA vaccination with hantavirus M segment elicits neutralizing antibodies and protects against seoul virus infection. Virology 255 (2), 269–278.

Hooper, J.W., Larsen, T., Custer, D.M., Schmaljohn, C.S., 2001. A lethal disease model for hantavirus pulmonary syndrome. Virology 289 (1), 6–14.

Huang, C., Jin, B., Wang, M., Li, E., Sun, C., 1994. Hemorrhagic fever with renal syndrome: relationship between pathogenesis and cellular immunity. J. Infect. Dis. 169 (4), 868–870.

Jonsson, C.B., Schmaljohn, C.S., 2001. Replication of hantaviruses. Curr. Top. Microbiol. Immunol. 256, 15–32.

Kang, J.I., Park, S.H., Lee, P.W., Ahn, B.Y., 1999. Apoptosis is induced by hantaviruses in cultured cells. Virology 264 (1), 99–105.

Koster, F., Vigil, J., Olson, D., Terajima, M., Ennis, F., Moehenkamp, C., Williams, T., 2001. Class I, II and III HLA alleles associated with severe hantavirus cardiopulmonary syndrome in the southwest US. Am. J. Trop. Med. 65 (3), 152 (meeting abstract).

Khaiboullina, S.F., Netski, D.M., Krumpe, P., St Jeor, S.C., 2000. Effects of tumor necrosis factor alpha on Sin Nombre virus infection in vitro. J. Virol. 74 (24), 11966–11971.

Khaiboullina, S.F., St Jeor, S.C., 2002. Hantavirus immunology. Viral Immunol. 15 (4), 609–625.

Kilpatrick, E.D., Terajima, M., Koster, F.T., Catalina, M.D., Cruz, J., Ennis, F.A., 2004. Role of specific CD8+ T cells in the severity of a fulminant zoonotic viral hemorrhagic fever, hantavirus pulmonary syndrome. J. Immunol. 172 (5), 3297–3304.

Lewis, R.M., Lee, H.W., See, A.F., Parrish, D.B., Moon, J.S., Kim, D.J., Cosgriff, T.M., 1991. Changes in populations of immune effector cells during the course of haemorrhagic fever with renal syndrome. Trans. R. Soc. Trop. Med. Hyg. 85 (2), 282–286.

Li, X.D., Kukkonen, S., Vapalahti, O., Plyusnin, A., Lankinen, H., Vaheri, A., 2004. Tula hantavirus infection of Vero E6 cells induces apoptosis involving caspase 8 activation. J. Gen. Virol. 85 (Pt. 11), 3261–3268.

Li, X.D., Makela, T.P., Guo, D., Soliymani, R., Koistinen, V., Vapalahti, O., Vaheri, A., Lankinen, H., 2002. Hantavirus nucleocapsid protein interacts with the Fas-mediated apoptosis enhancer Daxx. J. Gen. Virol. 83 (Pt. 4), 759–766.

Lidington, E.A., Moyes, D.L., McCormack, A.M., Rose, M.L., 1999. A comparison of primary endothelial cells and endothelial cell lines for studies of immune interactions. Transpl. Immunol. 7 (4), 239–246.

Linderholm, M., Elgh, F., 2001. Clinical characteristics of hantavirus infections on the Eurasian continent. Curr. Top. Microbiol. Immunol. 256, 135–151.

Maeda, K., West, K., Hayasaka, D., Ennis, F.A., Terajima, M., 2005. Recombinant adenovirus vector vaccine induces stronger cytotoxic T-cell responses than recombinant vaccinia virus vector, plasmid DNA, or a combination of these. Viral Immunol. 18 (4), 657–667.

Makela, S., Mustonen, J., Ala-Houhala, I., Hurme, M., Partanen, J., Vapalahti, O., Vaheri, A., Pasternack, A., 2002. Human leukocyte antigen-B8-DR3 is a more important risk factor for severe Puumala hantavirus infection than the tumor necrosis factor-alpha(-308) G/A polymorphism. J. Infect. Dis. 186 (6), 843–846.

Markotic, A., Dasic, G., Gagro, A., Sabioncello, A., Rabatic, S., Kuzman, I., Zgorelec, R., Smoljan, I., Beus, I., Zupanc, T.A., Dekaris, D., 1999. Role of peripheral blood mononuclear cell (PBMC) phenotype changes in the pathogenesis of haemorrhagic fever with renal syndrome (HFRS). Clin. Exp. Immunol. 115 (2), 329–334.

Markotic, A., Hensley, L., Geisbert, T., Spik, K., Schmaljohn, C., 2003. Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells. J. Gen. Virol. 84 (Pt. 8), 2197–2202.

Meyer, B.J., Schmaljohn, C.S., 2000. Persistent hantavirus infections: characteristics and mechanisms. Trends Microbiol. 8 (2), 61–67.

Milazzo, M.L., Eyzaguirre, E.J., Molina, C.P., Fulhorst, C.F., 2002. Maporal viral infection in the Syrian golden hamster: a model of hantavirus pulmonary syndrome. J. Infect. Dis. 186 (10), 1390–1395.

Mori, M., Rothman, A.L., Kurane, I., Montoya, J.M., Nolte, K.B., Norman, J.E., Waite, D.C., Koster, F.T., Ennis, F.A., 1999. High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. J. Infect. Dis. 179 (2), 295–302.

Mustonen, J., Helin, H., Pietila, K., Brummer-Korvenkontio, M., Hedman, K., Vaheri, A., Pasternack, A., 1994. Renal biopsy findings and clinicopathologic correlations in nephropathia epidemica. Clin. Nephrol. 41 (3), 121–126.

Mustonen, J., Partanen, J., Kanerva, M., Pietila, K., Vapalahti, O., Pasternack, A., Vaheri, A., 1998. Association of HLA B27 with benign clinical course of nephropathia epidemica caused by Puumala hantavirus. Scand. J. Immunol. 47 (3), 277–279.

Niikura, M., Maeda, A., Ikegami, T., Saijo, M., Kurane, I., Morikawa, S., 2004. Modification of endothelial cell functions by Hantaan virus infection: prolonged hyper-permeability induced by TNF-alpha of hantaan virus-infected endothelial cell monolayers. Arch. Virol. 149 (7), 1279–1292.

Nolte, K.B., Feddersen, R.M., Foucar, K., Zaki, S.R., Koster, F.T., Madar, D., Merlin, T.L., McFeeley, P.J., Umland, E.T., Zumwalt, R.E., 1995. Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. Hum. Pathol. 26 (1), 110–120.

Obert, G., Peiffer, I., Servin, A.L., 2000. Rotavirus-induced structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers. J. Virol. 74 (10), 4645–4651.

Pensiero, M.N., Sharefkin, J.B., Dieffenbach, C.W., Hay, J., 1992. Hantaan virus infection of human endothelial cells. J. Virol. 66 (10), 5929–5936.

Peters, C.J., Zaki, S.R., 2002. Role of the endothelium in viral hemorrhagic fevers. Crit. Care Med. 30 (Suppl. 5), S268–S273.

Schmaljohn, A.L., Li, D., Negley, D.L., Bressler, D.S., Turell, M.J., Korch, G.W., Ascher, M.S., Schmaljohn, C.S., 1995. Isolation and initial characterization of a newfound hantavirus from California. Virology 206 (2), 963–972.

Schmaljohn, C., Hjelle, B., 1997. Hantaviruses: a global disease problem. Emerg. Infect. Dis. 3 (2), 95–104.

Shaw, S.K., Bamba, P.S., Perkins, B.N., Luscinskas, F.W., 2001. Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. J. Immunol. 167 (4), 2323–2330.

Sundstrom, J.B., McMullan, L.K., Spiropoulou, C.F., Hooper, W.C., Ansari, A.A., Peters, C.J., Rollin, P.E., 2001. Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. J. Virol. 75 (13), 6070–6085.

Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J., Hashimoto, N., 1997. A case of tick-borne encephalitis in Japan and isolation of the virus. J. Clin. Microbiol. 35 (8), 1943–1947.

Temonen, M., Mustonen, J., Helin, H., Pasternack, A., Vaheri, A., Holthofer, H., 1996. Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. Clin. Immunol. Immunopathol. 78 (1), 47–55.

Terajima, M., Van Epps, H.L., Li, D., Leporati, A.M., Juhlin, S.E., Mustonen, J., Vaheri, A., Ennis, F.A., 2002. Generation of recombinant vaccinia viruses expressing Puumala virus proteins and use in isolating cytotoxic T cells specific for Puumala virus. Virus Res. 84 (1–2), 67–77.

Terajima, M., Vapalahti, O., Van Epps, H.L., Vaheri, A., Ennis, F.A., 2004. Immune responses to Puumala virus infection and the pathogenesis of nephropathia epidemica. Microbes Infect. 6 (2), 238–245.

Van Epps, H.L., Schmaljohn, C.S., Ennis, F.A., 1999. Human memory cytotoxic T-lymphocyte (CTL) responses to Hantaan virus infection: identification of virus-specific and cross-reactive CD8(+) CTL epitopes on nucleocapsid protein. J. Virol. 73 (7), 5301–5308.

Van Epps, H.L., Terajima, M., Mustonen, J., Arstila, T.P., Corey, E.A., Vaheri, A., Ennis, F.A., 2002. Long-lived memory T lymphocyte responses after hantavirus infection. J. Exp. Med. 196 (5), 579–588.

Vapalahti, O., Lundkvist, A., Vaheri, A., 2001. Human immune response, host genetics, and severity of disease. Curr. Top. Microbiol. Immunol. 256, 153–169.

Zaki, S.R., Greer, P.W., Coffield, L.M., Goldsmith, C.S., Nolte, K.B., Foucar, K., Feddersen, R.M., Zumwalt, R.E., Miller, G.L., Khan, A.S., et al., 1995. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am. J. Pathol. 146 (3), 552–579.

Zeier, M., Handermann, M., Bahr, U., Rensch, B., Muller, S., Kehm, R., Muranyi, W., Darai, G., 2005. New ecological aspects of hantavirus infection: a change of a paradigm and a challenge of prevention—a review. Virus Genes 30 (2), 157–180.

Zemmour, J., Little, A.M., Schendel, D.J., Parham, P., 1992. The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. J. Immunol. 148 (6), 1941–1948.

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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.

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.

References

[1] Peters CJ. Viral hemorrhagic fevers. In: Nathanson N, editor. Viral pathogenesis. Philadelphia: Lippincott-Raven; 1997. p. 779–99.

[2] Peters CJ, Zaki SR. Role of the endothelium in viral hemorrhagic fevers. Crit Care Med 2002;30:S268–73.

[3] Schnittler HJ, Mahner F, Drenckhahn D, Klenk HD, Feldmann H. Replication of Marburg virus in human endothelial cells. A possible mechanism for the development of viral hemorrhagic disease. J Clin Invest 1993;91:1301–9.

[4] Yanagihara R, Silverman DJ. Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses. Arch Virol 1990;111:281–6.

[5] Pensiero MN, Sharefkin JB, Dieffenbach CW, Hay J. Hantaan virus infection of human endothelial cells. J Virol 1992;66:5929–36.

[6] Khaiboullina SF, Netski DM, Krumpe P, St Jeor SC. Effects of tumor necrosis factor alpha on Sin Nombre virus infection in vitro. J Virol 2000;74:11966–71.

[7] Sundstrom JB, McMullan LK, Spiropoulou CF, Hooper WC, Ansari AA, Peters CJ, et al. Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. J Virol 2001;75:6070–85.

[8] Schmaljohn CS, Hooper JW. Bunyaviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 1581–602.

[9] Nichol ST. Bunyaviruses. In: Knipe DM, Howley PM, editors. Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 1603–33.

[10] Plyusnin A. Genetics of hantaviruses: implications to taxonomy. Arch Virol 2002;147:665–82.

[11] Schmaljohn C, Hjelle B. Hantaviruses: a global disease problem. Emerg Infect Dis 1997;3:95–104.

[12] Peters CJ, Simpson GL, Levy H. Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Annu Rev Med 1999;50:531–45.

[13] Khan AS, Young JC. Hantavirus pulmonary syndrome: at the crossroads. Curr Opin Infect Dis 2001;14:205–9.

[14] Peters CJ, Khan AS. Hantavirus pulmonary syndrome: the new American hemorrhagic fever. Clin Infect Dis 2002;34:1224–31.

[15] Koster FT, Jenison SA. Hantaviruses. In: Gorbach SL, Bartlett JG, Blacklow NR, editors. Infectious diseases. Philadelphia: WB Saunders; 1997.

[16] Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, et al. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am J Pathol 1995;146:552–79.

[17] Nolte KB, Feddersen RM, Foucar K, Zaki SR, Koster FT, Madar D, et al. Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. Hum Pathol 1995;26:110–20.

[18] Kang JI, Park SH, Lee PW, Ahn BY. Apoptosis is induced by hantaviruses in cultured cells. Virology 1999;264:99–105.

[19] Li XD, Kukkonen S, Vapalahti O, Plyusnin A, Lankinen H, Vaheri A. Tula hantavirus infection of Vero E6 cells induces apoptosis involving caspase 8 activation. J Gen Virol 2004;85:3261–8.

[20] Li XD, Lankinen H, Putkuri N, Vapalahti O, Vaheri A. Tula hantavirus triggers pro-apoptotic signals of ER stress in Vero E6 cells. Virology 2005;333:180–9.

[21] Markotic A, Hensley L, Geisbert T, Spik K, Schmaljohn C. Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells. J Gen Virol 2003;84:2197–202.

[22] Gavrilovskaya IN, Shepley M, Shaw R, Ginsberg MH, Mackow ER. Beta3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure. Proc Natl Acad Sci USA 1998;95:7074–9.

[23] Gavrilovskaya IN, Brown EJ, Ginsberg MH, Mackow ER. Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. J Virol 1999;73:3951–9.

[24] Tsukada H, Ying X, Fu C, Ishikawa S, McKeown-Longo P, Albelda S, et al. Ligation of endothelial alpha v beta 3 integrin increases capillary hydraulic conductivity of rat lung. Circ Res 1995;77:651–9.

[25] Niikura M, Maeda A, Ikegami T, Saijo M, Kurane I, Morikawa S. Modification of endothelial cell functions by Hantaan virus infection: prolonged hyper-permeability induced by TNF-alpha of hantaan virus-infected endothelial cell monolayers. Arch Virol 2004;149:1279–92.

[26] Gavrilovskaya IN, Peresleni T, Geimonen E, Mackow ER. Pathogenic hantaviruses selectively inhibit beta3 integrin directed endothelial cell migration. Arch Virol 2002;147:1913–31.

[27] Ennis FA, Cruz J, Spiropoulou CF, Waite D, Peters CJ, Nichol ST, et al. Hantavirus pulmonary syndrome: CD8+ and CD4+ cytotoxic T lymphocytes to epitopes on Sin Nombre virus nucleocapsid protein isolated during acute illness. Virology 1997;238:380–90.

[28] Terajima M, Vapalahti O, Van Epps HL, Vaheri A, Ennis FA. Immune responses to Puumala virus infection and the pathogenesis of nephropathia epidemica. Microbes Infect 2004;6:238–45.

[29] Kilpatrick ED, Terajima M, Koster FT, Catalina MD, Cruz J, Ennis FA. Role of specific CD8+ T cells in the severity of a fulminant zoonotic viral hemorrhagic fever, hantavirus pulmonary syndrome. J Immunol 2004;172:3297–304.

[30] Khaiboullina SF, St Jeor SC. Hantavirus immunology. Viral Immunol 2002;15:609–25.

[31] Maes P, Clement J, Gavrilovskaya I, Van Ranst M. Hantaviruses: immunology, treatment, and prevention. Viral Immunol 2004;17:481–97.

[32] Borges AA, Campos GM, Moreli ML, Souza RL, Aquino VH, Saggioro FP, et al. Hantavirus cardiopulmonary syndrome: immune response and pathogenesis. Microbes Infect 2006;8:2324–30.

[33] Mori M, Rothman AL, Kurane I, Montoya JM, Nolte KB, Norman JE, et al. High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. J Infect Dis 1999;179:295–302.

[34] Koster F, Vigil J, Olson D, Terajima M, Ennis F, Moehenkamp C, et al. Class I, II and III HLA alleles associated with severe hantavirus cardiopulmonary syndrome in the southwest US (meeting abstract). Am J Trop Med 2001;65:152.

[35] Hayasaka D, Maeda K, Ennis FA, Terajima M. Increased permeability of human endothelial cell line EA.hy926 induced by hantavirus-specific cytotoxic T lymphocytes. Virus Res 2007;123:120–7.

[36] Mustonen J, Partanen J, Kanerva M, Pietila K, Vapalahti O, Pasternack A, et al. Genetic susceptibility to severe course of nephropathia epidemica caused by Puumala hantavirus. Kidney Int 1996;49:217–21.

[37] Makela S, Mustonen J, Ala-Houhala I, Hurme M, Partanen J, Vapalahti O, et al. Human leukocyte antigen-B8-DR3 is a more important risk factor for severe Puumala hantavirus infection than the tumor necrosis factor-alpha(-308) G/A polymorphism. J Infect Dis 2002;186:843–6.

[38] Mustonen J, Partanen J, Kanerva M, Pietila K, Vapalahti O, Pasternack A, et al. Association of HLA B27 with benign clinical course of nephropathia epidemica caused by Puumala hantavirus. Scand J Immunol 1998;47:277–9.

[39] Temonen M, Mustonen J, Helin H, Pasternack A, Vaheri A, Holthofer H. Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. Clin Immunol Immunopathol 1996;78:47–55.

[40] Linderholm M, Ahlm C, Settergren B, Waage A, Tarnvik A. Elevated plasma levels of tumor necrosis factor (TNF)-alpha, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients with hemorrhagic fever with renal syndrome. J Infect Dis 1996;173:38–43.
[41] Makela S, Mustonen J, Ala-Houhala I, Hurme M, Koivisto AM, Vaheri A, et al. Urinary excretion of interleukin-6 correlates with proteinuria in acute Puumala hantavirus-induced nephritis. Am J Kidney Dis 2004;43:809–16.
[42] Araki K, Yoshimatsu K, Lee BH, Kariwa H, Takashima I, Arikawa J. Hantavirus-specific CD8(+)-T-cell responses in newborn mice persistently infected with Hantaan virus. J Virol 2003;77:8408–17.
[43] Araki K, Yoshimatsu K, Lee BH, Kariwa H, Takashima I, Arikawa J. A new model of Hantaan virus persistence in mice: the balance between HTNV infection and CD8(+) T-cell responses. Virology 2004;322:318–27.
[44] Araki K, Yoshimatsu K, Lee BH, Okumura M, Kariwa H, Takashima I, et al. Age-dependent hantavirus-specific CD8(+) T-cell responses in mice infected with Hantaan virus. Arch Virol 2004;149:1373–82.
[45] Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731–8.
[46] Zhao MQ, Stoler MH, Liu AN, Wei B, Soguero C, Hahn YS, et al. Alveolar epithelial cell chemokine expression triggered by antigen-specific cytolytic CD8(+) T cell recognition. J Clin Invest 2000;106:R49–58.
[47] Xu L, Yoon H, Zhao MQ, Liu J, Ramana CV, Enelow RI. Cutting edge: pulmonary immunopathology mediated by antigen-specific expression of TNF-alpha by antiviral CD8+ T cells. J Immunol 2004;173:721–5.
[48] Rothermel AL, Wang Y, Schechner J, Mook-Kanamori B, Aird WC, Pober JS, et al. Endothelial cells present antigens in vivo. BMC Immunol 2004;5:5.
[49] Johnson DR. Endothelial cell-mediated antigen presentation. Transfus Med Hemother 2006;33:58–70.
[50] Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, et al. Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. Eur J Immunol 2003;33:3117–26.
[51] Mazanet MM, Hughes CC. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. J Immunol 2002;169:3581–8.
[52] Eppihimer MJ, Gunn J, Freeman GJ, Greenfield EA, Chernova T, Erickson J, et al. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. Microcirculation 2002;9:133–45.
[53] Alff PJ, Gavrilovskaya IN, Gorbunova E, Endriss K, Chong Y, Geimonen E, et al. The pathogenic NY-1 hantavirus G1 cytoplasmic tail inhibits RIG-I- and TBK-1-directed interferon responses. J Virol 2006;80:9676–86.
[54] Spiropoulou CF, Albarino CG, Ksiazek TG, Rollin PE. Andes and Prospect Hill hantaviruses differ in early induction of interferon although both can downregulate interferon signaling. J Virol 2007;81:2769–76.
[55] Salazar G, Colombo G, Lenna S, Antonioli R, Beretta L, Santaniello A, et al. HLA-B35 influences the apoptosis rate in human peripheral blood mononucleated cells and HLA-transfected cells. Hum Immunol 2007;68:181–91.
[56] Rothman AL, Ennis FA. Immunopathogenesis of Dengue hemorrhagic fever. Virology 1999;257:1–6.

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