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A Novel Real-Time PCR System for Simultaneous Detection of Human Viruses in Clinical Samples From Patients With Uncertain Diagnoses

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A novel simultaneous detection system for human viruses was developed using a real-time polymerase chain reaction (PCR) system to identify causes of infection in clinical samples from patients with uncertain diagnoses. This system, designated as the "multivirus real-time PCR," has the potential to detect 163 human viruses (47 DNA viruses and 116 RNA viruses) in a 96-well plate simultaneously. The specificity and sensitivity of each probe–primer set were confirmed with cells or tissues infected with specific viruses. The multivirus real-time PCR system showed profiles of virus infection in 20 autopsies of acquired immunodeficiency syndrome patients, and detected frequently TT virus, cytomegalovirus, human herpesvirus 6, and Epstein–Barr virus in various organs; however, RNA viruses were detected rarely except for human immunodeficiency virus-1. Pathology samples from 40 patients with uncertain diagnoses were examined, including cases of encephalitis, hepatitis, and myocarditis. Herpes simplex virus 1, human herpesvirus 6, and parechovirus 3 were identified as causes of diseases in four cases of encephalitis, while no viruses were identified in other cases as causing disease. This multivirus real-time PCR system can be useful for detecting virus in specimens from patients with uncertain diagnoses. ***J. Med. Virol. 83:322–330, 2011.***

KEY WORDS: real-time PCR; acquired immunodeficiency syndrome (AIDS); virus; autopsy

INTRODUCTION

Polymerase chain reaction (PCR) is a powerful tool to detect viruses compared with some traditional methods such as the direct fluorescent-antibody assay or virus isolation in cell culture. Real-time PCR is a sensitive system to detect viral genomes, used commonly worldwide [Storch, 2000]. Moreover, multiplex PCR fluorescence techniques are able to identify several genes in one tube simultaneously. Some reports have described simultaneous detection systems for up to 20 viruses using real-time PCR or conventional PCR [Vet et al., 1999; Bellau-Pujol et al., 2005; Li et al., 2007; Mahony et al., 2007; Molenkamp et al., 2007; Nolte et al., 2007; van de Pol et al., 2007; Wada et al., 2009]. However, the number of viruses detectable in one tube is limited by fluorescence wavelength. On the other hand, microarray analysis can detect a large number of viruses simultaneously. The weak point of the microarray assay is its low sensitivity and specificity [Wang et al., 2002].

It has been demonstrated that many viruses are associated with human diseases, and such human pathogenic viruses include both DNA and RNA viruses. An ideal virus screening system may be a system capable of detecting all the human pathogenic viruses simultaneously. In the present study, a real-time PCR system capable of detecting more than one hundred human viruses in a 96-well reaction plate simultaneously was established, designated as the "multivirus real-time PCR" system. In this system, two viruses are detected in one well using a duplex TaqMan real-time reverse transcriptase (RT)-PCR system; since more than 82 different duplex real-time PCRs are performed in a 96-well plate except wells for standard curve and

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internal controls, theoretically 163 human viruses can be detected in a 96-well plate simultaneously. Using this system, the distribution and quantification of viruses were investigated in organ specimens from autopsies of 20 acquired immunodeficiency syndrome (AIDS) patients. In addition, clinical samples from patients with uncertain diagnoses were examined to identify the causes of infection.

MATERIALS AND METHODS

Probe–Primer Sets

A total of 163 human viruses were selected as targets (Table I). The choice of the viruses was based on their associations with human diseases, prevalence among humans, and possibility of the usages as vectors to human cells. Probe–primer sets for each virus were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) (Supplementary Table I). Probe–primer sets published elsewhere were employed for some of the viruses. Probes and primers were synthesized by Sigma Genosys (Sigma–Aldrich, St. Louis, MO). Probes were labeled with 6-carboxyfluorescein (FAM)—6-carboxytetramethylrhodamine (TAMRA) or hexacholoro-6-carboxyfluorescein (HEX)—non-fluorescent Black Hole Quencher (BHQ)-1. Each probe–primer set was confirmed to react with at least 10 copies of a positive control plasmid containing each virus fragment, using conventional TaqMan real-time PCR (Applied Biosystems).

Establishment of Multivirus Real-Time PCR

A duplex TaqMan real-time RT-PCR system was designed to detect many viruses in a 96-well plate. Design of this system, designated as the "multivirus real-time PCR," is shown in Figure 1A. Quantitect Multiplex Probe RT-PCR kit (Qiagen, Hilden, Germany), MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems), and MicroAmp Optical Adhesive Film (Applied Biosystems) were used as 2× master mix, 96-well plates, and adhesive film, respectively. Each well contains two probe–primer sets with 6-FAM- and HEX-labeled probes, allowing two viruses were to be detected in each well, and the 163 viruses listed in Table I to be detected in a 96-well plate simultaneously. A standard curve was established for nine wells of each plate (A1–A9), which contained FAM- or HEX-labeled probes and primers for green fluorescent protein and glutathione *S*-transferase genes with control plasmids at 10^1 to 10^7 copies. Thus, an approximate copy number of each virus could be calculated based on the standard curve. To use the system routinely, 2× probe–primer mix was stored in a 96-well plate at −20°C. For detection of viruses, DNA and RNA samples (50 ng per well) were added to 2× master mix with (for RNA) and without (for DNA) RT. When sufficient amounts of DNA or RNA were not obtained from clinical samples, <50 ng of DNA or RNA per well were applied in this system. Ten microliters of 2× probe–primer mix and 10 μl of 2× master mix with sample DNA (36 wells) or RNA (60 wells) were then

TABLE I. List of Target Viruses

DNA virus
Polyomavirus: JC virus, BK virus, Simian virus 40
Papillomavirus: Human papillomavirus 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73
Parvovirus: Adeno-associated virus 1, 2, 3, 5; Parvovirus B19; human bocavirus; adenovirus A, B, C, D, E, F
Herpes virus: Human herpesvirus 1–8, B virus
Poxvirus: Variola virus, Monkey pox virus, Molluscum contagiosum virus
Anellovirus: Torque teno virus
Hepadnavirus: Hepatitis B virus
Other: Mimivirus
RNA virus
Filovirus: Ebola virus, Marburg virus
Bunyavirus: Crimean–Congo hemorrhagic fever virus, hemorrhagic fever with renal syndrome virus (Hantaan, Dovrava, Puumala, and Seoul), Rift valley fever virus, Sin Nombre virus
Arenavirus: Lassa virus, Junin, Guanarito, Machupo, Sabia
Togavirus: Equine encephalitis virus (Venezuelan, Eastern, and Western), Sindbis virus, Mayaro virus, Getah virus, Chikungunya virus, Rubella virus
Enterovirus: Enterovirus 68, 71; Poliovirus 1,2,3; Coxsackievirus A2, A3, A4, A5, A6, A8, A9, A10, A16, A21, A24, B1, B2, B3, B4, B5, B6; Echovirus 5, 6, 7, 9, 11, 13, 14, 16, 17, 18, 25, 30; Parechovirus 1, 3; Rhinovirus A, B; rotavirus; reovirus 1–4; Melaka virus; Colorado tick borne fever virus
Flavivirus: Dengue virus 1, 2; Japanese encephalitis virus; Murray Valley encephalitis virus; St. Louis encephalitis virus; West Nile virus; Tick-borne encephalitis virus; Yellow fever virus
Orthomyxovirus: Influenza virus A, B, C; H5N1
Paramyxovirus: Parainfluenza virus 1–3; Hendra virus; Mumps virus; Measles virus; Sendai virus; RS virus A, B; metapneumovirus; Nipah virus
Rabdovirus: Rabies virus; Lyssavirus 5, 6; Chandipura virus; Duvenhage virus
Coronavirus: Coronavirus OC43, 229E, NL63, SARS virus
Calicivirus: Sapovirus, Norwalk-like virus 1, 2
Hepatitis virus: Hepatitis A virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, GB virus
Retrovirus: human immunodeficiency virus 1; human T cell leukemia virus 1, 2; human endogenous retrovirus K, H, W
Other: Astrovirus, Borna disease virus

Biosystems) or an Mx3005P (Stratagene, La Jolla, CA). The RT-PCR conditions were 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, and 60°C for 1 min. Quantitative results of viruses were obtained by generating standard curves for two plasmids in the A1–A9 wells. Real-time PCR using a condition for RT-PCR (50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min) had similar sensitivity to real-time PCR using usual DNA conditions (95°C for 5 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min) in the detection for some DNA viruses (Supplementary Fig. 1A). In addition, the duplex real-time PCR using Quantitect Multiplex Probe RT-PCR kit (Qiagen) had similar sensitivity to single real-time PCR procedures using Quantitect Probe RT-PCR kit (Qiagen) in several probe–primer sets (Supplementary Fig. 1B).

Gene Expression Image

A gene expression image was produced with TreeView and Cluster software by Michael Eisen, University of California at Berkeley (http://rana.lbl.gov/EisenSoftware.htm) [Eisen et al., 1998].

Determination of the Positivity and Copy Numbers of Viruses

The positivity and virus titer of all positive samples were confirmed with individual standard real-time (RT-) PCR systems using the same probe–primer sets. Virus DNA copy numbers per cell were calculated by dividing virus DNA copy numbers by half of beta-actin copy numbers, since each cell has two copies of DNA in two alleles [Asahi-Ozaki et al., 2006].

Patients and Samples

The study protocol was approved by the Institutional Review Board, National Institute of Infectious Diseases, Japan (Approval No. 156). Tissues were taken at autopsy from various organs of 20 patients with AIDS. All tissues were frozen immediately, and stored at −80°C. The clinical information of the patients is summarized in Table II. A total of 19 patients were male. The mean age of the patients was 41.8 years (range: 19–67 years), and the mean of CD4 counts was 17 cells/μl (range: 0–241). Risk factors for HIV infection in the patients were men who had sex with men (10), heterosexuality (5), and hemophilia (5). At least seven patients had lymphoma, and two had Kaposi's sarcoma. No patients received highly active anti-retroviral therapy (HAART). In addition, 40 clinical samples from patients with uncertain diagnoses were investigated (Table III). These clinical samples were sent to our department for virus diagnosis. Informed consents were obtained by the clinical doctors. Positive control

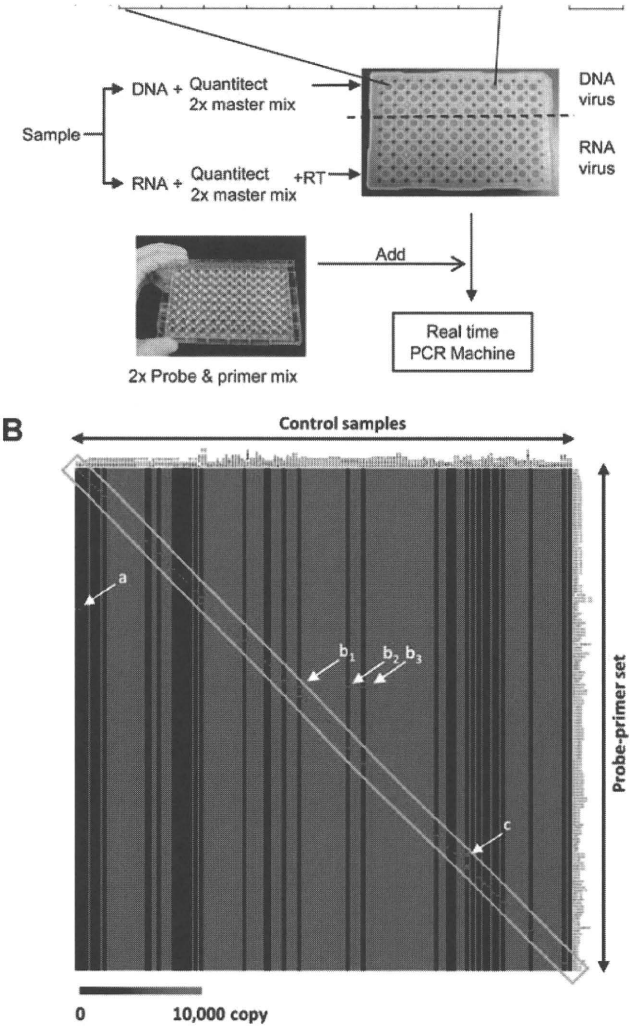

Fig. 1. Establishment and validation of multivirus real-time PCR. **A**: Procedure of the multivirus real-time PCR system. DNA sample was mixed with Quantitect 2× master mix, and RNA sample was mixed with Quantitect 2× master mix and reverse transcriptase (RT) mix. These mixtures were poured into each well in a 96-well plate at 10 μl per well. Ten microliters of 2× probe and primer mix were then added to each well in a premixed 96-well plate. Finally, the virus genes were amplified and detected in a real-time PCR machine for 2 hr. **B**: Validation of the multivirus real-time PCR. A gene expression image by TreeView software based on the results of the multivirus real-time PCR for control samples is shown. A horizontal line shows each probe–primer set and a vertical line is one sample of positive control. Gray vertical lines indicate no sample. A scale bar indicates copy number of color. A green box indicates specific reactions of target positive controls in specific probe–primer sets. Arrows of (a–c) also show specific signals. The arrow (a) shows positive signal for TTV in a brain sample with both JCV and TTV infection. The arrows (b1–3) show that a probe–primer set for pan-enterovirus reacted with poliovirus (b1), Coxsackievirus B3 (b2), and Echovirus 6 (b3) positive samples. The arrow (c) shows that a probe–primer set for influenza virus A reacted with H5N1 influenza virus. Details of positive controls were listed in Supplementary Table II.

DNA or RNA samples extracted from virus-infected cells or tissues were kindly provided by many researchers in National Institute of Infectious Diseases (Supplementary Table II).

Age	Sex	Risk factor	Complications	CD4[a]	Detected viruses by multivirus real-time PCR
49	M	MSM	PCP, aspergillus	NT	TTV, HBV, HERV-H
37	M	MSM	CMV, toxoplasma, PCP	NT	HSV-1, CMV, TTV, HIV-1, HERV-H
29	M	Drug	PCP	1	JCV, Adv-B, HSV-1, EBV, CMV, HHV-6, HHV-7, TTV, HBV, HIV-1, HERV-H
37	M	Blood product	MAC, CMV	1	EBV, CMV, HHV-6, TTV, HIV-1, HERV-H
43	M	Heterosexual	ML, CMV, cryptococcus	5	B19, Adv-A, EBV, CMV, HHV-6, TTV, HIV-1, HERV-H
54	M	MSM	CMV	NT	EBV, CMV, HHV-6, TTV, HBV, HIV-1, HERV-H
33	M	MSM	CMV	4	B19, CMV, HHV-6, TTV, HIV-1, HERV-H
47	M	MSM	HIV-encephalitis, KS, ML	1	BKV, CMV, HHV-6, TTV, HIV-1, HCV
35	M	Blood product	CMV	1	B19, CMV, TTV, HIV-1, HERV-H
27	F	Heterosexual	ML, MAC, CMV	3	BKV, EBV, CMV, TTV, HIV-1, HERV-H
50	M	MSM	HIV-encephalitis, ML, cryptococcus, CMV	0	B19, CMV, HHV-6, TTV, HIV-1, HERV-H
19	M	Blood product	HIV-encephalitis	2	BKV, TTV, HIV-1, HERV-H
26	M	Blood product	PML	3	JCV, BKV, B19, Adv-B, EBV, HHV-6, HHV-7, TTV, Echo6, HCV, HERV-H
67	M	MSM	ML	241	JCV, CMV, HHV-6, TTV, HIV-1, HERV-H
62	M	MSM	CMV, PCP	4	AAV-2, B19, EBV, CMV, HHV-6, TTV, HIV-1, HERV-H
28	M	Blood product	CMV, MAC	3	JCV, BKV, B19, CMV, HHV-7, TTV, HBV, HIV-1, HERV-H
46	M	Heterosexual	ML, aspergillus, CMV	5	BKV, AAV-2, B19, Adv-D, EBV, CMV, HHV-6, TTV, RSV-B, HIV-1
47	M	MSM	PEL, CMV	7	JCV, B19, EBV, CMV, HHV-6, HHV-8, TTV, HIV-1, HERV-K, HERV-H
60	M	MSM	ML, CMV, KS	1	B19, CMV, TTV, HIV-1, HERV-H
40	M	Heterosexual	CMV	NT	BKV, AAV-2, CMV, HHV-6, HHV-7, TTV, HBV, HERV-H

ino-associated virus; Adv, adenovirus; B19, parvovirus B19; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HBV, B virus; HCV, hepatitis C virus; HERV, human endogenous retrovirus; HHV, human herpesvirus; HIV-1, human immunodeficiency ISV, herpes simplex virus; JCV, JC virus; KS, Kaposi's sarcoma; MAC, mycobacterium avium-intracellulare complex; ML, malignant a; MSM, Men who have sex with men; NT, not tested; PCP, Pneumocystis pneumonia; PML, progressive multifocal leukoencephal- SV, respiratory syncytial virus; TTV, Torque teno virus.
nts per μl.

DNA and RNA Extraction

eic acid extraction methods differed according ype of samples. Each frozen tissue sample was in two, one part for DNA extraction and another A extraction. For DNA extraction, the samples omogenized with Multi-Beads Shocker (Yasui Tokyo, Japan) in TEN buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, and 100 mM NaCl) with ml proteinase K and 0.1% sodium dodecyl DNA was extracted from the homogenized using the phenol–chloroform method. Total as extracted from frozen tissues using Isogen n Gene, Tokyo, Japan). The samples were homogenized in the Isogen with Multi-Beads Shocker, and the extraction was performed according to the manufacturer's instructions. For small samples including tissue biopsy, blood, serum and cerebral fluid, both DNA and RNA were extracted simultaneously with All Prep Kit (Qiagen). All RNA samples were treated with DNase (Turbo DNA-Free, Ambion, Austin, TX) for 20 min according to the manufacturer's instructions.

RESULTS

Validation of Multivirus Real-Time PCR

To validate the sensitivity and specificity of each probe and primer set used in the system, DNA or RNA samples

TABLE III. Identification of Pathogenic Virus in Clinical Samples From Patients With Uncertain Diagnoses

	n	Samples	Identified pathogens (cases)
s	9	Liver biopsy	Parvovirus B19 (2), HHV-6 (3), TTV (2)
litis	11	Brain biopsy, serum, cerebral fluids	HSV-1 (2), HHV-6 (1), parechovirus 3 (1)
litis	6	Heart autopsy	Parvovirus B19 (1), TTV (2)
death	4	Blood, serum	TTV (1)
	10	Tissue, blood, serum	Parvovirus B19 (1), EBV (1), CMV (1), HHV-7 (1), TTV (2)
	40	—	—

l viruses in the cases are underlined.

extracted from virus-infected cells, supernatants, body fluids, or tissues were examined in this multivirus real-time PCR system (Supplementary Table II). Each probe and primer set amplified a gene fragment of target virus specifically (Fig. 1B and Supplementary Table II). When using supernatants of virus-infected cells, the reactions were specific. Some samples of supernatants were positive for two or three viruses, because the target virus belonged to several categories. For example, the RNA sample extracted from supernatants of H5N1 influenza virus-infected cells was positive in the wells with probe and primer sets for both H5N1 influenza virus and influenza-A virus. Some clinical samples, such as pathological samples and body fluids, were positive for other viruses as well as target viruses because of the presence of such viruses in the samples (Supplementary Table II and Fig. 1B, arrow (a)). Although not all positive control samples could be collected, the results confirmed the adequate specificity of each probe–primer set for its target virus for virus screening. The multivirus real-time PCR system also detects human internal control genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, DNA, and mRNA), beta-actin (DNA), and beta-2-microgloblin (mRNA) (Supplementary Tables I and II). It is known that certain specimen such as serum may have inhibitory effects on PCR [Vandenvelde et al., 1993; Willems et al., 1993]. Copy numbers of internal controls would be informative to know cell numbers and inhibitory effect by the sample.

Detection of Viruses in AIDS Autopsies

Using the multivirus real-time PCR, the presence of viruses was investigated in 20 AIDS autopsies. The multivirus real-time PCR detected 15 DNA viruses: JC virus (JCV), BK virus (BKV), adeno-associated virus (AAV)-2, parvovirus B19, three subgroups of adenovirus (A, B, and D), herpes simplex virus 1 (HSV-1), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus (HHV)-6, -7, -8, TT virus (TTV), and

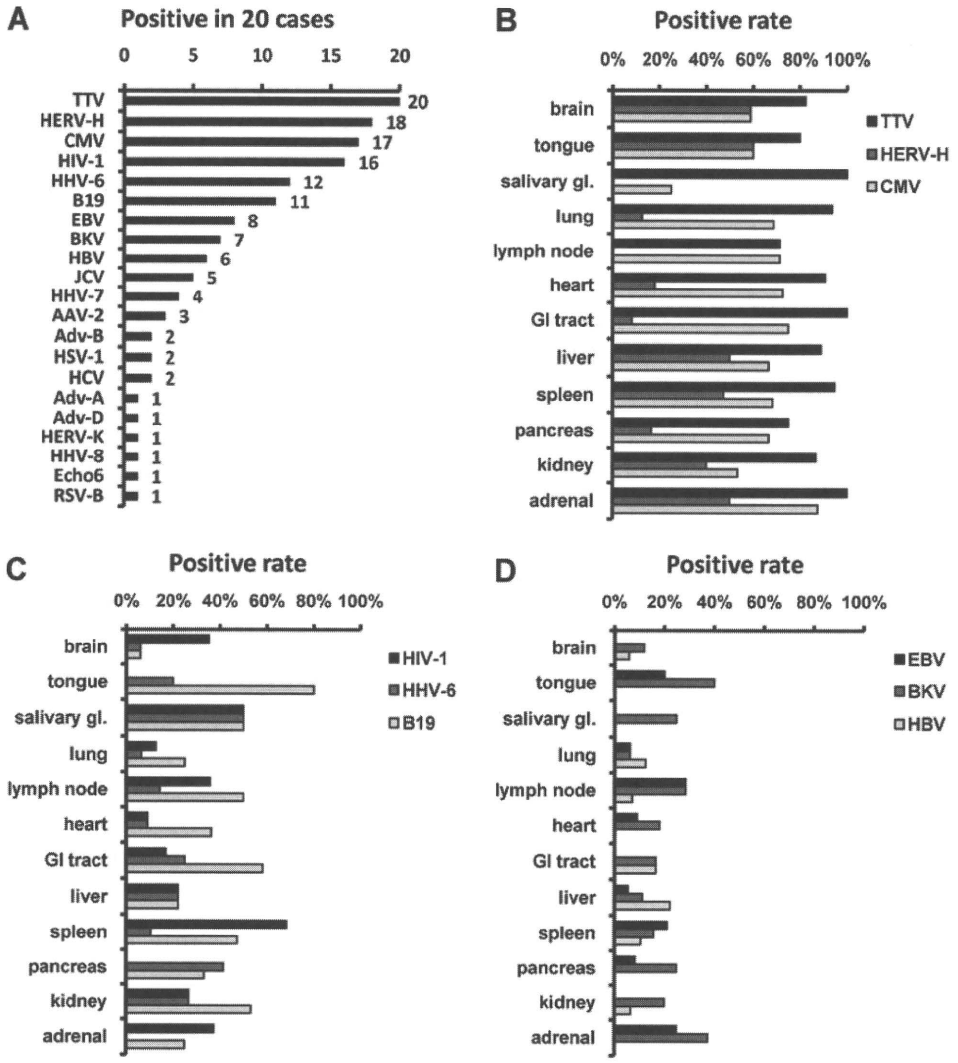

Fig. 2. Viruses detected in autopsied organs of AIDS patients. **A**: Positive number of each virus in the 20 cases of AIDS autopsy. **B–D**: Positive rates of CMV, HIV-1, and HHV-6 in organs. GI, gastrointestinal.

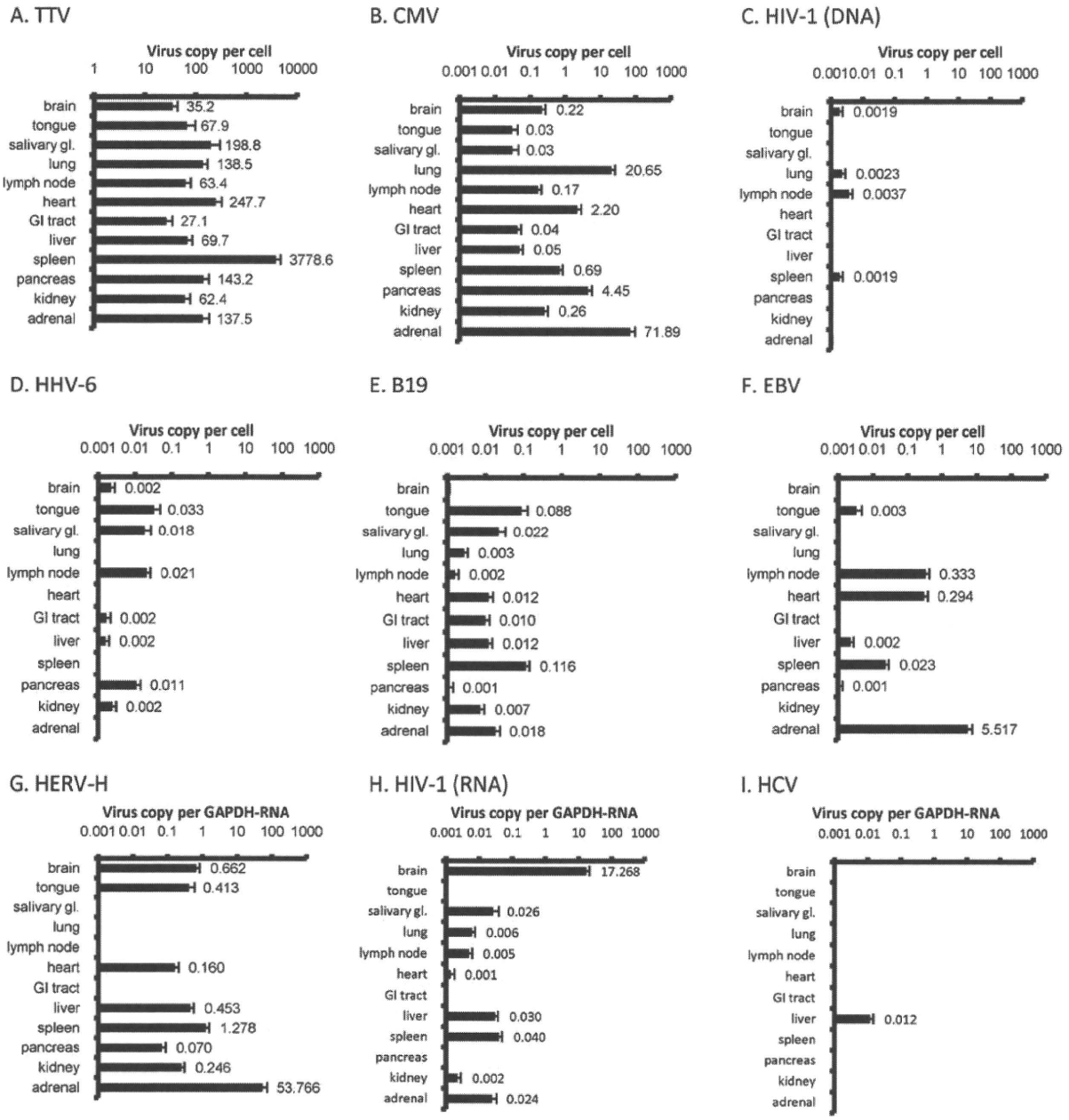

Fig. 3. Mean values of virus copy numbers in organs. Mean of copy numbers per cells are shown in DNA samples (**A**–**F**). Mean ratios of virus copy number per hGAPDH-RNA copy number are shown in RNA samples (**G**–**I**). Error bars show standard errors. One brain sample contained HIV-associated encephalopathy (C,H), and one of the heart and adrenal sample contained EBV-associated lymphoma (F).

hepatitis B virus (HBV). It also detected six RNA viruses: echovirus 6, respiratory syncytial (RS) virus type B, hepatitis C virus (HCV), HIV-1, and human endogenous retrovirus (HERV)-H and -K, in 20 cases of AIDS autopsies (Fig. 2). A few other viruses were detected at low copies in some samples, but additional individual standard real-time (RT-) PCR systems using the same probe–primer sets showed negative results, indicating that they were false positive. Although HIV-1 infections were confirmed clinically in all the patients, HIV-1 was not detected in four of the autopsy cases, even using both DNA and RNA samples. TTV, HERV-H, CMV, HIV, HHV-6, parvovirus B19, EBV, BKV, and HBV were detected in many organs, suggesting broad distribution (Fig. 2B–D). On the other hand, the positive rate of each virus differed among organs. CMV was detected most frequently in the adrenal gland, but HIV-1 was most common in the spleen, HHV-6 in the salivary gland, and HBV in the liver.

The multivirus real-time PCR also revealed copy numbers of each virus in AIDS autopsy (Fig. 3). High numbers of TTV copies were detected frequently in various organs without any symptoms, suggesting a ubiquitous distribution in the samples and no associa-

tion with any specific diseases (Fig. 3A). High numbers of CMV copies were detected in adrenal gland, lung, and pancreas (Fig. 3B). To confirm the results of the real-time PCR, CMV positivity was investigated using inclusion bodies in the pathological samples. Inclusion bodies of CMV were detected frequently in the adrenal gland, pancreas, and lung of AIDS autopsy cases (Supplementary Fig. 2). These results correlated with those of the real-time PCR. HHV-6 and parvovirus B19 showed low copy numbers in all the organs tested (Fig. 3D,E). High numbers of EBV copies were detected in the heart and adrenal gland, as well as lymph node and spleen (Fig. 3F). However, the heart and adrenal samples included lesions of EBV-associated lymphomas. Thus, EBV was detected in the lymphomas in those samples. The lymphomas in adrenal glands also included high numbers of HERV-H copies, affecting the results of HERV-H copy numbers in adrenal glands (Fig. 3G). High numbers of HIV-1-RNA copies, but not HIV-1-DNA, were also found in the brain of one case with HIV-1 encephalopathy (Fig. 3C,H). HCV was detected only in the liver of two patients (Fig. 3I).

Identification of Virus in Clinical Samples From Patients With Uncertain Diagnoses

Using the multivirus real-time PCR, clinical samples from 40 patients with uncertain diagnoses were examined to identify their causes of infection (Table III). The multivirus real-time PCR system identified HSV-1, HHV-6, or parechovirus 3 as a possible cause of infection in 4 out of 11 patients with encephalitis. HSV-1 was identified in brain biopsy tissues from two patients with encephalitis. A high copy number of HHV-6 was detected in the serum of a patient (1.5×10^7 copies/ml in the serum). In another patient, parechovirus 3 were detected in cerebral fluids. The presence of these viruses in the samples was confirmed by individual real-time PCR specific for each virus, and conventional (RT-) PCR. Clinical manifestations of these four patients were compatible with the virus infections. In addition, 29 samples from patients with other diseases such as myocarditis, hepatitis, and sudden death were examined. Parvovirus B19, EBV, CMV, HHV-6, HHV-7, and TTV were detected in the samples; however, the titers of these viruses were low. In addition, immunohistochemistry and in situ hybridization could not detect the viruses in the samples. It was therefore concluded that these viruses were not the causes of diseases in the cases.

DISCUSSION

In the present study, a new real-time PCR system was developed, designated as the "multivirus real-time PCR," that had the potential to detect 163 viruses simultaneously. This multivirus real-time PCR can detect 47 DNA viruses and 116 RNA viruses on a 96-well plate theoretically. This system revealed the anatomical distributions of human pathogenic viruses in AIDS autopsy cases. In addition, viruses were identified in four cases of encephalitis as the cause of infection. This multivirus real-time PCR system could be a useful technique for detection of virus in specimens from patients with uncertain diagnoses.

Real-time PCR is a sensitive detection system for the diagnosis of virus infection. TaqMan PCR has a high specificity compared with other systems because of its specific fluorescence probes. In addition, recent multiplex fluorescence technology is able to detect several genes in each tube without non-specific cross-reactions. Since one-step real-time RT-PCR employs specific primers as RT primers, with targets shorter than 100 bp, this system can detect short fragments of RNA viruses specifically with high sensitivity. The sensitivity and specificity of this system are equivalent to those of standard real-time PCR systems (Supplementary Fig. 1), and its sensitivity would be much higher than in microarray systems. In addition, the multivirus real-time PCR system requires only 5 μg each of DNA and RNA for detecting 163 viruses.

One disadvantage of this system is cost. To establish this system, 176 probe–primer sets should be prepared. Moreover, about 1 ml of Quantitect 2× master mix was used in single reaction of 96-well plate, which costs about 25,000 yen (approximately 263 U.S. dollars; containing probe–primer sets: ¥36 × 176 sets = ¥6,336, Quantitect 2× master mix: ¥16,000, filtered tips, 96-well reaction plate and seal: ¥2,664) per sample in a 96-well plate reaction to test. However, once the system is established, the procedure is very easy and takes 3 hr to obtain the results. Thus, the newly developed multivirus real-time PCR could be a useful tool for detecting pathogens in specimens from patients with uncertain diagnoses.

There is little current information about quantification of pathogenic viruses in immunocompromised hosts. Chen and Hudnall [2006] described anatomical mapping of herpes viruses in eight autopsy cases, including two AIDS cases. The multivirus real-time PCR showed that 21 of the 163 probe–primer sets for virus produced positive reactions in AIDS autopsy samples. Many RNA viruses were negative in all cases. Although the low detection rate of RNA viruses might be associated with the quality of extracted RNA, these results suggest that the AIDS patients in the present study were infected with limited types of viruses. TTV and HHV-6 were detected frequently in AIDS autopsy samples and some clinical samples. TTV was identified from a hepatitis patient as a hepatitis-associated virus [Nishizawa et al., 1997]. However, TTV, a ubiquitous virus, was shown to be present in various tissues [Okamoto, 2009]. Although TTV titers were relatively high compared with those of other viruses, broad TTV distribution suggests that it is not associated with specific diseases in immunocompromised hosts. HHV-6 is another ubiquitous virus with which almost 100% of adults are infected. Primary infection of HHV-6 causes exanthema subitum in children [Yamanishi et al., 1988]. Reactivation of HHV-6 may cause hepatitis, pneumonia, and encephalitis in immunocompromised hosts, such as

transplant patients [Ljungman, 2002]. The average number of HHV-6 copies in the HHV-6-positive samples in AIDS autopsy was 0.008 copies per cell, suggesting low numbers of HHV-6 copies in the organs. On the other hand, HHV-6 was identified as a possible cause of infection in a clinical case of encephalitis because of extremely high numbers of copies in the serum and clinical manifestations [Ogata et al., 2010]. Thus, a virus's copy number is important information to estimate its etiology. CMV was frequently detected in AIDS autopsy samples by the multivirus real-time PCR system. High CMV copy numbers in the adrenal gland, pancreas and lung were associated with the occurrence of CMV-associated adrenitis, pancreatitis, and pneumonia. CMV was detected frequently in the adrenal gland [Pulakhandam and Dincsoy, 1990]. The results of CMV detection in multivirus real-time PCR were correlated highly with the frequency of CMV inclusion bodies on the slides, suggesting that the occurrence of CMV-associated diseases is associated with virus titers of CMV in organs.

The multivirus real-time PCR failed to detect HIV-1 DNA or RNA in 4 cases out of 20 AIDS autopsies. There are several possible explanations for these results. Although none of the patients received HAART, HIV-1 titers always change in AIDS patients [Ho et al., 1989]; the autopsy samples might have had insufficient HIV-1 titers for detection by real-time PCR. Also, the probe and primers used in this system might not detect the HIV-1 because of mutations in the target regions. Mutations in HIV-1 occur so frequently that it is difficult to detect HIV-1 using one or two probe–primer sets [Desire et al., 2001; Yun et al., 2002].

Consequently, a multivirus real-time PCR system with the potential to detect 163 viruses simultaneously has been established in the present study. Although the system has some disadvantages with regard to cost and procedure, it will be a powerful tool for virus screening of clinical samples in laboratories. Since it is relatively easy to change probe–primer sets in the 96-well plate, it is possible for this system to change detectable viruses, implying that a new system detecting new viruses can be established quickly. Future refinement of its operation, such as higher throughput and microfluid techniques, may resolve the disadvantages of this system.

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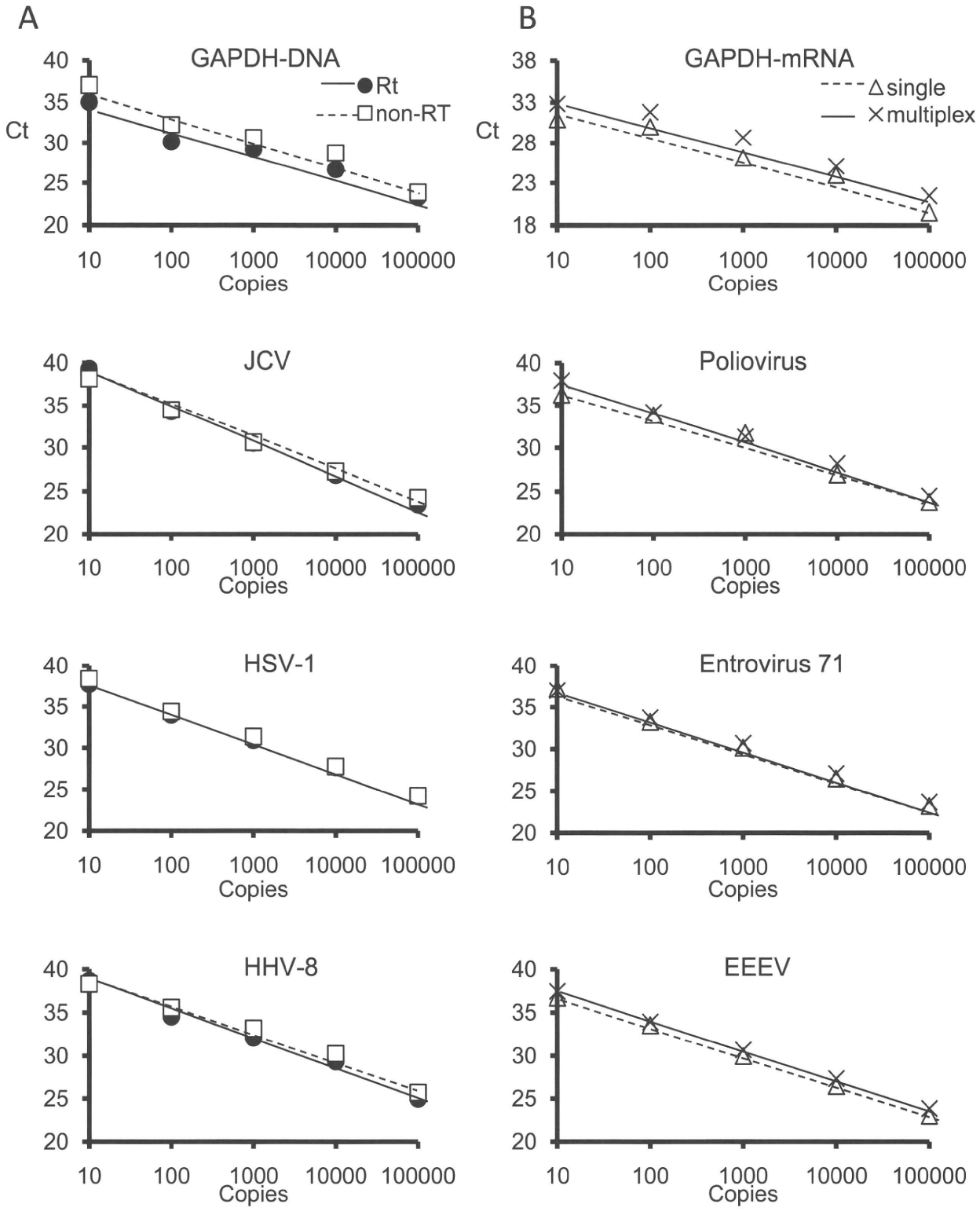
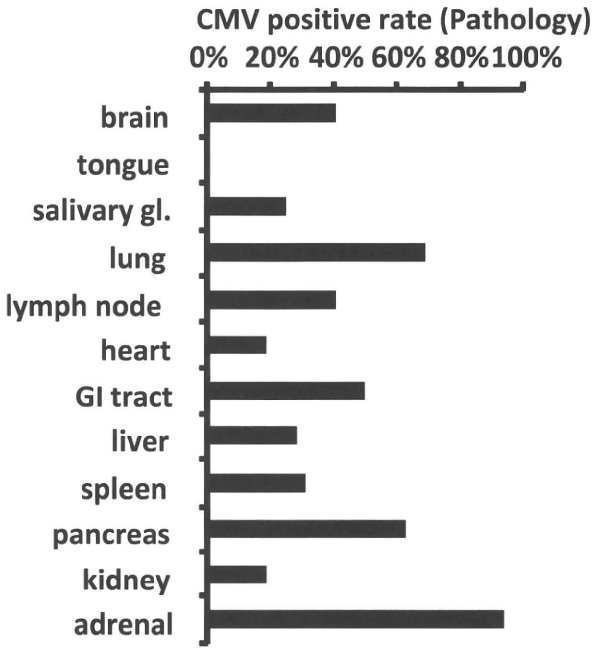
Supplementary Figure 1




CMV positive rate (Pathology)
0% 20% 40% 60% 80% 100%
brain
tongue
salivary gl.
lung
lymph node
heart
GI tract
liver
spleen
pancreas
kidney
adrenal

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Immune response to intranasal and intraperitoneal immunization with Kaposi's sarcoma-associated herpesvirus in mice

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ABSTRACT

A vaccine for Kaposi's sarcoma-associated herpesvirus (KSHV) is not currently available. To obtain the fundamental data in animals for vaccine development, KSHV particles were immunized to Balb/c mice through intraperitoneal and intranasal routes in the present study. Intranasal immunization with KSHV induced IgA to KSHV in not only serum, but also nasal wash fluid and saliva. A neutralization assay using recombinant KSHV that expressed green fluorescent protein revealed that nasal wash fluid and saliva from the KSHV-immunized mice neutralized KSHV infection to human embryonic kidney 293 cells in vitro in a dose-dependent manner to KSHV copies immunized. The serum and nasal wash fluid of KSHV-encoded K8.1 protein-immunized mice neutralized KSHV infection to 293 cells in vitro. These data suggest a possibility of mucosal vaccine for prophylaxis of KSHV infection.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) was identified as a causative agent of Kaposi's sarcoma (KS) in 1994 [1]. Since KSHV has been detected in all cases of KS, there is no doubt about the association between KS pathogenesis and KSHV infection [2]. More than 15 years after the discovery of KSHV, KS is still an important complication in AIDS patients. KS occurs frequently among human immunodeficiency virus (HIV)-infected men who have had sex with men (MSM), suggesting that homosexual behavior in males is an important risk factor for KS and KSHV infection [3]. Although vaccine is available for other herpes viruses, such as varicella zoster virus, KSHV vaccine is not available so far. There are several reasons why KSHV vaccine has not yet been developed.

First, most HIV-infected MSM are already infected with KSHV [3]. For example, an epidemiological study revealed that about 60% of HIV-infected MSM were positive for serum antibody to KSHV in Japan, suggesting widespread KSHV infection among MSM [4]. Immunodeficiency condition may cause some problems for vaccine to work in HIV-infected individuals [5]. However, vaccination of influenza vaccine to asymptomatic HIV-infected patients showed similar antibody production to uninfected group [6], suggesting possibility of vaccine strategy for KSHV in HIV-infected adults.

Second, immunohistochemical studies revealed that, while almost all KS cells express a latent protein, latency-associated nuclear antigen 1 (LANA-1), only a very small number (less than 1%) of KS cells express lytic proteins encoded by KSHV. This implies that replication of KSHV is very rare in KS regions, and latent KSHV infection is predominant and important in the pathogenesis of KS [7]. Generally, vaccine can prevent *de novo* infection or reactivation of virus in human bodies, but will not suppress function of latently infected virus. However, it is demonstrated that some lytic proteins encoded by KSHV such as K1, vGPCR, and vIL-6, promote KS development and angiogenesis. Condition with immunodeficiency is also required for KS pathogenesis. Thus, while LANA-1 may become a target of anti-tumor drug [8], KSHV vaccine may play a certain role in the suppression of lytic protein expression.

Third, it is difficult to evaluate a newly developed KSHV vaccine. Although it was recently demonstrated that common marmosets can be infected with KSHV [9], there is no convenient animal model in which KSHV can infect and replicate.

However, the occurrence of KS among MSM may still be prevented using a vaccine strategy. Although the details of infectious routes of KSHV are unknown, the mucosae in the oral cavity and rectum are possible entrances for KSHV, because saliva contains high copy numbers of KSHV, and because epidemiological studies have shown that KSHV infection is associated with homosexual behaviors [3,10]. Many studies have demonstrated that mucosal vaccine is a promising tool for prevention for viral and bacterial infections [11–16]. Those studies showed that the secreted form of IgA plays an important role in the mucosal immunity, and that

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mucosal immunity from IgA is more effective and cross-protective against viral infections than systemic immunity induced by serum IgG [17,18]. If the mucosae are main routes of KSHV infection, mucosal vaccine could become a tool to prevent the spread of KSHV among MSM.

Another reason for using vaccines for KSHV infection is that KS occurs frequently in HIV-infected MSM [19]. About 40% of HIV-infected MSM may be serologically negative for KSHV; they could be the target group for a KSHV vaccine [4]. Limiting use of an efficacious KSHV vaccine to $KSHV^{-}HIV^{+}$ MSM patients or $KSHV^{-}HIV^{-}$MSM could prevent KS efficiently.

However, for vaccine development, there is little information about immune responses to KSHV infection in human and animals. KSHV infection in humans induces the production of serum antibodies to KSHV-encoded proteins [4,20]. Such serum antibodies recognize K8.1, ORF59, ORF65, and ORF73 (LANA-1) proteins encoded by KSHV as immunogens [4]. KSHV infection also induces CD8 T cells in the region of KS, which play an important role in the regression of KS in AIDS patients receiving highly active anti-retroviral therapy [21]. This information suggests that KSHV induces similar immune responses in human as do other herpes viruses. Nevertheless, KSHV does not infect normal mice or macaques [22–25]. There are few reports offering detailed descriptions of immune responses against KSHV in KSHV-immunized animals [9].

The aim of this study was to obtain fundamental data in animal experiments for KSHV vaccine development. To estimate immune responses against KSHV in animals, Balb/c mice were immunized intranasally or intraperitoneally with KSHV particles, and their immunoreactions were investigated. In addition, an *in vitro* neutralization assay was performed using green fluorescent protein-expressing recombinant KSHV and the serum, nasal wash fluid (NW), and saliva from the KSHV-immunized mice.

2. Materials and methods

2.1. Viruses

KSHV particles were prepared from BCBL-1 cells stimulated with phorbol 12-myristate-13 acetate (PMA; Sigma, St. Louis, MO) as described previously [26]. Briefly, BCBL-1 cells were stimulated with PMA at 20 ng/mL for 72 h. The supernatant of BCBL-1 cells was collected and filtered through a 0.8-μm-pored membrane. Filtered supernatant was ultracentrifuged at 20,000 × *g* for 2 h. The pellet was dissolved in one-fiftieth volume of RPMI 1640. Virus copy number was measured with a real-time PCR as described previously [27]. A green and red fluorescent protein (GFP/RFP)-expressing recombinant KSHV, rKSHV.219 (kindly provided by Dr. Jeffrey Vieira, Washington University), was collected for the neutralization assay as described previously [28].

2.2. Immunization with virus or proteins

Female 8-week-old Balb/c mice were purchased from Clea Japan (Tokyo, Japan) and were kept under specific-pathogen-free conditions. All animal experiments were performed in accordance with the Guidelines for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of NIID (approvals No. 108056 and 209072). Five mice for each experimental group were anesthetized with isoflurane and immunized primarily by dropping 5 μl of phosphate buffered saline (PBS) containing 10^6–10^8 copies of KSHV or 10 ng of KSHV-encoded proteins with 10 μg of poly(I:C) (Sigma) into each nostril [29]. For immunization to the peritoneal cavity, 100-μl aliquots of PBS containing the viruses (10^6–10^8 copies) or proteins (100 ng) with poly(I:C) were immunized to the mice's peritoneal cavities. Additional immunizations were performed twice, 2 and 3 weeks later. Samples of blood, spleen, and NW were obtained from mice that were sacrificed under anesthesia with isoflurane 1 week after the final immunization. NW samples were taken as previously described [17]. Saliva samples were obtained using intraperitoneal administration of pilocarpine (150 μL of 1 mg/ml in PBS per mouse, P-6503, Sigma).

2.3. Real-time RT-PCR

Copy numbers of mouse IFN-γ, CD8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined with real-time RT-PCR using probe-primer sets described previously [30]. Total RNA was extracted from 1×10^7 spleen cells of each mouse with Isogen RNA isolation kit (Nippon Gene, Toyama, Japan). Real-time RT-PCR was performed with one-step Quantitect probe RT-PCR kit (Qiagen, Hilden, Germany).

2.4. Detection of IFN-γ by ELISA

Spleen cells were cultured in RPMI 1640 for 8 h and mouse IFN-γ in supernatant was measured with ELISA (Quantikine Mouse IFN-γ Immunoassay Kit, R&D systems, Minneapolis, MN).

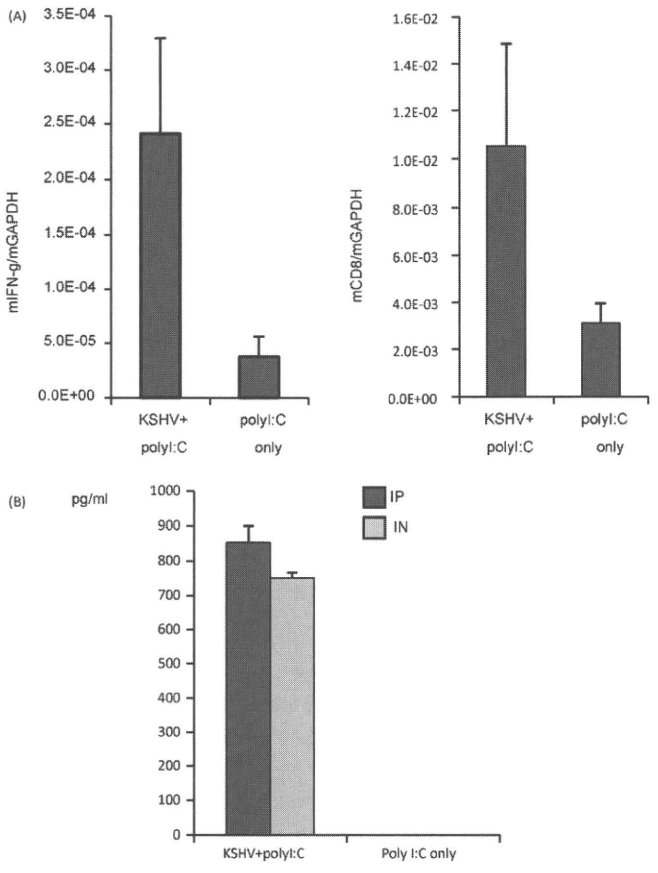

Fig. 1. Induction of cellular immune response in KSHV-immunized mouse. (A) Real-time RT-PCR for IFN-γ and CD8 mRNA. Copy numbers of IFN-γ, CD8 and GAPDH were measured with real-time RT-PCR in spleen cells. The ratio to GAPDH copy number is indicated on the *y*-axis. (B) IFN-γ production from spleen cells of KSHV-immunized mice. Error bars indicate standard deviations of triplicate experiments. IP: Intraperitoneal immunization. IN: Intranasal immunization.

2.5. Immunofluorescence assay

Titers of antibody to KSHV were determined by immunofluorescence assay (IFA) using PMA-stimulated TY-1, a KSHV-infected primary effusion lymphoma cell line [31]. TY-1 cells were stimulated with PMA for 48 h and smeared on slides. After acetone fixation, the smear slides were stored at −25 °C. Serum, NW, or saliva were diluted by dilution factors 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 for IgA, and 50, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, and 25,600 for IgG in Block Ace (Snow-Brand, Tokyo, Japan). Diluted samples were applied on the smear slides, and incubated at room temperature for 1 h. After washing with PBS, the slides were reacted with FITC-conjugated anti-mouse IgG or IgA antibody (BD Bioscience) for 30 min. Followed by washing and mounting, the slides were observed with a fluorescence microscope. Antibody titers were determined at the dilution of positive signals. For identification of immunogens in KSHV-immunized mice, dual-labeled IFA was performed. The mouse serum and anti-KSHV ORF K8, K8.1, ORF26, ORF59, ORF65, or ORF73 (LANA-1) rabbit polyclonal antibodies were reacted with the smear slides as the primary antibodies [7]. After washing, the slides were reacted with Alexa 488-conjugated anti-mouse IgG antibody and Alexa 568-conjugated anti-rabbit IgG antibody (Molecular Probe, Eugene, OR) as the secondary antibodies. After washing and mounting, the slides were observed with a confocal microscope (FV-1000, Olympus, Tokyo, Japan).

2.6. Neutralization assay

One hundred μl of 1000× diluted serum or 10× diluted NW or saliva were incubated with 10^6 copies of rKSHV.219, which contained about 100 infectious units, in DMEM in tubes at 37 °C for 2 h [28]. After the incubation, 100 μl of the virus solution was added to human embryonic kidney 293 cells (293 cells) in a 96-well plate. The plate was centrifuged for a short time at a low speed, and incubated for 2 h in a CO_2 incubator. After removing the supernatant, fresh media was added, and the cells were cultured at 37 °C. Five days after infection, the number of GFP^+ cells in each well was counted under a fluorescence microscope.

2.7. Recombinant proteins and western blotting analysis

Glutathione S-transferase (GST)-fusion proteins of K8, K8.1, ORF26, ORF59, ORF65, and ORF73 were synthesized as described previously [4]. Fifty nanograms of each GST-fusion protein was applied to western blotting. Since molecular sizes of these GST-fusion proteins range 41–60 kDa, 50 ng protein is corresponding to 0.8–1.2 pmol. The serum from mice and anti-GST rabbit polyclonal antibody were used as the primary antibodies. Anti-mouse or rabbit IgG antibodies (BD Bioscience) were used as the secondary antibodies; signals were detected with a chemiluminescence solution (Westdura, Pierce Biotechnology, Rockford, IL).

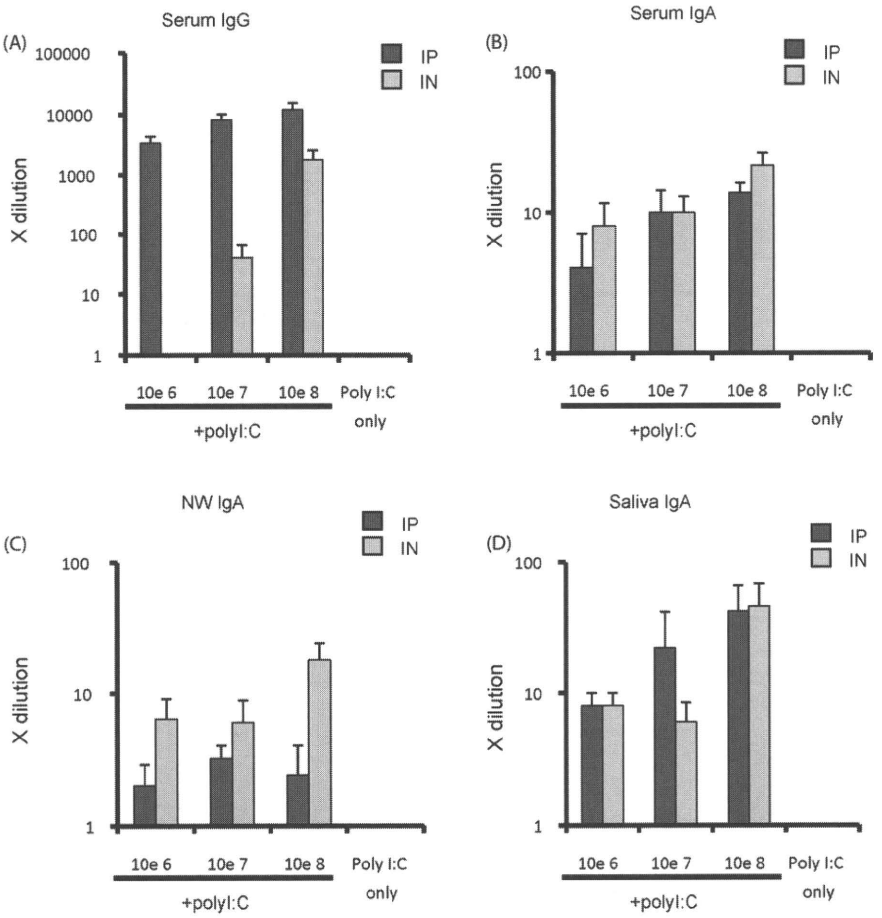

Fig. 2. Induction of humoral immunity to KSHV in KSHV-immunized mice. (A) Titers of serum IgG (A), serum IgA (B), nasal wash fluid (NW) IgA (C), and saliva IgA (D) were determined by IFA using TPA-stimulated TY-1. Copy numbers of immunized KSHV are shown in the *x*-axis. Error bars indicate standard deviations of five mice in each group. IP: Intraperitoneal immunization. IN: Intranasal immunization.

2.8. Statistical analysis

Student's *t*-test was applied for the comparison of mRNA levels and the KSHV neutralization assay.

3. Results

3.1. IFN-γ production in KSHV-immunized mice

To know whether KSHV induces cellular immune responses in mice, 10^8 copies of KSHV particles were immunized intranasally or intraperitoneally to Balb/c mice with poly(I:C) as an adjuvant. Poly(I:C) is a synthetic double-stranded RNA; it has been demonstrated to be an effective mucosal adjuvant for not only RNA viruses such as influenza virus, but also DNA viruses such as herpes virus and human papillomavirus [29,32]. Real-time RT-PCR showed that KSHV immunization to the peritoneal cavity increased mRNA levels of IFN-γ and CD8 in the spleen cells compared with poly(I:C)-immunized control mice (Fig. 1A). Similar data were obtained from the spleen cells of mice immunized intranasally with KSHV (data not shown). An ELISA to detect IFN-γ showed that both intranasal and intraperitoneal immunizations induced release of IFN-γ in the supernatant of the spleen cells after 8 h of culture (Fig. 1B). Release of IFN-γ was not observed in the spleen cells from poly(I:C)-immunized mice. These data suggest that both intranasal and intraperitoneal immunization with KSHV induced IFN-γ production in mice as a cellular immune response to KSHV.

3.2. Humoral response in KSHV-immunized mice

IgA plays an important role in protection from virus in the mucosae [33]. To know whether KSHV immunization induces humoral responses, including IgA expression, in mice, IgA and IgG titers in body fluids were measured in KSHV-immunized mice. There is currently no gold standard to measure the antibodies to KSHV, because the immunogens of KSHV are not constant in KSHV-infected individuals [34]. Therefore, IgA and IgG titers were determined with IFA using KSHV-infected lymphoma cells. IFA revealed that both intranasal and intraperitoneal immunization induced IgG and IgA to KSHV in serum (Fig. 2A and B). Titers of serum IgG and IgA increased in a dose-dependent man-

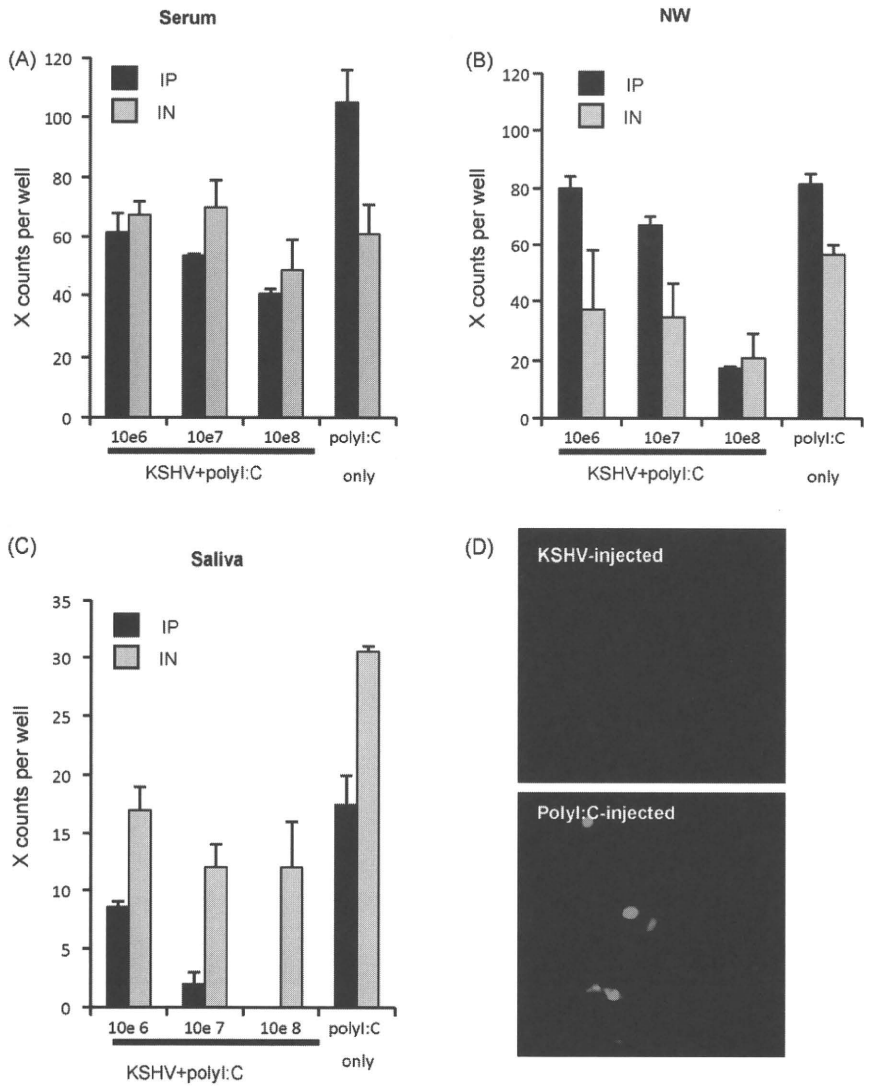

Fig. 3. Neutralization assay for KSHV using body fluids of KSHV-immunized mice. Serum (A), nasal wash fluid (NW) (B), and saliva (C) were used to neutralize GFP-expressing-KSHV infection to 293 cells. Numbers in the *y*-axis indicate counts of GFP^+ cells in each well. Error bars indicate standard deviations of triplicate experiments. IP: Intraperitoneal immunization. IN: Intranasal immunization. (D) Fluorescent images of neutralization assay using NW from 10^8 copies of KSHV-immunized (upper panel) and poly(I:C)-immunized (lower panel) mice.

ner to KSHV copies. In addition, IgA was detected in NW and saliva in mice immunized with KSHV intranasally (Fig. 2C and D), whereas the IgA titer in NW from intraperitoneally immunized mice with was low ($P<0.01$, in 10^8 copies of KSHV-immunized mice). These data indicate that both intranasal and intraperitoneal immunization with KSHV induced humoral response in mice, and IgA in the NW was induced effectively through the intranasal immunization.

3.3. Neutralization of KSHV infection with the body fluids of KSHV-immunized mice

To estimate the neutralization activity to KSHV of the serum, NW, and saliva, neutralization assay was performed using GFP-expressing recombinant KSHV, rKSHV.219, and 293 cells [28]. The serum of mice immunized intraperitoneally with 10^8 copies of KSHV showed reduced numbers of GFP^+ cells in 293 cells compared with serum of poly(I:C)-immunized mice ($P<0.05$, Fig. 3A). However, incubation with serum of intranasally immunized mice did not statistical significantly reduce the number of GFP^+ cells. The NW and saliva of mice immunized intraperitoneally or intranasally with 10^8 copies of KSHV showed reduced numbers of GFP^+ cells in a dose-dependent manner to KSHV copies immunized, compared with poly(I:C)-immunized mice ($P<0.05$, Fig. 3B–D). The numbers of GFP+ cells in saliva from poly(I:C)-immunized mice (Fig. 3C) was smaller than those in serum from poly(I:C)-immunized mice (Fig. 3A), implying that general humoral components in saliva reduced KSHV infection to 293 cells. Consequently, these data suggest that the body fluids from KSHV-immunized mice are able to reduce the efficacy of *in vitro* KSHV infection to 293 cells.

3.4. Immunogens encoded by KSHV in mice

Some of the KSHV-encoded proteins were identified as immunogens in human so far [4,34]. Among them, six KSHV-encoded proteins, K8, K8.1, ORF26, ORF59, ORF65, and ORF73 (LANA-1) were synthesized in *E. coli* as GST-fusion proteins to ascertain immunogens in KSHV-immunized mice [4]. Western blot revealed that GST-K8.1 and ORF59 proteins reacted more strongly with the serum from KSHV-intraperitoneally immunized mice than did other proteins (Fig. 4A). The serum also produced faint bands in the lanes of K8, ORF26, and ORF65 proteins, but not of ORF73C and ORF73N. Immunofluorescence assays using the serum and anti-KSHV-encoded protein antibodies demonstrated that the stain of the serum overlapped with those of K8.1 and ORF 59 frequently, of ORF26 and ORF65 partially, but not of K8 and ORF73. These data suggest that the serum of KSHV-immunized mice recognized mainly K8.1 and ORF59 protein, partially ORF26 and ORF65, but not K8 and ORF73.

3.5. Neutralization with the body fluids of KSHV-encoded protein-immunized mice

To know whether the KSHV-encoded proteins induce humoral immunity in mice, these proteins with poly(I:C) were immunized intranasally and intraperitoneally to mice. IFA using KSHV-infected cells revealed that intranasal and intraperitoneal immunization with the protein induced serum IgG and IgA to KSHV in the mice (Fig. 5A and B). Intranasal immunizations with the proteins also induced IgA to KSHV in the NW and saliva, as effectively as immunization with KSHV particles and ORF73 protein (Fig. 5C and D). The neutralization assay revealed that the serum from mice intraperitoneally immunized with GST-K8.1 reduced the numbers of KSHV-infected 293 cells in this assay ($P<0.05$), whereas the serum from mice intraperitoneally immunized with ORF59 and ORF73 proteins did not reduce them significantly ($P=0.55$, Fig. 6A). Neutralization activity of body fluid of K8.1-immunized mice was also shown in the NW of mice intranasally immunized with K8.1 protein ($P<0.01$, Fig. 6B). These data suggest the neutralization activity of the antibodies to K8.1 *in vitro*.

4. Discussion

In the present study, we demonstrated that KSHV immunization resulted in cellular and humoral immune response in mice. Spleen cells from KSHV-immunized mice produced IFN-γ, and the serum, NW and saliva of KSHV-immunized mice neutralized KSHV infection to 293 cells *in vitro*. The serum of KSHV-immunized mice recognized KSHV-encoded K8.1 and ORF59 proteins. The serum and NW from K8.1-immunized mice neutralized KSHV infection to 293 cells *in vitro* as effectively as the serum from KSHV-immunized mice. These results suggest a possibility of mucosal vaccine using inactivated KSHV particles or recombinant K8.1 protein for the prophylaxis of KS.

The animal experiments in the present study suggest that intranasal immunization of KSHV induced similar immune responses to intraperitoneal immunization in the production of serum IgA and saliva IgA (Fig. 2B and D). IgA level in NW of intranasally immunized mice is higher than those of intraperi-

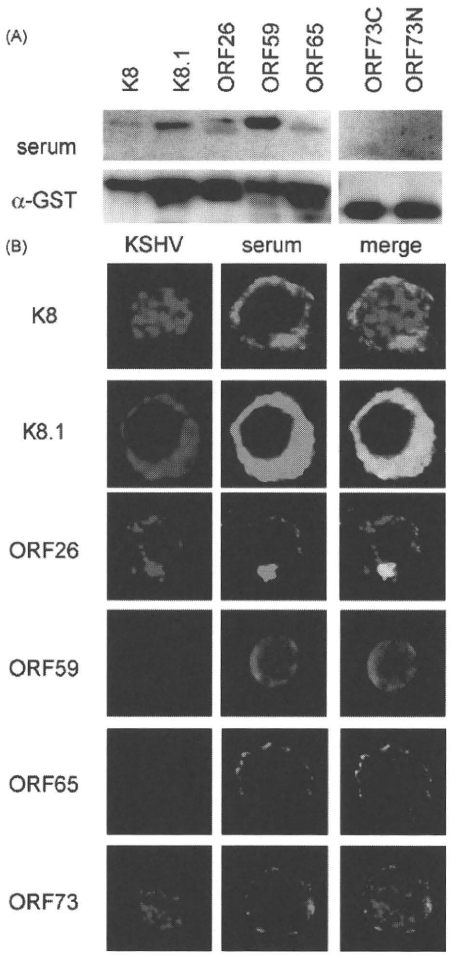

Fig. 4. Immunogens recognized by serum of KSHV-immunized mice. (A) Western blot. Seven KSHV-encoded proteins were synthesized as GST-fusion proteins and applied for western blot using serum of KSHV-immunized mice (upper panel) or anti-GST antibody (lower panel). (B) Dual-labeled immunofluorescence assay. KSHV-encoded proteins were labeled with Alexa-568 (red, left panels), and the mouse serum was labeled with Alexa-488 (green, middle panels). Merged images are shown in the right panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

toneally immunized mice (Fig. 2C). Considering that KSHV infects humans through the mucosae in the oral cavity or rectum, vaccination to the mucosae seems effectively to induce cellular and humoral immunity in human. Although it is unknown if intranasal immunization would induce similar immunity to a route using the rectum or oral cavity, the nasal or oral cavity is a promising candidate as a route of KSHV vaccination.

Immunogens of KSHV are important for development of KSHV vaccine. In this study, we identified the KSHV-encoded proteins, K8.1 and ORF59, as immunogens to which mouse serum reacted (Fig. 4A). K8.1 protein, a glycoprotein composing of virion membrane, was contained by virion, while ORF59 protein, a processivity factor for viral DNA polymerase, is not detected in KSHV virions [35]. Recognition of the serum to ORF59 protein suggests a possibility that KSHV entered in mouse cells and expressed the protein for a short period. In this study, several mice immunized with KSHV were autopsied, and all organs were investigated histopathologically. However, there was no specific disease to KSHV like KS or lymphoma, and immunohistochemistry for LANA-1 or ORF59 did not detect any positive signal in any organ, suggesting that ORF59 protein expression occurred for a very short period or at a very low rate in mice. In any case, serum from mice immunized with

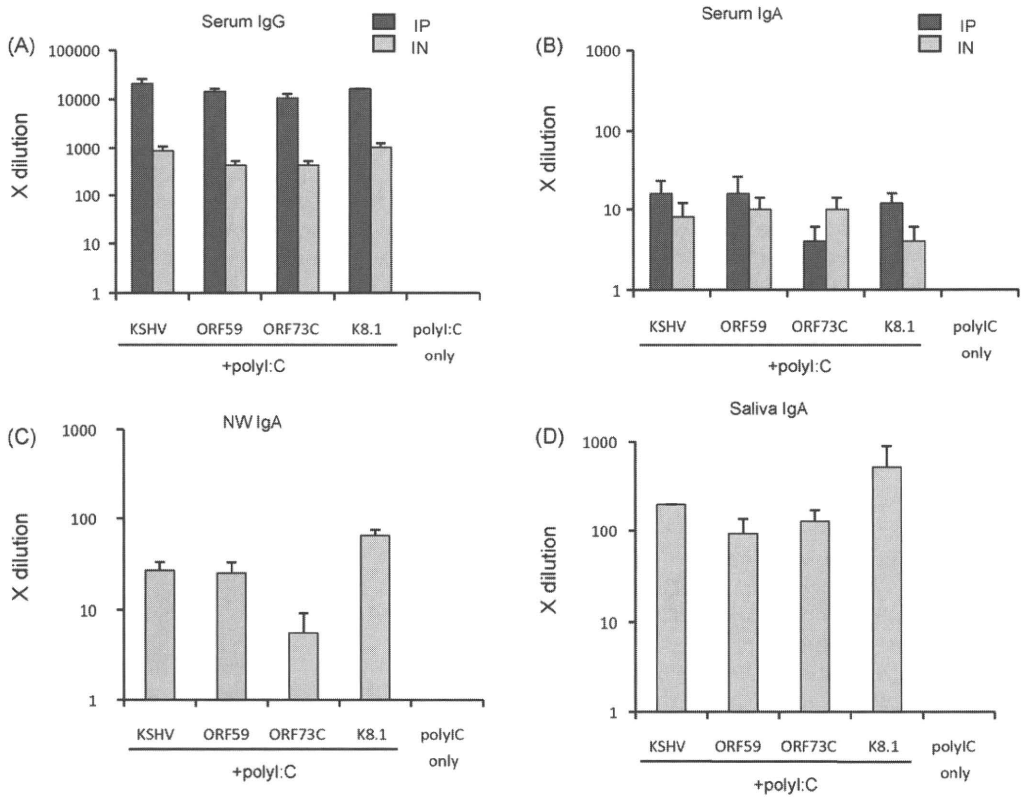

Fig. 5. Serum antibody titers of KSHV or KSHV-encoded protein-immunized mice by IFA. Titers of serum IgG (A), serum IgA (B), nasal wash fluid (NW) IgA (C), and saliva IgA (D) were determined by IFA using TPA-stimulated TY-1. Immunogens (10^8 copies of KSHV particles, and 10 ng of GST-ORF59, ORF73c, and K8.1) are shown in the *x*-axis. Error bars indicate standard deviations of five mice in each group. IP: Intraperitoneal immunization. IN: Intranasal immunization.

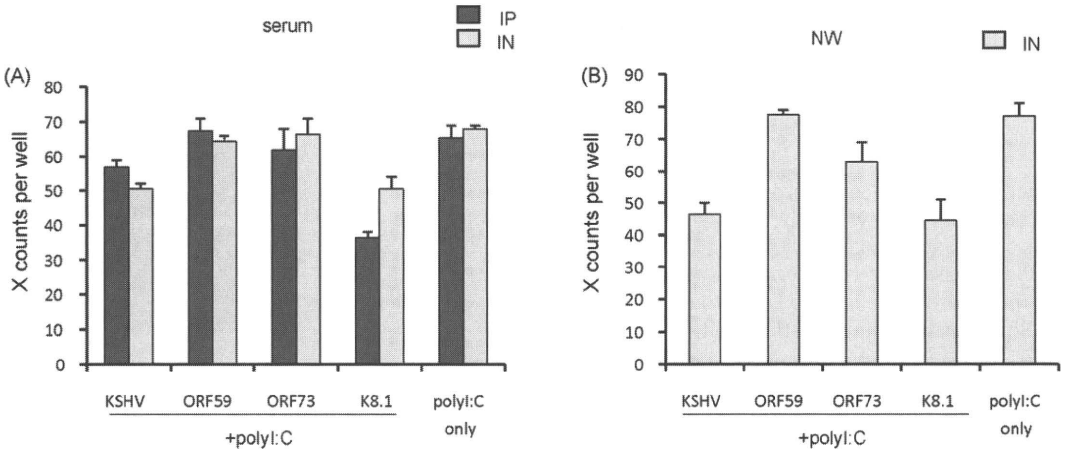

Fig. 6. Neutralization assay for KSHV using body fluids of KSHV or KSHV-encoded protein-immunized mice. Serum (A) and nasal wash fluid (NW) (B) were used to neutralize GFP-expressing-KSHV infection to 293 cells. Numbers in the *y*-axis indicate cell counts of GFP^+ cells in each well. Error bars indicate standard deviations of triplicate experiments. The serum was obtained from mice with intraperitoneal immunization; nasal wash fluid was obtained from mice with intranasal immunization.

the K8.1 protein, but not ORF59 protein, showed some effects for prevention of KSHV infection *in vitro* (Fig. 6). It is already shown that K8.1 protein interacts with cellular heparin sulfate, suggesting that K8.1 protein plays an important role in the attachment of KSHV to cell surfaces [36]. Like the serum from KSHV-immunized mice, the serum from K8.1-immunized mice reduced the number of $KSHV^+$ 293 cells partially, but not completely. The GST-fusion system cannot produce glycosylation modification, which may be one of the reasons why the serum protected 293 cells from KSHV infection partially. In addition, some previous studies demonstrated that one or a few proteins encoded by KSHV are not sufficient to detect serum antibodies to KSHV in humans, implying that single or a few recombinant viral proteins may not be sufficient for vaccine [4,34]. Although it is possible that some KSHV-encoded proteins may become vaccine targets [37,38], our data suggest that K8.1 may be one of suitable vaccine targets.

The selection of adjuvant is another issue for development of KSHV vaccine. Although poly(I:C) worked well in this study, the adjuvant should be selected considering the route of vaccination, volume of vaccine, and characterization of vaccine product. In addition, an animal model able to contract KSHV infection is required for development of KSHV vaccine. KSHV infects only humans, but no other species, including mice [22–25]. One study demonstrated that repeated intravenous immunizations of KSHV to NOD/SCID mice resulted in the establishment of latent KSHV infection; LANA-1 was immunohistochemically detected in the spleen of the mice in that report [24]. A recent study showed KSHV infected common marmosets [9]. However, there is currently no report describing successful KSHV infection in immunocompetent small animals. Thus, development of a new animal model is an important issue to estimate the efficacy of KSHV vaccine.

The seroprevalence of KSHV among the general population is extremely low compared with other herpes viruses [4,20]. Seropositivity of KSHV among the Japanese general population is about 1%, whereas many adults have antibodies to herpes simplex virus-1 (55–63%), varicella zoster virus (almost 100%), Epstein-Barr virus (>90%), cytomegalovirus (95% in pregnant women), and HHV-6 (79%) in Japan [4,39–43]. Since vaccine is generally effective for prevention of *de novo* infection of virus, a vaccine strategy could be effective for the prevention of KSHV infection in KSHV-uninfected individuals. Epidemiological data revealed that KSHV is widespread among MSM [3]. However, 40% of HIV-infected MSM were KSHV-uninfected in Japan [4]. In addition, vaccine should have some effect on the prevention of virus reactivation. In that sense, KSHV vaccine may have some effects on KSHV-infected individuals to prevent occurrence of KS. Thus, KSHV vaccine should be a promising tool for prophylaxis of KS. The present study provides a part of the fundamental data of animal experiments on KSHV. Further studies are required to develop the KSHV vaccine.

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Genotypic and Clinicopathological Characterization of Kaposi's Sarcoma-Associated Herpesvirus Infection in Japan

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Kaposi's sarcoma-associated herpesvirus (KSHV) is related causally to Kaposi's sarcoma, primary effusion lymphoma, and a subset of cases of multicentric Castleman's disease. As the numbers of acquired immunodeficiency syndrome (AIDS) patients have increased, KSHV-associated diseases have also increased in Japan. Sporadic cases of classic Kaposi's sarcoma have also been reported in Japan. In the present study, the clinicopathological characteristics of 75 samples, comprising 68 cases of Kaposi's sarcoma, 5 cases of primary effusion lymphoma, and 5 cases of multicentric Castleman's disease were investigated. All of these cases were positive for KSHV by immunohistochemistry or PCR analysis. All fifty-two of the AIDS-associated Kaposi's sarcoma cases were males, whereas 7 of the 13 non-AIDS-associated Kaposi's sarcoma cases were females. The mean age of patients with AIDS-associated Kaposi's sarcoma or primary effusion lymphoma was 46 years, whereas the mean age of patients with non-AIDS-associated Kaposi's sarcoma or primary effusion lymphoma was 71.8 and 97.5, respectively. KSHV genotypes were determined based on the sequence of variable region 1 in the *K1* gene. Genotypes A and C of KSHV were detected in both AIDS- and non-AIDS-associated Kaposi's sarcoma. Genotype A was detected more frequently in AIDS-associated cases than non-AIDS-associated cases, suggesting that genotype C is broadly distributed in Japan, and genotype A spreads among AIDS patients. Genotype D was detected only in non-AIDS-associated Kaposi's sarcoma. These data confirmed the difference between AIDS- and non-AIDS-associated KSHV diseases with regard to age of onset, gender, and genotypes in Japan. ***J. Med. Virol. 82:400–406, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: Kaposi's sarcoma; Kaposi's sarcoma-associated herpesvirus; AIDS; genotype

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus-8, HHV-8) was identified from a Kaposi's sarcoma (KS) specimen by representational difference analysis in 1994 [Chang et al., 1994]. KSHV has been detected in KS, primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (MCD) cases [Moore and Chang, 2001]. KS was first described in 1872 by Moriz Kaposi, a Hungarian dermatologist, as an idiopathic, multipigmented sarcoma of the skin [Kaposi, 1872]. KS is classified into four types: classic, endemic, iatrogenic, and acquired immunodeficiency syndrome (AIDS)-associated KS (AIDS-KS) [Antman and Chang, 2000]. Classic KS affects typically elderly men in Mediterranean littoral, endemic KS affects typically people in Africa, iatrogenic KS affects most commonly organ-transplant recipients receiving immunosuppressive therapy and AIDS-KS is mainly associated with homosexual men infected with human immunodeficiency virus (HIV). In Japan, KS was a very rare condition prior to 1980 [Fujii et al., 1986]. A few patients with adult

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