When measles was introduced to the USA, extensive transmission was controlled within two generations of secondary transmission by maintaining high-level vaccine coverage, effective surveillance, and a prompt outbreak response [CDC, 2008a,b]. The two-dose strategy has eliminated measles without indigenous measles transmission chain [Janaszek et al., 2003; Meissner et al., 2004]. Thus, the proportion of countries offering children a second dose of measles vaccine is increasing, and 168 (88%) countries implement the two-dose strategy [WHO, 2006]. The new goal is a 90% reduction of measles mortality by 2010 compared with the mortality in 1999 [WHO, 2006].

As for the reporting system for measles surveillance in Japan, through 3,000 sentinel clinics or hospitals for pediatric infectious diseases and 450 clinics for adult measles surveillance, patients with clinically suspected measles are reported to the Regional Health Care Center mostly without any systematic virological studies. The last measles outbreak was observed in 2001 in Japan. Among 33,812 reported cases, most patients were under 5 years of age and had not been vaccinated. Through a vaccination campaign to increase immunization coverage at 1 year of age, the number of reported cases decreased to 545 in 2005. The Japanese Government implemented a two-dose, combined measles and rubella vaccine (MR) for children at 1 and 6 years of age in 2006 [National Institute of Infectious Diseases, 2007]. Therefore, elimination of measles would be expected. However, patients with measles were reported increasingly in March 2007, and this outbreak expanded subsequently throughout the Japanese districts, peaking in the middle of May. Further, several reports indicated measles transmission by Japanese travelers or participants in an international sporting event [Delaportel et al., 2007; CDC, 2008a].

This outbreak showed different characteristics, demonstrating that most patients were young adults or adolescents attending high school and university students, with a much lower proportion of young infants, at the early stage of the outbreak. Finally, cases of measles were reported in all age groups, and a total of 3,105 pediatric cases and 959 adult patients were reported in 2007 and the outbreak is still ongoing. The actual number of cases of measles was estimated to be 10 times higher than the number of reported cases for pediatric measles, and 50–60 times higher for measles in adults. Thus, the number of patients with measles was suspected to be approximately 31,000 for pediatric and 50,000 for measles in adults. The age distribution was quite different from the previous outbreak in 2001. The number of patients with measles was the highest at 1–4 years of age, accounting for 40–50% in 2001, which decreased to 22% in the outbreak in 2007. A significant shift in the age distribution of cases of measles in 2007 was observed to be 10–14 years or higher, accounting for 44% in 2007 [National Institute of Infectious Diseases, 2007]. A relatively large proportion of adult patients with measles had a previous immunization history and, thus, typical measles symptoms were not observed, with patients only with mild fever and skin eruptions. In this report, virus isolation and detection of the measles virus genome by the reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) were examined in clinical samples. The genotyping and antigenicity of current circulating viruses were also investigated.

MATERIALS AND METHODS

Materials

Nasopharyngeal swabs were obtained from 22 patients suspected of measles on day 1 or 2 of the onset of the rash. Four patients had typical symptoms of measles with post-pigmentation, and 18 had atypical symptoms with fever for less than 3 days and mild eruptions, which did not satisfy the criteria of clinical measles by WHO, [2006].

Virus Isolation

B95a cells were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) and 0.1 ml of clinical samples was used to inoculate a monolayer of B95a cells in a 24-well plate. After two passages, samples without cytopathic effect (CPE) were considered negative. Seven strains were isolated in this study. MVi/Aichi.JPN/44.06 [D9] was isolated from a sporadic case in Nagoya City, a central district of Japan, in 2006. MVi/Tokyo.JPN/17.07 [D5] and MVi/Tokyo.JPN/18.07 [D5] were isolated in Tokyo and MVi/Mie.JPN/19.07 [D5] and.MVi/Mie.JPN/23.07 [D5] in Mie Prefecture, a central district of Japan, in 2007. In addition, MVi/Mie.JPN/41.07 [D5] was obtained in the middle of the outbreak in 2007 and MVi/Mie.JPN/03.08 [D5] was isolated in 2008.

RT-PCR and Sequence Analysis

Total RNA was extracted from 200 μl of clinical samples with a magnetic bead RNA extraction kit (TOYOBO Co., Ltd., Osaka, Japan), and the RNA pellet was suspended in 30 μl of distilled water. The pellet was subjected to RT-nested PCR and RT-LAMP targeting the C-terminus of the N protein region, known as the most variable region [WHO, 2001]. The measles virus genome was first converted to cDNA with the N-430 (+) primer (5′-ATTAGTAGTGATCAATCCAGG) with AMV reverse transcriptase (Life Technologies Inc., Gaithesburg, MD). The first PCR was performed with a set of N-850 (+) (5′-TAGAAACTATGTATCCTGCT-3′) and MPX (−) (5′-AGGCCTGATTGAACCATGAT-3′) and the nested PCR was conducted with N1200 (+) (5′-GATCCAGCATATTTTAGATTAG-3′) and NP-P2 (−) (5′-AGGGTAGGCGGATGTTGTTCT-3′). PCR was performed using 1.25 U of *Taq* DNA polymerase (TaKaRa Bio-Medicals, Tokyo, Japan) with a TaKaRa thermal cycler (TaKaRa BioMedicals), with 30 rounds of thermal cycling conditions: denaturing at 93°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2.5 min. PCR products were confirmed by

electrophoresis through 1.5% agar stained with ethidium bromide, as previously reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003]. PCR products were excised from the gel and applied to sequence analysis by the dye terminator method using ABI 3130 (Applied Biosystems, Tokyo, Japan).

Measles Virus Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method is characterized by auto-cycling strand displacement DNA synthesis with *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) and a specially designed set of primers. Six LAMP primers were synthesized, recognizing eight different regions: F3 (5′-ACATTGGCATCTGAACTC), B3 (5′-TCCTCGACTCTGTTTGAC), FIP (5′-TGTCCTCAGTAGTATGCATTGCAGGTATCACTGCCGAGGATG), BIP (5′-AGCCCAAGTGTCATTTCTACACGGTGTCCTATCTTCCTTGCCCCCC), F Loop (5′-ATCTCTGAAACAAG), and B Loop (5′-CAAAGTGAGAATGAGCT). For the LAMP reaction, the mixture was made up to a total of 25 μl of reaction mixture, containing 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3, 20 pmol (each) of Loop F and Loop B, 1.4 mM each of dNTPs, 0.8 M betaine, 20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, 0.5 U AMV reverse transcriptase (New England Biolabs), 8 U *Bst* DNA polymerase (New England Biolabs), and 5 μl of sample RNA. The reaction mixture was subjected to real-time turbidimeter LA200 (TERAMECS, Kyoto, Japan) [Mori et al., 2004; Fujino et al., 2005] and the LAMP reaction was carried out at 63°C for 60 min. The turbidity measurement was closely related to the amplification of DNA and the turbidity >0.1 was considered LAMP-positive [Mori et al., 2004].

Neutralization Test (NT) Antibody

A total of 32 serum samples were used, which were obtained in April 2007, just before the peak of the outbreak, as part of a sero-epidemiological study on entering primary school at the age of 6 years, having received two doses of measles vaccine. The AIK-C vaccine strain [A], MVi/Tokyo.JPN/31.00-K [D5], MVi/Tokyo.JPN/17.07 [D5], and MVi/Aichi.JPN/44.06 [D9] were used as the challenge virus. Sera were treated at 56°C for 30 min to inactivate the complement activity and serially diluted by twofold, starting from 1:4 dilutions. Diluted sera were mixed with approximately 100 TCID50 of challenge virus at 37 C for 90 min and the mixture was placed on a monolayer of B95a cells. NT antibody titers were expressed as the reciprocal of the serum dilutions that neutralized the appearance of the CPE of measles virus [Okafuji et al., 2006].

RESULTS

Laboratory Examination of Patients With Non-Typical Measles Illness

From March 2007, several patients with measles were observed in outpatient clinics and 22 clinical samples were examined for virus isolation and genome detection and the results are shown in Table I. Four patients had typical measles with a marked fever for more than 3 days and typical measles eruptions with post-pigmentation. Three patients were adults and one had a history of vaccination. One patient was 8 years old and complicated with Gitelman syndrome during the measles illness. Two measles strains (MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07) were isolated and the measles genome was detected in all patients by RT-PCR and RT-LAMP (Table Ia).

During the measles outbreak, 18 clinical samples were obtained from patients with non-typical measles with mild febrile illness and eruptions for less than 3 days. They were over 15 years of age and had a past history of single-dose measles immunization at 1–3 years of age. Measles virus was not isolated but the measles genome was detected in 9 (50%) by RT-PCR and in 12 (67%) by RT-LAMP (Table Ib). All RT-PCR positive samples were also positive on RT-LAMP.

MVi/Aichi.JPN/44.06, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, and MVi/Mie.JPN/41.07 were isolated from patients with modified measles and MVi/Mie.JPN/03.08 was isolated from typical pediatric measles patient. Detection of the measles genome was not examined directly from the clinical samples in these five cases.

Genotype Analysis

Seven measles strains were isolated and examined for sequence analysis of the C-terminal of the N protein region, as recommended by WHO, [2005a]. MVi/Aichi.JPN/44.06 was isolated from a sporadic case

TABLE I. Results of Laboratory Examinations

Case	Vac	V. Iso.	RT-PCR	LAMP
a. Four typical measles cases				
1 (23Y)	+	+	+	+
2 (19Y)	–	–	+	+
3 (18Y) Encephalitis	–	–	+	+
4 (8Y) Gitelman syndrome	–	+	+	+
	V. Iso. (+)	RT-PCR (+)	LAMP (+)	
b. 18 cases with mild fever and rash	0/18	9/18	12/18	

Vac: Past history of measles vaccination
V. Iso: Virus isolation

in Nagoya in 2006, a central district of Japan, before the nationwide outbreak, and this strain did not cause further transmission. MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07 were isolated in Tokyo. MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated in Mie Prefecture, a central area of Japan, during the outbreak 2007–2008. The results of phylogenetic analysis are shown in Figure 1. MVi/Aichi.JPN/44.06 was D9 and two stains isolated in Tokyo at the beginning of the outbreak in 2007 were identified as genotype D5. Four strains isolated in Mie Prefecture were also identified as genotype D5. MVi/Gunma.JPN/19.07 was isolated in Gunma Prefecture, located in the north direction of Tokyo, was also D5 [Morita et al., 2007]. Measles outbreaks last for more than a year and a relatively homologous strain has been circulating throughout Japan.

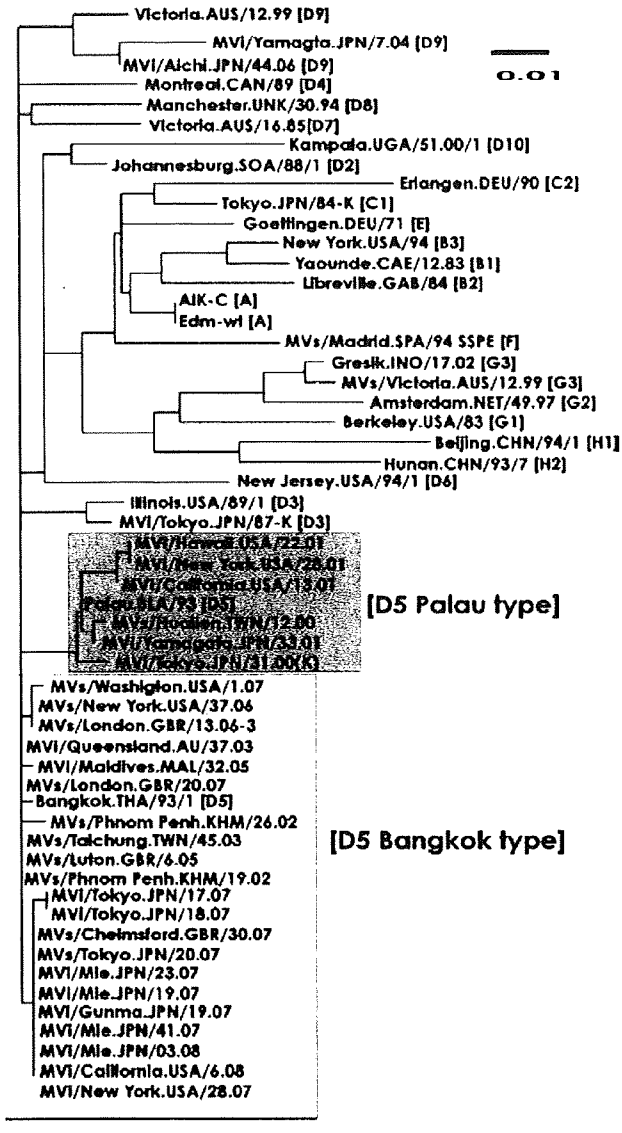

Fig. 1. Phylogenetic analysis of measles virus in the N gene. MVi/Aichi.JPN/44.06 was isolated in 2006 and MVi/Tokyo.JPN/17.07, MVi/Tokyo.JPN/18.07, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated during the outbreak 2007–2008 in this study. MVi/Gunma.JPN/19.07 was reported by Morita et al. [2007] and other strains were registered in GeneBank. The Palau type D5 strains are shown in gray square and the Bangkok type D5 strains in open square.

Two different reference strains, MVi/Bangkok.THA/93/1 and MVi/Palau.BLA/93, were identified as genotype D5 and the Palau type D5 was transmitted via a Japanese traveler in 1993 [Rota et al., 1998]. The Palau type D5 was a major circulating genotype from 1990 to 1997 and from 2002 and afterward [Nakayama et al., 2003; Zhou et al., 2003; Okafuji et al., 2006]. However, the D5 strains in 2007–2008 belonged to the Bangkok type D5. Among D5 sequences obtained in 2007–2008, there was one nucleotide difference in the target of the N gene. Accession numbers of the partial N gene sequence of the isolates in this study are AB426895–99.

In Switzerland, 11 cases of measles were reported between March and April 2007 and, thereafter, a large nationwide outbreak was reported with further expansion to other European countries and the USA [Delaporte et al., 2007; Richard et al., 2008; CDC, 2008b]. MVi/New York.USA/28.07 and MVi/California.USA/6.08 were registered as imported from Switzerland and classified into the Bangkok type D5. In 456 nucleotides of partial N gene, there was no difference among isolates in outbreak in Japan, 2007–2008 and those prevalent in Europe and the USA. The epidemiological linkage was not identified but would be suspected by molecular epidemiological data.

The entire length of the H gene was sequenced and analyzed together with reference strains and data from the previous reports. Four strains isolated in 2007 were classified as the Bangkok type D5, not Palau type (Fig. 2). Differences in the nucleotide and amino acid sequences of the H gene are shown in Table II. Sequence variations of the H gene consisted of 52 (2.8%) to 59 (3.2%) nucleotide differences among genotypes A (AIK-C vaccine strain) and D5 strains, and 66 (3.6%) between genotypes A and D9. There were 16–19 (2.6–3.1%) amino acid changes in D5 or D9 in comparison with genotype A. In the same D5 strains, MVi/Tokyo.JPN/17.07 [Bangkok type] showed 35–43 (1.9–2.3%) nucleotide differences in comparison with the Palau type D5 strains, MVi/Palau.BLA/93 or MVi/Tokyo/JPN/31.00-K, but 16 (0.9%) in comparison with MVi/Bangkok.THA/93/1. Accession numbers for the entire H gene sequence of the isolates in this study are AB426900–04.

Antigenic Differences

MVi/Tokyo.JPN/18.07 [Bangkok type D5], MVi/Tokyo.JPN/31.00-K [Palau type D5], MVi/Aichi.JPN/44.06 [D9], and the AIK-C vaccine strain [A] were used for the analysis of antigenicity. Five or six serum samples for each NT antibody titer were selected, for which the NT titers against the AIK-C strain had already been checked, and a total of 32 sera were used for the analysis of antigenicity. The challenge viruses were

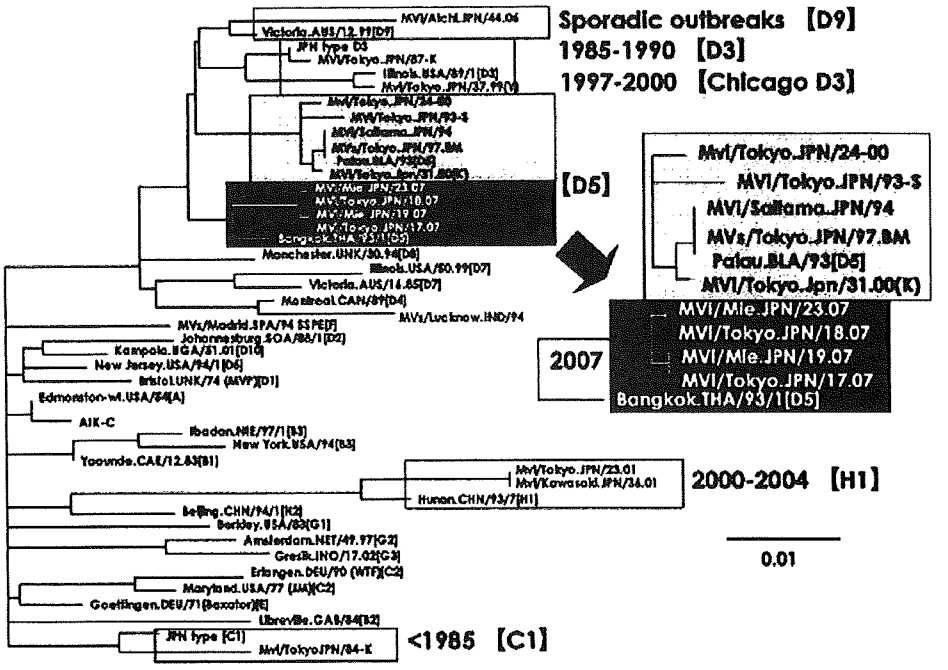

Fig. 2. Phylogenetic analysis of measles virus in the H gene. Strains in gray square are Palau type D5 and those in black square Bangkok type D5.

adjusted to 100 TCID50 and the results are shown in Figure 3. There was no significant difference in NT antibody titers within fourfold dilutions against the different genotypes of A, Palau type D5, Bangkok type D5, and D9.

Characteristics of Wild-Type Measles Virus

Seven wild-type measles virus strains were isolated and the current wild type did not induce cell fusion in Vero cells. D5 and D9 strains infected and replicated in Vero cells without demonstrating typical cell fusion. Infectious titers in Vero cell culture fluid detected 7 days after infection were similar or 1/2 or1/3 lower than those in the B95a cell culture (data not shown).

Measles virus grew well at 35–37°C and virus growth at 39°C differs from strain to strain [Nakayama et al., 2003]. Virus growth of MVi/Aichi.JPN/44.06 at different temperatures of 33, 35, 37, and 39°C was investigated in B95a cells, and the results are shown in Figure 4. It grew well at 33, 35, and 37°C, but the infective titers at 39°C decreased to approximately 1/100 in comparison with those observed at 33 or 35°C. Three D5 strains isolated in 2007 and MVi/Tokyo.JPN/93-S [Palau type D5] were cultured in B95a cells at 35°C and 39°C and the infective titers on day 7 of culture are also shown in Figure 4. As previously reported, MVi/Tokyo.JPN/93-S grew well at 39°C as it did at 35°C, but the infectivity of the Bangkok type D5 was lower at 39°C than at 35°C.

DISCUSSION

The WHO global measles and rubella laboratory network (LabNet) has been established and the Western Pacific Region adopted the goal of measles elimination by increasing the use of laboratory testing as an integral component of its surveillance. They recommended that effective surveillance comprised laboratory confirmation by the detection of IgM antibody in single serum, together with virus isolation for genotyping. However, the sensitivity of virus isolation is low and depends on the timing of sample collection and transporting conditions. The detection of measles virus-specific IgM antibodies in serum is a standard serological diagnostic

TABLE II. Nucleotide and Amino Acid Differences in D5 and D9 Strains

	AIK-C	Palau	Bangkok	Tokyo, 2000	Tokyo, 2007	Aichi, 2006
AIK-C		52 (2.8%)	53 (2.9%)	59 (3.2%)	57 (3.1%)	66 (3.6%)
Palau	16 (2.6%)		25 (1.3%)	10 (0.5%)	35 (1.9%)	45 (2.4%)
Bangkok	18 (2.9%)	8 (1.3%)		33 (1.8%)	16 (0.9%)	39 (2.1%)
Tokyo.JPN/2000	17 (2.8%)	5 (0.8%)	11 (1.8%)		43 (2.3%)	54 (2.9%)
Tokyo.JPN/17.07	16 (2.6%)	8 (1.3%)	4 (0.7%)	11 (1.8%)		50 (2.7%)
Aichi.JPN/44.06	19 (3.1%)	11 (1.8%)	10 (1.6%)	14 (2.3%)	10 (1.6%)	

Values present diagonally below in the table body indicate amino acid differences and the values present diagonally above in the table body indicate nucleotide differences.

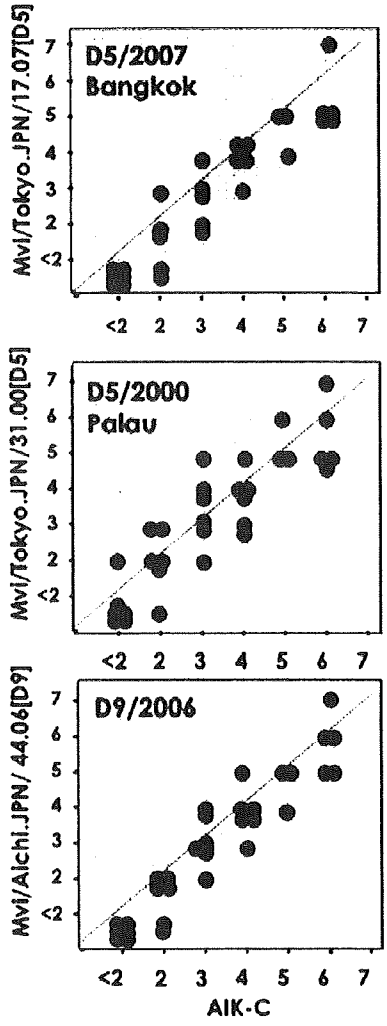

Fig. 3. Neutralization test antibodies against the AIK-C vaccine strain (genotype A), MVi/Tokyo.JPN/17.07 (genotype D5 Bangkok type), MVi/Tokyo.JPN/31.00 (genotype D5 Palau type), and MVi/Aichi. JPN/44.06 (genotype D9).

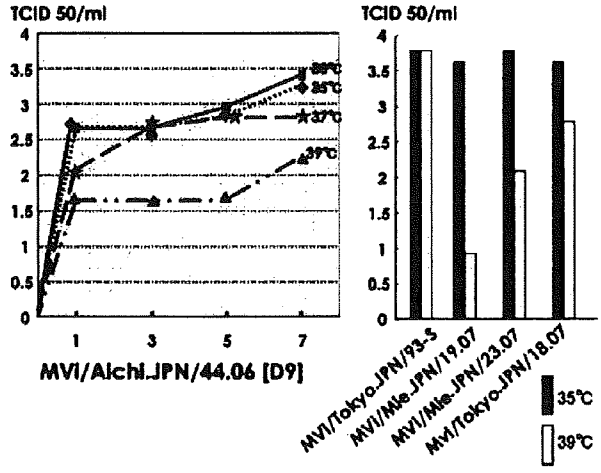

Fig. 4. Virus growth at different temperatures of 33, 35, 37, and 39°C.

method when serum samples are obtained on day 4 or 5 of rash onset and later [WHO, 2005b]. These methods were effective in the case of the primary infection causing typical measles illness. However, in the case of modified or subclinical measles, the detection rate of serum IgM antibodies was reported to be extremely low and the genomic detection by RT-PCR should be examined [Mosquera et al., 2005]. In this report, serum IgM antibody against measles virus was initially examined in several patients, but it was negative because all patients visited the clinics within day 1 or 2 after rash onset. In addition, the patients over 15 years of age having a single dose of measles immunization demonstrated very mild febrile illness with eruptions. Among 18 patients, 12 were diagnosed by RT-LAMP. The remaining six patients were not confirmed, because paired sera were obtained rarely from adults with mild symptoms. Detection of the measles genome is reliable and sensitive method and these six patients were supposed to have some other virus infections.

van den Hof et al. [2003] examined 791 serum samples from Dutch persons aged 2–49 years. The sero-prevalence and mean titers of measles EIA and NT were lower in the vaccinated populations than in older individuals infected naturally, and vaccine-acquired immunity weakened year by year. Glass and Grenfell [2004] developed a mathematical model to predict clinical and subclinical measles cases. Vaccination levels dropped from >90% in the 1990s to 84% in 2001/2002 in England, and they considered that clinical measles cases would remain fairly stable over time if vaccine coverage is maintained at 90%, but that there would be a pronounced increase in the numbers of clinical cases if vaccine coverage is around 84% or below. In any case, they predicted an increase in the number of subclinical measles. In the measles outbreak in Japan 2007–2008, a large proportion of cases of measles comprised adults or teenagers with very mild illness, and many cases went undiagnosed. These cases were confirmed by detection of the measles genome in this study and the diagnostic approach is now combining genome detection with virus isolation and the detection of IgM antibodies [CDC, 2008c].

Circulating wild-type measles virus genotypes have been investigated since 1984 and dynamic changes in the major circulating genotype have been reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003; Nakayama et al., 2004; Okafuji et al., 2006]. The dominant circulating genotypes changed drastically in large outbreaks in 1984, 1987–1988, 1991–1993, and 2001–2002 and each outbreak was caused by a different genotype in Japan, as summarized in Figure 5. Genotype C1 was an indigenous strain for a long period before 1985, D3 was involved in the 1987–1988 outbreak, and D5 in 1990–1993. The genotype of measles virus was studied in India from 1994 to 1997. The indigenous strain in India was D4, and a large outbreak was observed in 1997, caused by the Chicago type D3 strain [Nakayama et al., 2004]. In 1997, the Chicago type D3 was isolated in Japan, which was a different cluster from

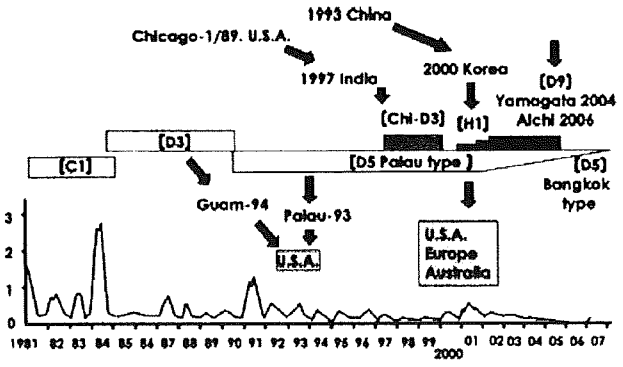

Fig. 5. Major circulating measles genotypes since 1984.

the D3 strains in 1987–1988. The Chicago-type D3 in 1997–1999 was supposed to have been imported from outside, and it was replaced by D5 in 2000, similar to the previous Palau type D5 before 1997. The H1 genotype was isolated from adult measles patients, and this genotype became a dominant strain after 2000 [Zhou et al., 2003]. In 2004, a sporadic outbreak was reported at a junior high school in Yamagata Prefecture and several measles strains were isolated. They were identified as genotype D9, which had not previously been reported in Japan [Mizuta et al., 2005]. In 2005, sporadic outbreaks were reported in Chiba and Ibaraki Prefectures, caused by the Palau type D5. In Asian countries, several different indigenous genotypes have been reported: H1 in China [Xu et al., 1998], G2 in Indonesia and Malaysia [Rota et al., 2000], D4 in India [Wairagkar et al., 2002], D3 in Papua New Guinea [Miki et al., 2002], and D7 in Australia [Chibo et al., 2003]. D8 in Nepal [Truong et al., 2001] and D9 in Australia [Chibo et al., 2003] were also reported. The sporadic outbreak caused by D9 in Yamagata in 2004 was suggested to have been imported from outside. In this study, D9 was also isolated in a sporadic outbreak in Aichi Prefecture in 2006, and seven nucleotide differences in the partial N gene (7/456: 1.5%) were observed between the two D9 strains of the MVi/Yamagata.JPN/7.04 and MVi/Aichi.JPN/44.06. There was no information on the isolation of D9 during 2004–2006 and no epidemiological linkage. Thus, they seemed to have been imported independently from other Asian counties, considering the narrow transmission chain in Japan.

The Palau type D5 was an indigenous strain since 1990 in Japan but was interrupted by outbreaks of the Chicago type D3 from 1997 to 2000 and of H1 from 2001 to 2004. Genotype D5 was detected in a sporadic outbreak in 2006 around Tokyo and transmitted to Okinawa, which was identified as the Bangkok type D5, which was different from the Palau type circulating during1990–2005 in Japan [National Institute of Infectious Diseases, 2007; Morita et al., 2007]. The Bangkok type D5 isolated in Japan in 2007 would have been imported from other Asian countries rather than being a result of the accumulation of mutations of the indigenous Palau type D5. Similar Bangkok type D5 strains (MVi/Queensland.AU/37.03, MVi/Maldives. MAL/32.05, MVs/Phnom Penh.KHM/19.02, MVs/Taichung. TWN/45.03) were reported in Asian countries and also in the UK (MVs/Chelmsford.GBR/30.07).

In 2007 and 2008, similar strains were reported in Europe and the USA and epidemiological linkage to the outbreak in Switzerland was identified [Delaportel et al., 2007; Richard et al., 2008; CDC, 2008b]. In this report they were thought to have been transmitted from Japan, demonstrating high-level sequence homology to Japanese isolates. Asia is the major reservoir of the wild measles virus and several Asian genotypes have been detected in the USA. and Europe. Global knowledge on the distribution of genotypes is imperative in identifying the geographical regions where more aggressive vaccination campaigns should be implemented in order to eliminate measles.

Minor antigenic changes in the Chicago type D3 and H1 strains were reported in comparison with the NT titers against the AIK-C vaccine strain [Zhou et al., 2003; Nakayama et al., 2004]. In this study, 1.3–1.8% amino acid differences were observed between the D5 Bangkok and Palau types. Therefore, the difference in the antigenicity was investigated and there were no significant differences in NT antibody titers against the Bangkok type D5, Palau type D5, and D9 strains in comparison with those against the AIK-C vaccine strain. No significant difference was demonstrated in terms of antigenicity. Some virus strains of the Chicago type D3 and Palau type D5 grew as well at 39°C as they did at 33, 35, and 37°C. However, the Bangkok type D5 and D9 showed poor growth at 39°C. The responsible genomic region(s) for virus growth at different temperatures is now under investigation.

Recommendations to prevent further transmission from the index case of importation noted that the outbreak investigation was conducted when the index case of a Japanese boy had a measles-like illness and only six additional cases were identified [CDC, 2008a]. Transmission was interrupted because of the high vaccination coverage rates of two-dose MMR among children and adolescents. The prompt response was sustained by strong and effective surveillance systems. The WHO has recommended that all children should be provided with a second opportunity for measles vaccination [WHO, 2006]. This second opportunity is scheduled just before entry to primary school in most countries, and a two-dose schedule of combined measles–rubella vaccine was launched in 2006 in Japan. The scheduled timing of the two doses is at the age of 12–24 months and 5–6 years. Supplemental immunization for the other populations was not considered, and most school children (over 8 years of age) did not have the benefit of the two-dose schedule. Okafuji et al. [2006] reported that NT antibodies decreased to undetectable levels in approximately 10% of vaccine recipients 6–7 years after vaccination when the measles outbreak was controlled. The outbreak in Japan 2007 highlighted the inadequacy of the immunization strategy, leading to a gap in immunization among teenagers without

supplemental immunization. Additionally, several had no immunization history because of a distrust of the vaccine due to the MMR scandal from 1989 to 1993 in Japan [Ueda et al., 1995]. All school children and young teenagers should have a second dose of measles vaccine to attain the goal of measles elimination. Thus, the Japanese government has decided to launch a catch-up campaign targeting young teenager at 13 and 18 years of age for the next 5 years, anticipating elimination of measles by 2012. The infrastructure for the surveillance system based on laboratory-based diagnosis is now in preparation.

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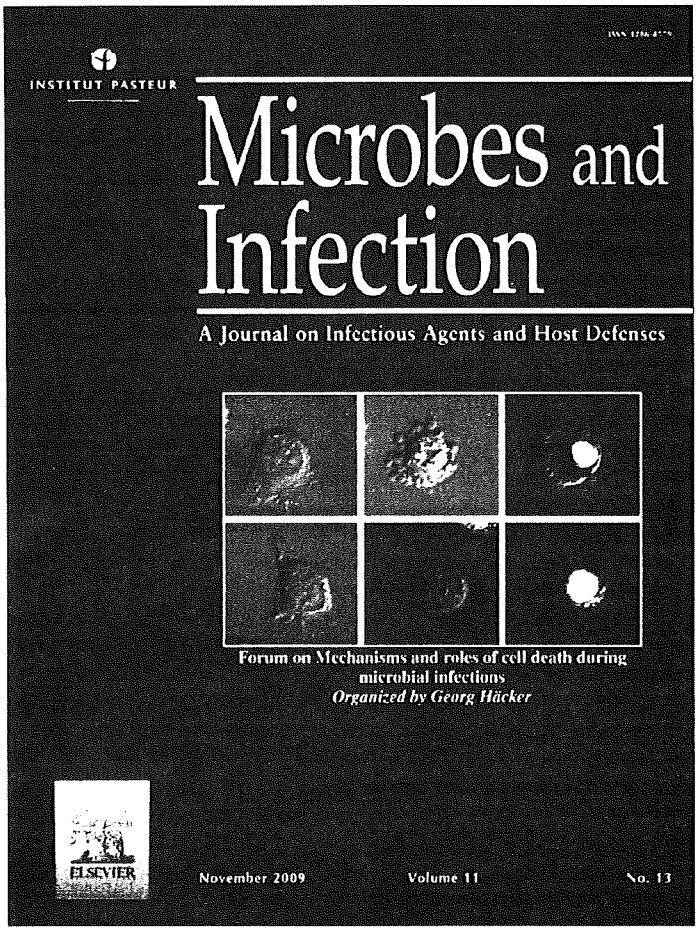

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Original article

Recombinant human monoclonal antibodies to human cytomegalovirus glycoprotein B neutralize virus in a complement-dependent manner

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Abstract

Human antibodies specific for HCMV are currently considered as potential anti-HCMV therapeutic agents. In this study, we used a combinatorial human antibody library to isolate and characterize complete human monoclonal antibodies that effectively neutralize HCMV in a complement-dependent manner. One hundred and six clones were isolated in two independent screens using HCMV virions and recombinant glycoprotein B, gB654, as antigens. All of the clones recognized the same molecule gB and were classified into 14 groups based on the amino acid sequence of the V_H region. Seven representative clones from these 14 groups had a strong gB654 binding affinity by surface plasmon resonance (SPR). A pairwise binding competition analysis suggested that there were three groups based on differences in the gB recognition sites. Although Fab fragments of the seven groups showed strong affinity for gB, none of the Fab fragments neutralized HCMV infectivity *in vitro*. In contrast, complete human IgG_1 antibodies of at least three groups neutralized HCMV in a complement-dependent manner. These data suggest that potent therapeutic antibodies can be obtained from a human antibody library, including most of the functional antibodies that mediate humoral immunity to the selected pathogen.

Keywords: HCMV; Human antibody; Phage display; Antiviral agents

1. Introduction

Primary human cytomegalovirus (HCMV) infection generally occurs in infancy or early childhood but is asymptomatic in immunocompetent hosts. However, HCMV is a major cause of morbidity and mortality in immunocompromised individuals, including transplant recipients, AIDS patients and newborn infants [1]. Additionally, congenital HCMV infection can cause severe neurological and visceral manifestations and in rare instances is fatal. Due to the number and severity of these infections, HCMV infection is associated with a large economical burden. Antiviral drugs (Ganciclovir, GCV; Foscarnet, PFA; Cidofovir, CDV; etc.) have been used to treat patients with HCMV-related diseases [2]. Although these drugs reduce the risk of HCMV-related diseases in patients, they can induce serious side effects, including myelosuppression and renal toxicity [3]. Another treatment strategy is cytomegalovirus-hyper immune globulin (CMVIG), which is used as a pre-emptive treatment for post-transplantation patients [4,5]. A meta-analysis performed by Bonaros et al. suggested that prophylactic administration of CMVIG effectively prevents CMV disease and CMV-associated deaths in patients after solid organ transplantation [6]. Moreover, it was

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suggested that treating pregnant women with CMV-hyperimmune globulin is an effective treatment and prevention strategy for congenital CMV infection [7]. These data suggest that administering anti-HCMV immunoglobulin is a promising treatment option for HCMV infection. However, as immunoglobulin is obtained from donated blood, the possibility of using contaminated or infectious blood is not completely eliminated. Thus, effective monoclonal antibodies that prevent HCMV infection and are free from the risk of infection may be useful to treat patients.

Because of recent developments in antibody engineering, a number of antibody preparations are currently used as clinical treatments [8]. There are two major technologies used to isolate human or humanized monoclonal antibodies: hybridoma technology used to produce mouse-human chimeric antibodies and phage display antibody library technology [9]. The anti-HCMV human monoclonal antibody, MSL-109 (Protein Design Laboratories, CA, USA), is a product of hybridoma technology that uses a non-producing murine-human hybrid myeloma fused to human B-lymphocytes that are stimulated *in vitro* [10]. However, when the efficacy of MSL-109 was evaluated in several clinical trials, this monoclonal antibody showed no significant reduction in CMV infection or CMV disease [11,12]. It remains unclear why MSL-109 was ineffective in clinical trials despite the robust neutralizing activity *in vitro*. By contrast, phage-displayed library technology enables various antibody clones to be selected [9]. We previously established a human antibody library that contains a large number of clones from surgically resected tonsillar tissues and obtained over three hundred antibody clones against carcinoma-specific antigens, and also obtained antibodies against rotavirus and varicella zoster virus [13–15].

It is widely thought that establishing a safe and effective antibody treatment may be important for improving treatment strategies for HCMV infections. In order to produce effective human monoclonal antibodies, we thought that a phage library established from lymphocytes from a healthy individual with a high anti-HCMV antibody titer would be suitable. Therefore, in the present study, we established a large antibody library derived from a pediatrician with a high anti-HCMV antibody titer. Thus, it is expected that comprehensively screening this large antibody library would yield many suitable types of anti-HCMV-neutralizing antibodies.

2. Materials and methods

2.1. Cells and viruses

The human embryonic lung fibroblasts cell line MRC-5 was maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum. The human embryonic kidney cell line HEK293T was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. HCMV laboratory strain AD169 was distributed from the Research Institute for Microbial Diseases (Osaka, Japan) and propagated in MRC-5 cells. HCMV strain Towne was propagated in HEL cells. 91S and 93R were clinically isolated viruses propagated from previously reported viruses [16].

2.2. Construction of gB654 expression plasmids

gB654 is a truncated form of glycoprotein B, encoded by HCMV UL55. gB654 consists of 654 amino acids and excludes the N-terminal 24 amino acids that encode the signal peptide and amino acids 680 to 906. The gB654 expression plasmid was constructed using standard methods. The UL55 gene was directly amplified by pfu DNA polymerase (Fermentus) following the manufacturer's instructions, using the HCMV AD169 genome as a template. We used the following primer set, which contains enzyme recognition site (italicized), (5′-primer, GGGCCC*AAGCTT*GTCTTCTAGTACTT CCCATGC; and 3′-primer, CGCCGC*GGATCC*GAGAGG TCAAAAACGTTGCTGG). The amplified fragments were digested with HindIII and BamHI enzymes and then cloned into the pSecTag2A (Invitrogen, Carlsbad, CA) vector. The pSecTag2A vector contained a 6×His-tag and was used for soluble protein expression.

2.3. Expression and purification of gB654 6×His-tagged protein

The gB654 expression plasmid, pgB654-SecTag2A, was extracted from transformed *Escherichia coli*, competent cells JM109 (Promega) using the Plasmid Maxi Kit (QIAGEN, Germantown, MD). 27 μg of purified plasmid was transfected into 293 T cells in 15-cm dishes using Lipofectoamine 2000 reagent (Invitrogen). After a 48-h incubation with serum-free medium, the supernatant of transfected cells was harvested, and affinity-purified using 6×His-tag with Ni-NTA agarose (QIAGEN) following the manufacturer's instructions. The purified gB654-myc-His protein was detected by Western blotting with a rabbit polyclonal anti-myc antibody (MBL, Nagoya) diluted 1:2000, a mouse monoclonal anti-Histidine-tag antibody (MBL) diluted 1:5000, and a goat polyclonal anti-HCMV antibody (Biogenesis, England) diluted 1:2000.

2.4. Antibody library

The human antibody library "A-library" was used as the antibody source. The library was constructed using the lymphocyte-rich fraction of peripheral blood donated by a volunteer who had a high HCMV-neutralizing antibody titer. Mononuclear cells were collected by apheresis from the equivalent of 3 L of blood from an HCMV-seropositive pediatrician. The resulting cell population contained approximately 10^6 B-lymphocytes. This library is a combinatorial antibody library, which was made from V_H and V_L DNAs reverse-transcribed using total RNAs from lymphocytes as described previously [13, 15]. An Fab antibody fused to cp3 (Fab-cp3) was expressed on the phage surface. The plasmid, which includes the V_H, C_H1, V_L and C_L coding regions and the

cp3 protein coding region, was called "phagemid". The "A-library" consisted of approximately 10^{11} phagemids.

2.5. Screening for CMV binding antibodies using a human antibody library

The phages exhibiting antigen-binding activity were selected using a panning method as described previously [13,15]. Two antigens (purified HCMV virions and gB654 protein) were used to perform the panning. The phage was recovered by the third round of panning in the screen, and were infected into *E. coli* without helper-phages and spread onto plates. The clones that expressed a Fab-cp3 protein that bound the antigen were selected as neutralizing HCMV human antibody candidates. Finally, the V_H and V_L regions of the selected clones were sequenced with a T7-primer (TAATACGACTCACTATAGG) and CH1 J-primer (TTCTATTTCAAGGAGACAGTCAT) respectively. To determine the reactivity of the Fab-clones to recombinant gB (gB654) and HCMV virions, enzyme-linked immunosorbent assay (ELISA) was used. The conditions of ELISA were essentially the same as described previously [17].

2.6. Preparation of various forms of antibodies

Three forms of antibodies were prepared in this study: Fab-cp3, Fab-PP (P indicates an Fc-binding domain of protein A), and IgG_1. The Fab-cp3 molecules initially accumulated in the periplasm of *E. coli* (DH12S) and then were gradually secreted and/or released into the culture medium (crude Fab-cp3). On average, 1 μg of Fab-cp3 molecules/ml was present in the culture fluid. The Fab-cp3 molecules were purified with anti-cp3 mAb-conjugated Sepharose beads. The conversion from Fab-cp3 to Fab-PP was described previously [13]. Fab-PP molecules were purified with IgG-conjugated Sepharose beads, using the affinity between protein A (Fab-PP) and the IgG Fc domain (Sepharose). Conversion from a Fab to a human IgG_1 was performed as described previously [13]. IgG_1 molecules were expressed by transfecting the IgG_1 expression vectors into 293T cells using Lipofectamine 2000 (Invitrogen) or the LONZA system (LONZA, IFA).

2.7. Surface Plasmon Resonance (SPR) analysis

Interaction kinetics and pairwise competition of Fab-antibodies with gB654 were analyzed by SPR, using Biacore 3000 (Biacore, Inc.). The flow cells of a carboxymethylated dextran (CM5) sensor chip were used with activation following manufacturer's instruction. gB654 was immobilized at a flow rate of 5 μl/min for 5 min. Fab-PP antibodies were affinity-purified using IgG Sepharose, prepared at a 1 μM concentration and serially diluted with HBS-EP running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20; Biacore Inc.). Diluted antibody solutions were injected over the gB654 immobilized sensor chips. Dissociation occurred in HBS-EP running buffer for 5 min following the injection. The doubly referenced data were analyzed and fit globally to the 1:1 binding model using BIA evaluation 3.1 software.

2.8. Virus neutralizing assay

HCMV-neutralizing activity was examined by a plaque reduction assay. Briefly, 150 plaque-forming units (pfu) of cell-free virus in 75 μl of virus solution was mixed with 75 μl of various antibody concentrations. The mixtures were incubated at 37 °C for 1 h, and then 50 μl of the mixture was inoculated into MRC-5 cells in one well of a 24-well microtiter plate. After a 1-hour incubation, the virus-antibody mixture was aspirated, fresh medium (5% fetal bovine serum-supplemented MEM) was added, and the plate was incubated for eight days. The neutralizing activity was expressed as the concentration necessary to reduce the number of plaques by 50% (IC_{50}). To compare the neutralizing antibody concentrations, we used human gamma-globulin, Kenketu Venilon®-I (Kaketsuken, Kumamoto, Japan) as a control. The original concentration of the human gamma-globulin was 50 mg/ml. The monoclonal antibody against habu-venom, which was prepared by using same phage display procedure, was used as a negative control.

3. Results

3.1. Isolation and classification of Fab-clones from the human antibody library

As described previously, two antigens, a recombinant truncated glycoprotein B (gB) and HCMV purified virion, were used to perform the panning. The purity and expression of the recombinant truncated gB protein, gB654, was confirmed by Western blotting using an anti-myc antibody and polyclonal anti-HCMV antibodies (Fig. 1). The purified virion preparation consisted of intact, infectious particles in order to obtain antibodies that recognize neutralizing epitopes on envelope glycoproteins. In the gB654 screen, we isolated 80 clones that were classified into 10 groups based on the amino acid sequences of the V_H region (Table 1). In the HCMV virion screen, we isolated 27 clones that were classified into eight groups. Four groups with the same V_H sequence were isolated in both screens (Table 1). Thus, we obtained 107 clones from two independent screenings methods that were classified into 14 groups, H01–H14, based on their V_H sequences.

3.2. Analysis of the binding activities to recombinant gB protein (gB654) or HCMV virions

The crude Fab-cp3 solution of each clone was used to determine the gB654 or HCMV virion binding activities in an enzyme-linked immunosorbent assay (ELISA). Of the Fab-cp3 mAbs classified by their V_H sequences, four clones (H11, H12, H13, H14) isolated only from the HCMV virion screening reacted with both HCMV virions and gB654 (Fig. 2A).

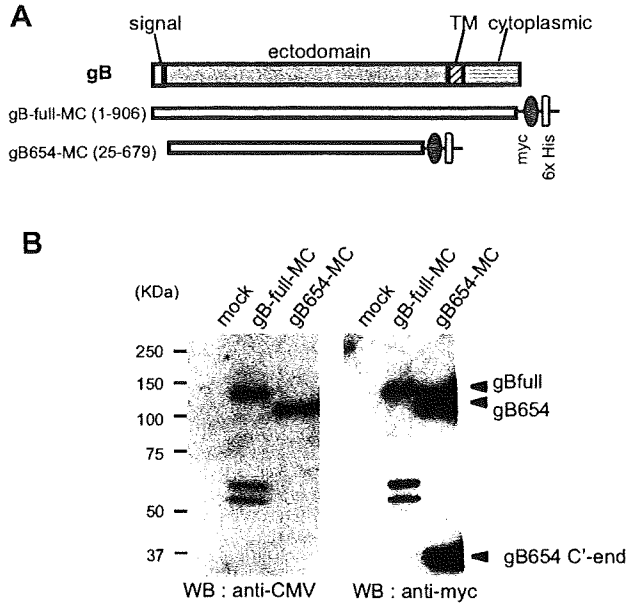

Fig. 1. Transient expression of recombinant glycoproteins in mammalian cells. (A) Schematic illustration of the recombinant gB proteins. Wild type (WT) gB-myc protein consisted of full-length gB, and gB654 consisted of the ectodomain of gB (amino acids 25–679). These recombinant proteins were myc- and hexametric histidine-tagged at the C-terminus. (B) Detection of recombinant gB proteins by Western blotting using an anti-CMV polyclonal antibody and anti-myc monoclonal antibody. WT gB-myc or gB654-myc expression plasmids were transiently transfected into HEK293 cells. Whole cell lysates were separated by SDS-PAGE and blotted with the indicated antibodies. Mock cells were transfected with the pSecTag2A vector.

3.3. Pairwise binding competition analysis reveals three potential epitopes on gB654

In order to evaluate the gB654 binding activity of the isolated clones, we measured the affinity constants using surface plasmon resonance (SPR). As we hypothesized that the clones, which were isolated multiplicity, were important for prevention viral infection, 7 (H02, H03, H04, H05, H08, H09, and H14) of the 14 clones were selected to perform this experiment. Fab-PP antibodies, which are another type of monomeric Fab antibody, for the seven groups were expressed in *E. coli* and affinity-purified as described in the Materials and Methods. The k_a, k_d and K_D were determined and are shown in Table 2. The K_D value ranged from 0.53 nmol/l (nM) to 9.3 nM for the seven groups, suggesting that the Fab-antibodies of these seven groups had strong gB654 binding affinities.

Table 1
Number of isolated Fab-clones by the screenings using two antigen; (I) gB654 and (II) HCMV virion.

V_H	Number of isolated clones	
	(I) gB654	(II) HCMV virion
H01	4	0
H02	6	3
H03	31	8
H04	7	4
H05	14	0
H06	1	0
H07	1	0
H08	11	3
H09	3	0
H10	2	0
H11	0	1
H12	0	1
H13	0	2
H14	0	5
Total	80	27

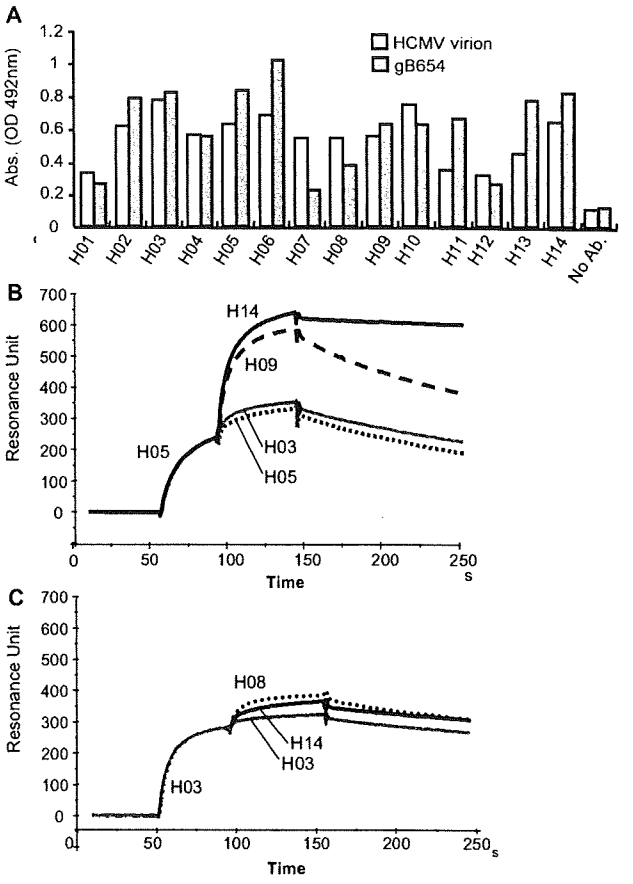

Fig. 2. Antigen-binding analysis in ELISA and pairwise competition. (A) Enzyme-linked immunosorbent assay of monoclonal antibodies using the screening antigens, purified HCMV virions and gB654. Fourteen clones were selected as representative clones from each V_H group based on the major combination of V_H and V_L in each group. Affinity-purified Fab-PP antibodies of each clone were used in this assay. White and gray bars indicate the binding activities against HCMV virions and gB654, respectively. The reactivity of the 14 clones was similar, suggesting that all of the clones could bind gB654. (B and C) Pairwise mapping studies with monoclonal antibodies using Biacore. During the first injection, the primary antibody (500 nM) was applied for 50–100 s, and it bound gB654 to almost complete saturation. The second injection from 100–150 s contained the primary antibody (500 nM) and a second antibody (500 nM), and was used to determine if the second antibody could bind a different epitope. (B) The H05 antibody was used as the first antibody, followed by H14 (black solid line), H09 (dashed line), H03 (gray solid line) and H05 (dotted line) as the second antibody. (C) The H03 antibody was used as first antibody, while H14 (black solid line), H08 (dotted line) and H03 (gray solid line) were used as the second antibody.

We were also interested in the epitopes recognized on gB654. Therefore, we next performed a competitive binding analysis by SPR with gB654 and examined the consecutive binding of two antibodies. The signal of second antibody's binding would increase only if the binding site of the second antibody differed from the first, whereas the signal would not increase or increase only slightly if the binding sites were the same or overlapping. We analyzed previously used seven groups. When H05 was used as the first antibody, only the binding of H09 and H14 antibodies were not inhibited (Fig. 2B), vice versa (data not shown). In addition, H09 and H14 should recognize similar or overlapping epitopes as they inhibit the binding of each other (data not shown). These results suggest that the H05 epitope was completely different from the epitopes recognized by H09 and H14. In addition, H05 binding could not completely block the binding of H03 nor H02, H04, and H08 antibodies (Fig. 2B). Antibodies from six groups except H05 could inhibit binding of each other to varying degrees (Fig. 2C, data not shown). As a result of this competition analysis, the seven groups were divided into three groups, consisting of group I (H05), group II (H09 and H14) and group III (H02, H03, H04 and H08).

3.4. Three representative IgG_1 antibodies exhibit complement-dependent neutralizing activity against HCMV (Towne)

The Fab form was converted into an IgG_1 antibody in order to evaluate the neutralizing activities of the IgG_1 antibodies for three selected clones, H05, H08 and H14. Although these three IgG_1 antibodies could not neutralize HCMV without complement, all three IgG_1 antibodies neutralized HCMV when the assay conditions were supplemented with 5% guinea pig complement. The monoclonal antibody against habu-venom prepared by using same phage display procedure did not show neutralizing activities (data not shown) against HCMV. As indicated in Fig. 3, the 50% inhibition concentration (IC_{50}) of H05 was 3.9 μg/ml and that of H14 was approximately 20.8 μg/ml. These results suggested that the IC_{50} of H05 was similar to that of human gamma-globulin, which is a clinically used human immunoglobulin product (see Section 2). Additionally, we investigated the neutralizing activity against clinically isolated strains, 91S and 93R. The 91S strain was derived from a Ganciclovir (GCV)-sensitive strain (91-7S), and 93R was derived from a GCV-resistant strain (93-1R) [16]. As indicated in Fig. 4, the H05 IgG_1 antibody diluted 1:512 (1.95 μg/ml) protected more than 50% of cells from the cytopathic effects (CPE) caused by either 91S or 93R infection.

Table 2
Affinity constants for antigen-binding of seven Fab-antibodies.

V_H	Affinity constants		
	k_a (1/Ms)	k_d (1/s)	K_D (nM)
H02	8.1×10^5	4.7×10^{-4}	0.58
H03	1.3×10^5	1.0×10^{-3}	8.5
H04	7.9×10^4	3.7×10^{-4}	4.7
H05	8.2×10^5	7.6×10^{-4}	9.3
H08	2.5×10^5	1.8×10^{-4}	0.73
H09	8.9×10^4	5.7×10^{-4}	6.5
H14	1.2×10^6	6.5×10^{-4}	0.53

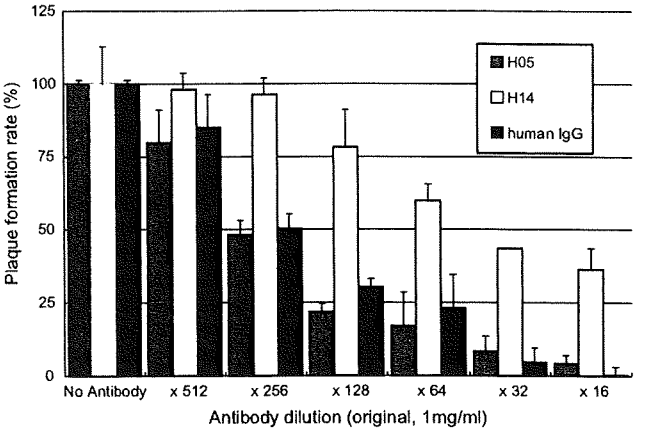

Fig. 3. The complement-dependent neutralization of two IgG monoclonal antibodies was measured using a plaque reduction assay. The virus used in this assay was Towne strain. H05 and H14 IgG antibodies were incubated with 5% guinea pig complement before inoculation. Plaques were counted eight days post inoculation. A plaque formation rate of 100% corresponded to the number of plaques without antibody (Control). The IC_{50} values of H05 IgG, H14 IgG, and human gamma-globulin were 3.9 μg/ml, 20.8 μg/ml and 3.9 μg/ml, respectively.

4. Discussion

In this study we attempted to produce ideal anti-HCMV monoclonal antibodies using a phage display system, which is advantageous because multiple monoclonal antibody clones can be obtained at once. Although many human monoclonal antibodies against several viruses have been produced using phage display technology [13,15,18,19], this is the first study

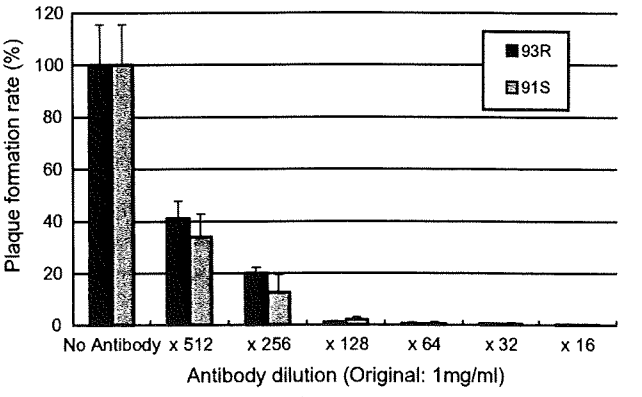

Fig. 4. The complement-dependent neutralization of H05 IgG-antibody against two clinical isolates, 93R and 91S, was measured using a plaque reduction assay. 91S and 93R were clinically isolated viruses propagated from previously reported viruses [16]. H05 antibody was incubalted with 5% guinea pig complement before inoculation. Plaques were counted eight days post inoculation. A plaque formation rate of 100% corresponded to the number of plaques without antibody (control).

to use this method to generate several human anti-HCMV monoclonal antibodies. The source of lymphocytes used to generate the library is crucial in order to obtain reliable monoclonal antibodies. Lymphocytes collected during the convalescent phase in virus-infected patients [20] or vaccine recipients [21] have been used to establish phage display libraries. In order to obtain ideal HCMV monoclonal antibodies, we used lymphocytes collected from a healthy pediatrician with a high HCMV-neutralizing antibody titer.

Several envelope glycoproteins, including glycoprotein B (gB, UL55), glycoprotein H (gH, UL75), glycoprotein L (gL, UL115), glycoprotein M (gM, UL100) and glycoprotein N (gN, UL73), have been identified as targets for neutralizing antibodies [22]. Specifically, gB is one of the most abundant envelope proteins and is involved in virus entry, particularly attachment and fusion [23]. gB is considered to be a major target of the humoral immune response because anti-gB antibodies, which include HCMV-neutralizing antibodies, have been mostly detected in the sera of infected individuals [24] and are a large component of the neutralizing antibody response to HCMV [25]. Therefore, in order to obtain effective neutralizing antibodies using a human antibody library derived from an HCMV-seropositive individual, purified HCMV virions and a truncated gB, gB654, were used for the screening. However, all 14 different clones isolated from screening both of these antigens showed binding activities against gB654 by ELISA (Fig. 2A). Although it has been demonstrated that there are several neutralizing epitopes in gH or gM [22,26], which are other HCMV envelope glycoproteins, all isolated clones had an affinity for gB. During the biological enrichment for antibody clones in the panning rounds, the number of major clones may increase exponentially, while the number of minor clones decreases rapidly. It is also possible that antibodies with strong affinity are specifically selected by the panning rounds. Therefore, it is possible that anti-gH and anti-gM antibodies, which may have been much smaller in number than anti-gB antibodies, were present in the original repertoire. In order to obtain anti-gH and anti-gM/gN antibodies in the future, it will be necessary to use synthesized gH and gM/gN complexes for the panning rounds.

Recent study has demonstrated that pathways for HCMV entry into target cells were different between endothelial or epithelial cells and fibroblasts [27]. gB independent pathway has been suggested to be important for viral entry into epithelial or endothelial cells. Further depth analysis such as neutralization tests using endothelial cells and epithelial cells is necessary to elucidate the mechanism of virus neutralization by our monoclonal antibodies. The authors have also demonstrated that antibodies against gH play an important role in inhibition of cell-to-cell spread of HCMV [27]. Meanwhile, the latest data has suggested that anti-gB antibodies might prevent not only viral entry but also virus spreading [28]. In order to determine whether our monoclonal antibodies can prevent cell-to-cell spread, activities of our monoclonal antibodies against HCMV infected cells are now under investigation. In any case, in addition to anti-gB antibodies, as anti-gH or gL antibodies are considered to be useful for prevention of HCMV infection, modification of our screening system should be attempted to isolate anti-gH or gL clones in the future study.

SPR analysis of the binding kinetics revealed that the representative clones had a strong affinity for gB654. The K_D values for at least seven types of V_H ranged from 0.53 nmol/l (nM) to 9.3 nM (Table 2), which are equivalent to that of a general IgG-antibody, which ranges from 0.1 to 10 nM. Moreover, three potential epitopes were identified with a sequential competitive binding assay using SPR analysis. The epitopes of group III (H02, H03, H04 and H08) were inhibited when competitive binding assays were performed with members in this group. This finding suggests that the epitopes recognized by each antibody in this group are located in close proximity, or that antibody-binding induces conformational alterations that block the binding of another antibody. On the other hand, the H05 (group I) antibody did not inhibit H09 and H14 (group II), nor did H09 or H14 binding inhibit H05 binding. Thus, the epitopes of group I and group II are considered to be independent epitopes. It has been demonstrated that gB654 includes major antigenic domains of the native gB, notably antigenic domain-1 (AD-1) and AD-2. Compared to AD-2, AD-1 has been demonstrated to be the dominant target of neutralizing antibodies against gB [23] and to have several distinct antibody-binding sites [22]. We have not determined whether the epitopes of the three antibody groups, classified by the SPR competition analysis, are located in the AD-1 site or other sites. Therefore, molecular and biochemical analyses will be necessary to identify the epitopes recognized by these three independent clones and to prepare these antibodies for potential clinical applications.

We obtained 107 clones through two screens, and an ELISA using gB654 revealed that all of the isolated clones were anti-gB antibodies (Fig. 2A). Fab-antibodies of these clones, which were monovalent and synthesized in *E. coli*, did not show neutralizing activity, even though SPR analysis determined that they had high gB654 binding affinities. Finally, we constructed intact human IgG antibodies of three clones, H05, H08 and H14, and found that they had complement-dependent neutralizing activities against HCMV. It has been demonstrated that a neutralizing antibody to HCMV gB was exclusively complement-dependent, and the present data are consistent with this previous report [29]. Higher complement-activation potential can be generated by genetically engineering the IgG Fc-region [30]. Thus, additional molecular manipulation of these antibodies should be attempted to improve the efficacy of these monoclonal human antibodies in future analyses.

Although it has been demonstrated that prophylactic administration of immunoglobulin products can reduce HCMV infection in transplant recipients, MSL-109 (Protein Design Laboratories), which is an anti-gH monoclonal antibody produced by hybridoma technology, had potent *in vitro* neutralizing activity but was ineffective *in vivo*. It is thought that many neutralizing epitopes are critical to prevent HCMV infection *in vivo*. Therefore, immunoglobulin products containing polyclonal antibodies against HCMV might have

potent clinical efficacy [7]. In contrast to hybridoma technology, the phage display system can obtain various types of antibodies as illustrated in this study (Table 1). We demonstrated that at least three different monoclonal anti-gB antibodies isolated with this system had neutralizing activities *in vitro*. At least one of the two antibodies H05 antibody) had similar level of neutralizing activities to a commercially available human immunoglobulin product, which is generally used and has good prevention effect on CMV reactivation in Japan (Fig. 3). Moreover, because the H05 antibody showed higher neutralizing activity than the H14 antibody, we examined whether the H05 antibody could neutralize clinical isolates. As shown in Fig. 4, the 1:512 diluted H05 antibody (1.95 μg/ml) had more than 50% of plaque reduction activities against both Ganciclovir-sensitive and -resistant clinical isolates. Although the number of clones was limited, the present study suggested that human antibodies produced by the phage display system could effectively prevent HCMV infection. Furthermore, these antibodies were fully encoded by a human gene and matured in the immunized human body and were not humanized antibodies. In order to overcome the problems associated with the previous human monoclonal antibody treatment for HCMV infection, it will be necessary to obtain a large and diverse number of anti-HCMV clones. Gehrz RC et al. have demonstrated that a cocktail of HCMV monoclonal antibodies efficiently neutralizes HCMV [31]. Moreover, it has been demonstrated that a cocktail of several human monoclonal antibodies produced by the phage display system effectively prevents rabies virus infection by targeting several different strains [21,32]. Therefore, a cocktail of the three monoclonal antibodies examined in this study should be evaluated as a treatment to prevent HCMV infection *in vivo* in future clinical studies.

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Supplementary table

Supplementary table associated with this article can be found, in the online version, at doi:10.1016/j.micinf.2009.07.010.

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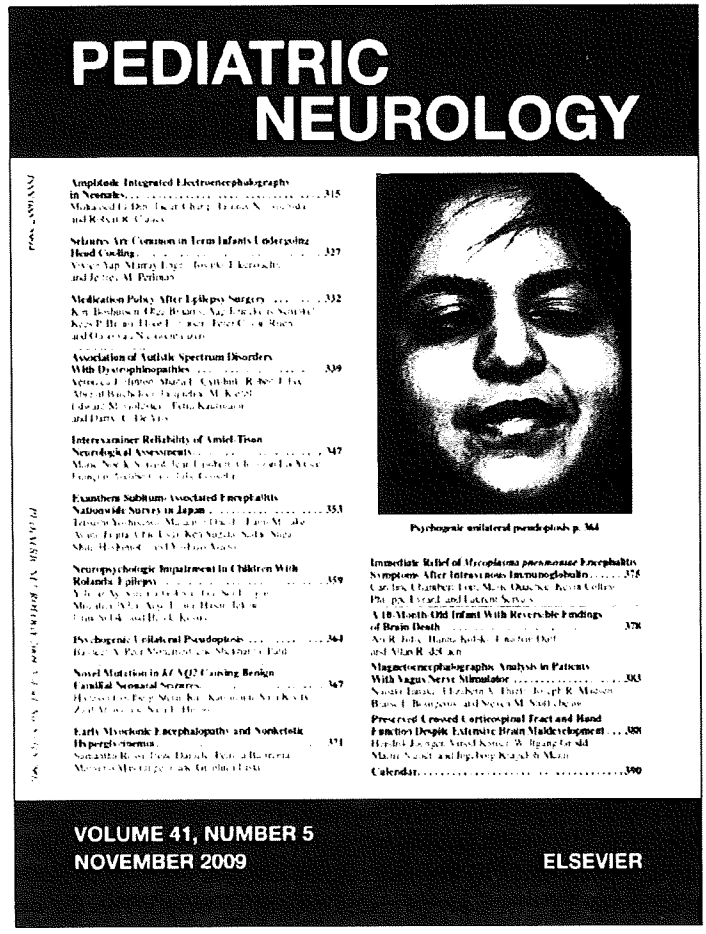

ELSEVIER

Exanthem Subitum-Associated Encephalitis: Nationwide Survey in Japan

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We sought to clarify clinical features of exanthem subitum associated-encephalitis/encephalopathy, generally caused by primary human herpesvirus-6 infection in Japan. A two-part questionnaire was sent to hospitals between January 2003-December 2004. Of 3357 questionnaires, 2357 (70.2%) were returned, and 2293 (68.3%) were eligible for analysis. Eighty-six cases of exanthem subitum-associated encephalitis/encephalopathy were reported. Seventy-seven (89.5%) of 86 patients were diagnosed with human herpesvirus-6 infection by virologic examination. Although 41 (50.6%) of 81 patients had no sequelae, 38 (46.9%) had neurologic sequelae. Moreover, two fatal cases (2.5%) were reported. Pleocytosis was evident in only 4 (7.5%) of 53 patients, and cerebrospinal fluid protein levels were within normal range (23.4 ± 14.6 mg/dL S.D.) in all patients. Human herpesvirus-6 DNA was detected in 21 (53.8%) of 39 patients. Abnormal computed tomography findings were a predictor of neurologic sequelae (P = 0.0097). As a consequence of this survey, we estimate that 61.9 cases of exanthem subitum-associated encephalitis occur every year. The disease prognosis was unexpectedly poor.

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Introduction

Primary human herpesvirus-6 infection can cause exanthem subitum in infants and young children [1]. Although the disease is generally a benign, febrile illness with a self-limiting clinical course [2], several severe manifestations, particularly in the central nervous system, can occur [3-13]. Exanthem subitum is associated with febrile seizures [14]. Moreover, we found that the incidence of severe forms of febrile seizures, e.g., hemiconvulsions, prolonged seizures, and repeated seizures, was high in cases of exanthem subitum-associated febrile seizures [15]. After human herpesvirus-6 was identified as an etiologic agent of exanthem subitum, human herpesvirus-6 encephalitis/encephalopathy was reported by several investigators [3-13]. Some studies reported on patients who recovered completely, whereas others manifested severe neurologic sequelae, including several cases with fatal outcomes [4,8,16]. Human herpesvirus-6 DNA was detected in the cerebrospinal fluid of several patients via polymerase chain reaction [5,6], suggesting direct viral invasion of the central nervous system. Saito et al. [17] detected viral antigens and DNA in postmortem brain tissues obtained from AIDS patients, which supports the concept of direct invasion of the virus into the central nervous system. Moreover, it was suggested that human herpesvirus-6 can infect not only neurologic cell lines [18-20] but also fetal astrocytes [21], and can alter cytokine synthesis in infected cells [22]. Thus, human herpesvirus-6 is recognized as a neuro-pathogen. Although a recent study from the United Kingdom indicated that human herpesvirus-6 and human herpesvirus-7 are associated with encephalitis or severe forms of febrile seizure [23], details of the clinical features and frequency of human herpesvirus-6 encephalitis/encephalopathy remain unclear. Therefore, we performed a nationwide survey to determine the frequency and clinical features of exanthem subitum-associated encephalitis/encephalopathy in Japan.

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Materials and Methods

A two-part questionnaire sought to determine the number of cases and clinical features of the disease over a 2-year period, between January 2003-December 2004. The first part included six questions. The first question asked if the hospital had hospitalized cases of exanthem subitum-associated encephalitis/encephalopathy, because all patients with the disease are thought to be admitted to hospitals for intensive treatment. Exanthem subitum-associated encephalitis/encephalopathy was defined as exanthem subitum in patients with stupor or convulsion. To exclude exanthem subitum patients with severe forms of febrile convulsion, abnormal findings of radiologic examinations were required for diagnoses of patients with only convulsions to indicate exanthem subitum-associated encephalitis/encephalopathy. If a hospital reported a case of exanthem subitum-associated encephalitis, five questions were asked: (1) age of patients, (2) sex of patients, (3) presence or absence of human herpesvirus-6 virologic examinations and the examination methods used (significant increase in human herpesvirus-6 IgG antibodies, positive human herpesvirus-6 immunoglobulin M, positive human herpesvirus-6 DNA in serum, and human herpesvirus-6 isolation), (4) detection of human herpesvirus-6 DNA in cerebrospinal fluid via polymerase chain reaction, and (5) the patients' prognosis. The second part of the questionnaire was subsequently sent to hospitals that reported cases of exanthem subitum-associated encephalitis/encephalopathy, and focused on clinical features such as (1) a febrile period, (2) time of skin-rash appearance, (3) onset of neurologic signs, (4) cerebrospinal fluid findings, including the presence or absence of human herpesvirus-6 DNA, (5) electroencephalogram findings, (6) radiologic findings, including computed tomography and magnetic resonance images, (7) treatments, including antiviral drugs, steroids, and gamma-globulins, and (8) the patients' prognosis.

In March 2005, the first questionnaire was mailed to the chiefs of pediatric departments in the 3357 hospitals with pediatric wards throughout Japan. A second mailing was sent to nonresponders in August 2005 to increase survey reliability. In December 2005, the second part of the questionnaire was sent to hospitals that reported patients with exanthem subitum-associated encephalitis/encephalopathy in the first questionnaire.

Data analysis was performed by members of our laboratory. An association between seven clinical factors, including sex, age, onset of neurologic signs, presence of human herpesvirus-6 DNA in cerebrospinal fluid, abnormal electroencephalogram findings, abnormal computed tomography findings, and abnormal magnetic resonance imaging findings and neurologic sequelae, were examined via χ^2 test. The statistical analysis was performed using StatView software, version J-5.0 (distributed by HULINKS, Inc.). The protocol of this study was approved by the Review Board of Fujita Health University.

Results

Results of First Questionnaire

Of 3357 questionnaires sent to hospitals, 2357 (70.2%) were returned. Fifty-seven (2.4%) pediatric wards were closed, and seven questionnaires (0.3%) were returned without any information, leaving 2293 (68.3%) eligible for analysis. Eighty-six patients (45 male [52.3%] and 41 female ([47.7%]) with exanthem subitum-associated encephalitis/encephalopathy were reported at 61 hospitals (2.7%), and no patients with encephalitis/encephalopathy were reported at the remaining 2232 hospitals (97.3%). Seventy-seven (89.5%) of 86 patients were diagnosed with human herpesvirus-6 infection according to virologic examinations, e.g., viral isolation (1 patient), serologic assay (53 patients), and plasma polymerase chain reaction (34 patients). Eleven patients were diagnosed with human herpesvirus-6 infection using two different virologic examinations. Only nine patients (10.5%) were diagnosed with exanthem subitum according to typical clinical features of the illness. The mean age of patients was 14.0 ± 8.8 months S.D. The youngest patients were 3 months old, and a peak in patient numbers was evident at age 10 months. Eight patients were older than 24 months. A prognosis was available for 81 (94.2%) of 86 patients. Although 41 (50.6%) of the 81 patients manifested no sequelae, 38 (46.9%) of 81 patients manifested neurologic sequelae. Moreover, two fatal cases (2.5%) were reported. Details of neurologic sequelae were examined in the second questionnaire.

Results of Second Questionnaire

To collect more precise information, a second questionnaire was sent to hospitals that had reported patients with exanthem subitum-associated encephalitis/encephalopathy in the first questionnaire. Data for the second questionnaire were collected regarding 60 of the 86 patients, and several analyses were performed on the data gathered from these 60 patients. To predict the pathogenesis of the exanthem subitum-associated central nervous system manifestations, a time correlation between the onset of neurologic signs and the appearance of a skin rash was evaluated in 56 patients, because data on disease onset were not available for four patients. We defined the day when a skin rash appeared as day zero. Neurologic signs occurred before and after the appearance of a skin rash in 37 (66.1%) and 11 (19.6%) patients, respectively. One patient (1.8%) manifested neurologic signs when a skin rash appeared. No skin rashes were evident in seven patients (12.5%), diagnosed according to virologic examinations. Forty-three (71.7%) of the 60 patients manifested stupor and convulsion, whereas the remaining 17 patients (28.3%) manifested only convulsions during the observation period. However, abnormal magnetic resonance imaging findings were evident in these 17 patients.

Table 1. Summary of abnormal findings in radiologic examinations

Examination	Patients With Abnormal Findings/Patients Examined	Abnormal Findings	Number of Patients
CT	21/54 (38.9%)	Brain edema	14
		Low density area	
		Frontal and temporal lobe	3
		Left hemisphere	1
		Bilateral striatum region	1
MRI	34/50 (65.4%)	Hyperintense on diffusion image*	21
		Brain atrophy†	10
		Hyperintense on T_2-weighted image	9
		Brain edema	5
		Subdural effusion	3
		Hyperintense on FLAIR	2
		Hyperintense on T_1-weighted image	1
		Hypointense on T_1-weighted image	1

* Without corresponding apparent diffusion coefficient result.
† Brain atrophy was evident late in time course (later than 10 days after onset of illness) in 9 of 10 patients.

Abbreviations:
CT = Computed tomography
FLAIR = Fluid-attenuated inversion recovery
MRI = Magnetic resonance imaging

Cerebrospinal fluid findings were available for 53 patients. Pleocytosis (20 cells/μL, 15 cells/μL, 11 cells/μL, and 51 cells/μL) was evident in only 4 (7.5%) of 53 patients, and the remaining 49 patients (92.5%) demonstrated normal cell counts. Cerebrospinal fluid protein levels were within normal range (23.4 $\pm$ 14.6 mg/dL) in all patients. The presence of human herpesvirus-6 DNA in cerebrospinal fluid was examined in 39 (65.0%) of 60 patients via polymerase chain reaction. Human herpesvirus-6 DNA was detected in 21 (53.8%) of those 39 patients. Electroencephalogram findings in the acute phase of the illness (within 10 days after onset of illness) were available for 55 patients. Abnormal findings (31 patients with high-voltage slow waves, 4 patients with spikes, 7 patients with other findings, and 14 patients without abnormal findings) were evident in 42 (76.4 %) of 55 patients. Computed tomography and magnetic resonance imaging of the brain were performed in 54 and 50 patients, respectively. The radiologic findings are summarized in Table 1. Computed tomography was performed 3.0 $\pm$ 2.1 days after the onset of illness. Abnormal findings (14 patients with brain edema, five patients with low-density areas, and two patients with other findings) were evident in 21 (38.9%) of 54 patients. Magnetic resonance imaging was performed 8.7 $\pm$ 5.5 days after the onset of illness, and 34 (68.0%) of 50 patients manifested abnormal findings, including 21 patients with hyperintensity on a diffusion-weighted image (without the corresponding apparent diffusion coefficient result).

Antiviral drugs were administered to 31 (51.7%) of 60 patients. Acyclovir was used in 28 patients, ganciclovir in one patient, and a combination of both drugs in two patients. Thirty-three (55.0%) of 60 patients received steroid treatments, including three who received steroid pulse therapy. Immune globulin was administered to 19 (31.7%) of 60 patients, including seven patients with high-dose γ-globulin treatment.

Neurologic sequelae were evident in 32 (53.3%) of 60 patients. As shown in Table 2, cases of severe neurologic sequelae were reported, including nine patients with spastic quadriplegia, eight patients with mental retardation, and seven patients with hemiplegia. Two fatal cases were reported from two different hospitals.

Association Between Clinical Factors and Neurologic Sequelae or a Fatal Clinical Course

As described in Table 3, an association between six clinical factors and neurologic sequelae or a fatal clinical course was evaluated. No statistical association was evident between sex, age, or onset of neurologic signs and neurologic sequelae. Although 9 (42.9%) of 21 patients with positive human herpesvirus-6 DNA in cerebrospinal fluid manifested neurologic sequelae, 13 (72.2%) of 18 patients without human herpesvirus-6 DNA in their cerebrospinal fluid manifested neurologic sequelae. Thus, patients with human herpesvirus-6 DNA in their cerebrospinal fluid exhibited less frequent neurologic

Table 2. Summary of neurological sequelae

Neurologic Sequelae	Number of Patients
Spastic quadriplegia	9
Psychomotor retardation	8
Hemiplegia	7
Motor retardation	3
Epilepsy	2
Speech disturbance	2
Facial nerve paralysis	1