Vaccine 32 (2014) 4529–4536

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Recombinant measles viruses expressing respiratory syncytial virus proteins induced virus-specific CTL responses in cotton rats

Yoshiaki Yamaji, Tetsuo Nakayama*

Laboratory of Viral Infection I, Kitasato Institute for Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 27 May 2014
Accepted 6 June 2014
Available online 18 June 2014

ABSTRACT

Respiratory syncytial virus (RSV) is a common cause of serious lower respiratory tract illnesses in infants. Natural infections with RSV provide limited protection against reinfection because of inefficient immunological responses that do not induce long-term memory. RSV natural infection has been shown to induce unbalanced immune response. The effective clearance of RSV is known to require the induction of a balanced Th1/Th2 immune response, which involves the induction of cytotoxic T lymphocytes (CTL). In our previous study, recombinant AIK-C measles vaccine strains MVAIK/RSV/F and MVAIK/RSV/G were developed, which expressed the RSV fusion (F) protein or glycoprotein (G). These recombinant viruses elicited antibody responses against RSV in cotton rats, and no infectious virus was recovered, but small amounts of infiltration of inflammatory cells were observed in the lungs following RSV challenge. In the present study, recombinant AIK-C measles vaccine strains MVAIK/RSV/M2-1 and MVAIK/RSV/NP were developed, expressing RSV M2-1 or Nucleoprotein (NP), respectively. These viruses exhibited temperature-sensitivity (*ts*), which was derived from AIK-C, and expressed respective RSV antigens. The intramuscular inoculation of cotton rats with the recombinant measles virus led to the induction of $CD8^+$ IFN-γ^+ cells. No infectious virus was recovered from a lung homogenate following the challenge. A Histological examination of the lungs revealed a significant reduction in inflammatory reactions without alveolar damage. These results support the recombinant measles viruses being effective vaccine candidates against RSV that induce RSV-specific CTL responses with or without the development of an antibody response.

1. Introduction

Respiratory syncytial virus (RSV) is the most common pathogen of viral lower respiratory tract infections and causes severe diseases in infants with congenital heart diseases, preterm infants with respiratory illnesses, immunodeficient patients, and the elderly. More than 70% of children have had RSV infections in their first year of life, and all children experienced RSV infections by two years of age [1–3].

A vaccine trial for RSV that used formalin-inactivated RSV (FI-RSV) failed in the 1960s. FI-RSV did not evoke protective humoral immunity and enhanced the severity of illnesses following RSV infection among FI-RSV vaccine recipients. The marked infiltration of eosinophils was observed in their lungs because of a skewed Th2-dominant immune response [4–6]. Therefore, a RSV vaccine need to be developed that elicits both humoral and cell-mediated immune responses against the virus [7,8].

IFN-γis an essential cytokine in Th1-type immune responses in most viral infections. Previous studies on mouse models demonstrated that IFN-γ was the most important cytokine for the clearance of virus-infected epithelial cells, and $CD8^+$ IFN-γ^+ T cells were found to inhibit the infiltration of eosinophils into the lungs when mice were challenged with RSV [9,10]. In the RSV vaccine strategy, M2-1 and nucleocapsid protein (NP) are known to induce a strong Th1-dominant immune response and inhibit the infiltration of eosinophils into the lungs, thereby inducing IFN-γin mouse models [11–16]. These M2-1 or NP peptides are presented on MHC-I molecule, recognized by $CD8^+$ T cells, and induce immune responses. Peptides with the M2-1 sequence have been used previously to induce IFN-γ or Th1-type immune responses for BALB/c mouse MHC. The epitope region presented on human MHC differs from that in mice. Although cell-mediated immunity is effective for the clearance of RSV-infected cells, previously studies reported the deterioration of

* Corresponding author. Tel.: +81 3 5791 6269; fax: +81 3 5791 6130.
E-mail address: tetsuo-n@lisci.kitasato-u.ac.jp (T. Nakayama).

http://dx.doi.org/10.1016/j.vaccine.2014.06.024
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lung injuries, with a weakened neutralizing antibody response [17–19].

In our previously study, the recombinant measles virus expressing RSV-fusion protein, called MVAIK/RSV/F, was developed. It elicited an antibody response against RSV and decreased the amount of infectious virus in lung homogenates obtained from cotton rats after a RSV challenge [20]. In the present study, two recombinant measles viruses expressing RSV-M2-1 and NP were developed, and the immune responses were investigated against RSV in cotton rats together with MVAIK/RSV/F.

2. Materials and methods

2.1. Cells and viruses

The AIK-C seed strain was used in the development of measles vaccine-vectored recombinant viruses. A wild-type strain of RSV subgroup A was isolated in HEp-2 cells. RSV Long strain (subgroup A) was propagated in HEp-2 cells.

293T and HEp-2 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Vero CCL cells were maintained in VP-SFM supplemented with 4 mM L-glutamine. B95a cells were maintained in RPMI-1640 medium supplemented with 10% FBS. All culture media were supplemented with 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin, and cells were propagated at 37 °C in 5% CO_2.

2.2. Cloning of the RSV/NP and M2-1 genes

Genomic RNA was extracted from RSV subgroup A (wild-type). Viral RNA was first converted to cDNA using the PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd.), as previously described [20]. The NP and M2-1 genes were amplified by PCR using the following primer sets: NP gene Fwd: (5′-CATATGGAATCCATGGCTCTTAGCAAAGTCAA-3′) and NP gene-Rev: (5′-ATGGAATTCCGCGGCCGCTCAAAGCTCTACATCATTAT-3′) for the NP gene, and M2-1-gene-Fwd: (5′-CATATGGAATCCATGGCACGA-AGGAATCCTTG-3′) and M2-1 gene-Rev: (5′-TCCAAGGCGGCCGC-TCATCAGGTAGTATCATTATTTT-3′) for the M2-1 gene. The NP and M2-1 genes were cloned into pMVAIK/20-77, which has already been developed in our laboratory for the construction of MVAIK/RSV/F using two restriction enzymes, *Nco* I and *Not* I (underlined sequences), as previously described [20].

2.3. Immunofluorescence staining and Western blotting

B95a cells grown on 8-well chamber plates were infected with rMVAIK, MVAIK/RSV/M2-1, or MVAIK/RSV/NP and then cultured for 3 days at 33 °C. Cells were fixed with 1% glutaraldehyde for 30 min at room temperature, followed by the permeabilization of membranes with 0.2% Triton X-100. Fixed cells were washed thoroughly with PBS and incubated for 60 min with M2-1 or NP-specific monoclonal antibody (Abcam, Cambridge, UK) diluted 1:1000 in PBS-1%FBS. RSV-M2-1 and NP were then stained using a secondary antibody conjugated with Alexa Flour 488. Measles virus-NP was stained using a secondary antibody conjugated with Alexa Flour 568 (Invitrogen, Carlsbad, CA).

B95a cells were infected with MVAIK/RSV/NP on 24-well plate, and were cultured for three days at 33 °C. Cells and culture fluid were collected and subjected to SDS-PAGE. Proteins were separated through SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to membranes. These membranes were then treated with blocking buffer (Nacalai Tesque, Inc., Kyoto, Japan.), incubated with a M2-1 or NP-specific monoclonal antibody (Abcam, Cambridge, UK), washed again, and incubated with goat anti-mouse IgG (H^+L) conjugated with HRP (Santa Cruz Biotechnology, Inc.). They were stained with a DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA, US) as recommended by the manufacturer and described previously [20].

2.4. Immunization and RSV challenge in cotton rats

Female outbred cotton rats (*Sigmodon hispidus*) were purchased from Harlan Sprague Dawley (Indianapolis, IN, US). Three cotton rats for each group were immunized intramuscularly with 1×10^6 $TCID_{50}$ of rMVAIK, MVAIK/RSV/F, MVAIK/RSV/M2-1, or MVAIK/RSV/NP. Cotton rats in the negative control group were injected with PBS (−). Serum samples were obtained immediately before and 1, 3, 6, and 12 weeks after immunization. Cotton rats were anesthetized and infected with 1×10^6 PFU/0.5 ml of RSV subgroup A (Long strain) though an intranasal route nine weeks after the first immunization (4 weeks after reimmunization) and were sacrificed four days after the challenge. The serum, broncho-alveolar lavage (BAL), spleen, thymus, and lung tissues were collected. Cotton rats in the reimmunization group were administered the recombinant viruses 8 weeks after the first immunization and then challenged with 10^6 PFU/0.5 ml of the Long strain (subgroup A) four weeks after the second immunization.

2.5. Intracellular cytokine staining (ICS)

Freshly isolated $3–4 \times 10^6$ splenocytes were stimulated with 1 μM of individual peptides or inactivated viruses in the presence of 20 μg/ml brefeldin A (Alomone labs, Israel) in 500 μl of culture medium (RPMI, 10% FCS) at 37 °C for 5 h. Peptides that corresponded to $CD8^+$ cell epitopes were synthesized (Medical & Biological Laboratories Co., Ltd, Japan). After the stimulation, cells were harvested, washed, and incubated with an anti-CD8 antibody (R&D Systems, USA) for 30 min. Cells were fixed with the Cytofix/cytoperm kit (BD Pharmingen, San Diego, CA), and intracellular cytokines were stained with a goat IgG polyclonal antibody against IFN-γ (R&D Systems, USA) and anti-goat IgG-PE-Cy7 (Santa Cruz Biotechnology, Inc., USA) for 60 min, as reported previously [9,11,19]. Cells were quantified with flow cytometry, *Cytomics* FC 500 (Beckman Coulter, Inc., USA).

2.6. Serology

Neutralization tests against RSV were performed with the 50% plaque reduction assay using the Long strain. Briefly, serum samples were serially diluted by four-fold, starting from a 1:10 dilution, and mixed with an equal volume of RSV (100 PFU) in MEM for 1 hour at room temperature, as previously described [20]. The virus-serum mixture was added on HEp-2 cells in 24-well plates. The virus was adsorbed for 4 h at 37 °C with regular shaking. The inoculum was removed, and cells were overlaid with MEM containing 5% FCS and 0.5% agar. After being incubated for a week in 5% CO_2 at 37 °C, cells were fixed with 1% formalin. Agar was removed and cells were stained with neutral red [21].

Particle agglutination (PA) antibodies against the measles virus were measured using a PA antibody detection kit (Serodia®-Measles, FujiRebio, Tokyo, Japan).

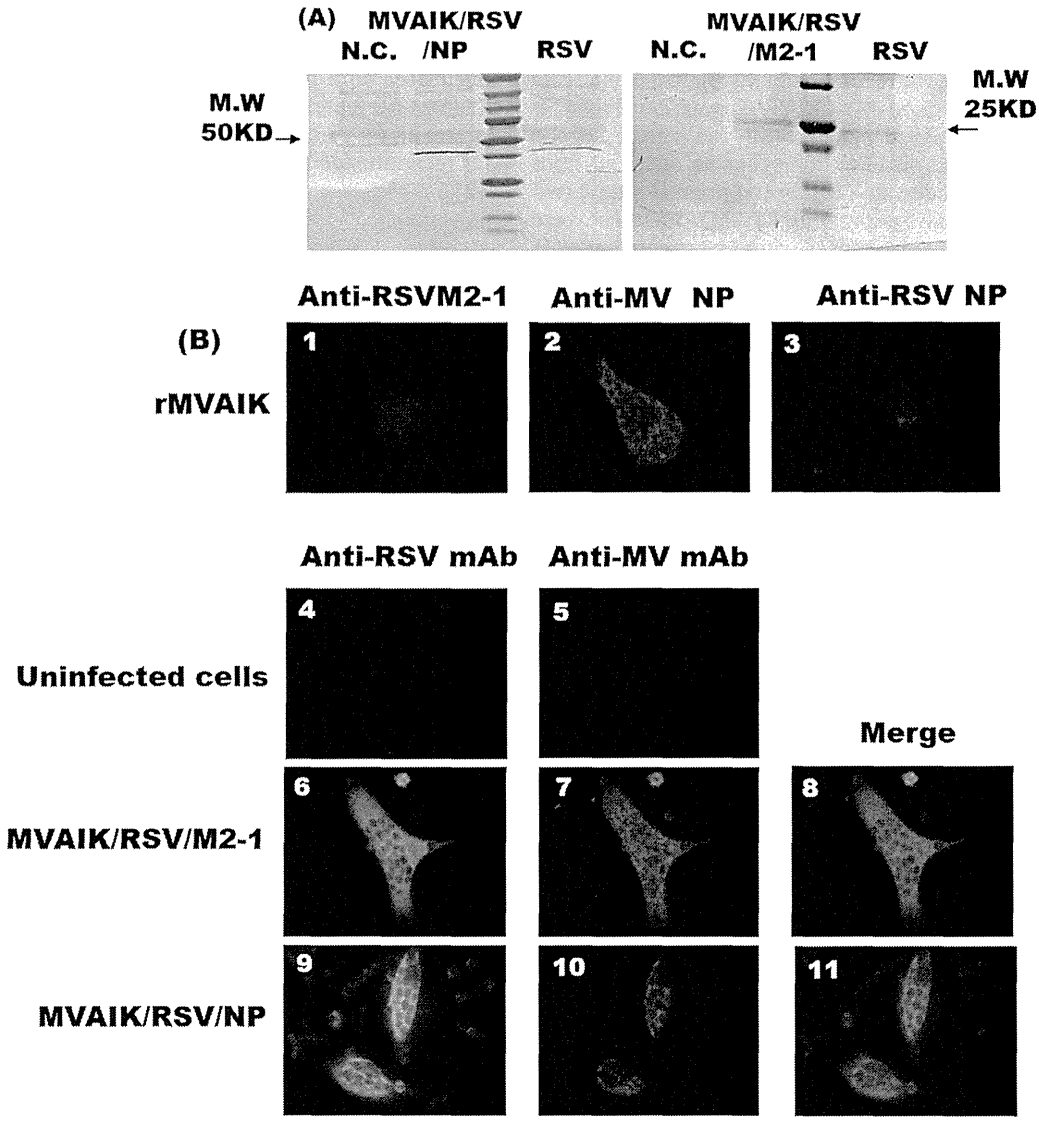

Fig. 1. *Expression of MV-N, RSV-M2-1, and N proteins.* (A): B95a cells were infected with MVAIK/RSV/M2-1 or MVAIK/RSV/NP, and were also mock infected (N.C.). The positive control was prepared in HEp-2 cells infected with the RSV Long strain. The results of western blotting were obtained using a monoclonal antibody against the RSV-N protein (left panel) and that against RSV-M2-1 protein (right panel). (B): B95a cells were infected with rMVAIK (panels (1)–(3)), or mock infected (panels (4) and (5)), MVAIK/RSV/M2-1 (panels (6)–(8)), or MVAIK/RSV/NP (panels (9)–(11)). The results of immunofluorescence staining with the monoclonal antibody against RSV are shown in panels (1), (3), (4), (6), and (9). The measles virus antigen was stained with the monoclonal antibody against the MV-N protein and the results are shown in panels (2), (5), (7), and (10). Merged images are shown in panels (8) and (11).

2.7. Detection of an infectious virus in lung tissues

Cotton rats were sacrificed 4 days after the challenge and lung tissues were obtained to detect RSV. 0.1 ml volume of serial 10-fold dilutions of lung tissue homogenates was placed on HEp-2 cells and incubated at 37 °C with shaking every 30 min for 4 h. MEM supplemented with 5% FBS with 0.5% agar was overlaid. Plaque numbers were counted after cells had been incubated for 6 days at 37 °C and infectivity was expressed as the number of plaques adjusted to 1 g lung tissue, as previously described [20].

2.8. HE staining and immunostaining

Lung tissue samples were fixed by formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Immunostaining was performed using a four clone blend of monoclonal antibodies against RSV P, F, and N proteins (Abcam, Cambridge, UK) and anti-mouse IgG conjugated with HRP (Dako North America, Inc.).

3. Results

3.1. Detection of RSV antigens

The expression of RSV antigens by the recombinant measles viruses was examined using western blotting, and the results are shown in Fig. 1A. M2-1 and NP were stained at a similar molecular weight to the cell lysate infected with RSV. Although M2-1 and NP were not detected in the culture supernatant, antigen-specific bands were detected on the lane of virus-infected cell lysates (data not shown).

RSV-M2-1 and NP were previously identified in cytoplasmic inclusions in RSV-infected cells [22]. RSV-M2-1 and NP were visualized with second antibodies conjugated with Alexa Flour 488, as shown in Fig. 1B. The negative staining of RSV-M2-1 and RSV-NP

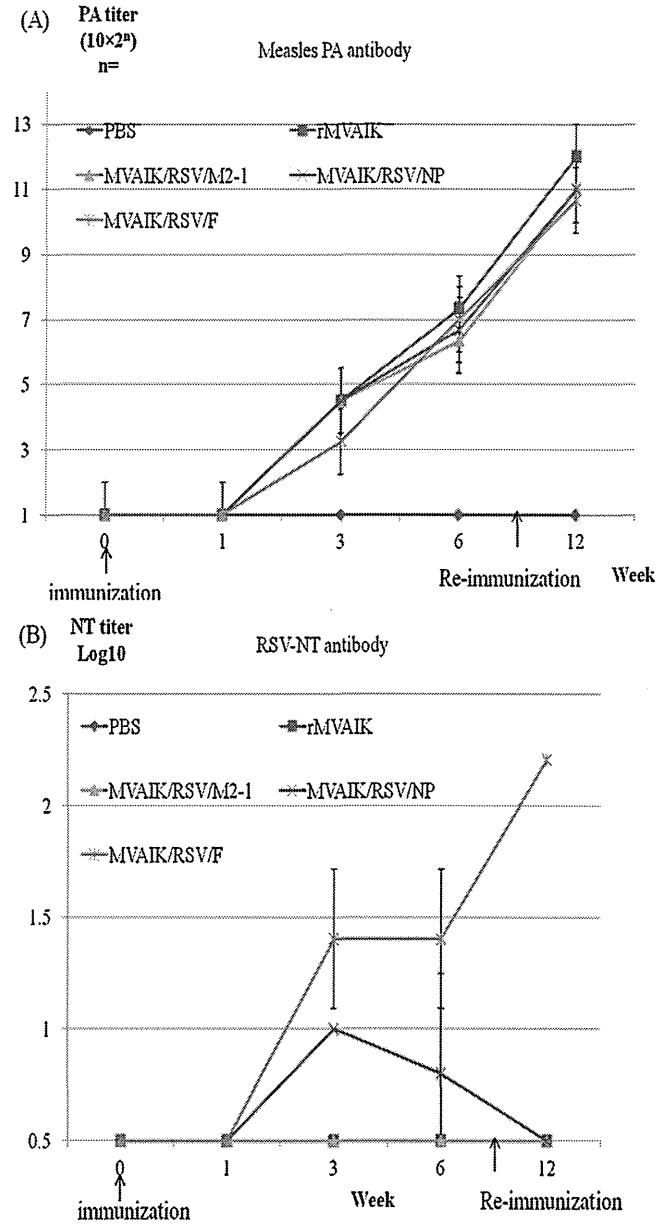

Fig. 2. *Serological responses of PA antibodies against MV and neutralizing antibody responses against RSV.* Serum samples were obtained before immunization and 1, 3, 6, and 12 weeks after immunization with mock (PBS), rMVAIK, MVAIK/RSV/M2-1, MVAIK/RSV/NP, and MVAIK/RSV/F. PA antibodies were expressed as 10×2^n (panel (A)). The NT antibody response against RSV was examined using the 50% plaque reduction assay with RSV subgroup A, Long strain (panel (B)). Each bar represents the standard deviation of the mean of three animals per group.

monoclonal antibodies was observed in panels 1 and 3 in B95 cells infected with rMVAIK. In the case of recombinant MVAIK/RSV/M2-1 and MVAIK/RSV/NP, M2-1 and NP were expressed in cells infected with the recombinant measles viruses, localized in the cytoplasm (panels 6, 7, 9, and 10).

3.2. Detection of a serum-neutralizing antibody response

The results of neutralizing antibody responses are shown in Fig. 2. PA antibodies against the measles virus were detected 3 weeks after the immunization of all animals. PA antibody levels, $10 \times 2^{6-7}$ (1:640–1:1280), were maintained. Cotton rats were reimmunized 8 weeks after the first immunization and sera were obtained 4 weeks after the reimmunization in each group. PA antibodies were 2.8 to 9.5-fold higher after reimmunization than before.

Table 1
Lists of peptides for the M2-1, F, and NP of RSV.

Position	Sequence	MHC
$M2\text{-}1_{82-90}$	SYIGSINNI	H-2K^d
$M2\text{-}1_{127-135}$	RVYNTVISY	H2-K^d
$M2\text{-}1_{151-159}$	RLPADVLKK	HLA A3
$M2\text{-}1_{64-72}$	AELDRTEEY	HLA B44
$F_{247-261}$	VSTYMLTNSELLSLI	Unknown
$F_{253-265}$	TNSELLSLINDMP	Unknown
$NP_{360-368}$	NGVINYSVL	H2-D^b

The results of neutralizing antibody responses against RSV are shown in Fig. 2B. Cotton rats immunized with MVAIK/RSV/F were used as the positive control. Antibody responses against RSV subgroup A were detected 3 weeks after the immunization of all cotton rats with MVAIK/RSV/F, with a mean titer of $10^{1.40\pm0.31}$. High titers were observed after 12 weeks, with a mean titer of $10^{2.20}$. Antibody responses were detected in the MVAIK/RSV/NP group at very low levels of 1:10 three weeks after the immunization. No detectable antibody response was induced 6 weeks later. Cotton rats in the MVAIK/RSV/M2-1 group were reimmunized after 8 weeks. No detectable antibody response was detected in cotton rats reimmunized with MVAIK/RSV/M2-1.

3.3. Assessment of the number of CD8$^+$ cells expressing IFN-γ in cotton rats immunized with recombinant measles viruses

To evaluate the development of cellular adaptive immunity by recombinant viruses, the number of CD8$^+$ cells expressing IFN-γ was determined by flow cytometry.

Splenocytes were obtained from cotton rats immunized with recombinant measles viruses following a challenge with RSV Long strain, and were stimulated with the M2-1, NP, and F peptides. Figs. 3 and 4 show the results of the flow cytometry analysis. Seven peptides were used in this study, as listed in Table 1. The number of CD8$^+$ IFN-γ$^+$ double positive cells was 0.5% after the stimulation with mock antigen (RPMI medium). On the other hand, the number of CD8$^+$ IFN-γ$^+$ cells increased upto 3.3% after the stimulation with the RSV-$F_{253-265}$ peptide (Fig. 3). The results of the induction of CD8$^+$ IFN-γ$^+$ cells in the splenocytes of cotton rats immunized with recombinant measles virus were shown in Fig. 4. Splenocytes in animals immunized with MVAIK/RSV/M2-1 induced CD8$^+$ IFN-γ$^+$ cells when stimulated with the four M2-1 peptides. The strongest response (3.4%) was detected after the stimulation with $M2\text{-}1_{151-159}$. The number of CD8$^+$ IFN-γ$^+$ cells was higher in animals immunized with MVAIK/RSV/NP or MVAIK/RSV/F. These responses were markedly enhanced by the respective reimmunization. The number of CD8$^+$ IFN-γ$^+$ cells increased upto 8.5% ($NP_{360-368}$) or 6.4% ($F_{253-265}$) in cotton rats reimmunized with MVAIK/RSV/NP or MVAIK/RSV/F when stimulated with the respective peptides (Fig. 4).

The number of CD8$^+$ IFN-γ$^+$ cells also increased in the thymocytes of cotton rats immunized with MVAIK/RSV/NP and MVAIK/RSV/F when stimulated with the respective peptides, but not in cotton rats immunized with MVAIK/RSV/M2-1. However, the number of CD8$^+$ IFN-γ$^+$ cells was lower than that observed in the splenocytes (data not shown).

3.4. Protection against a RSV challenge

The detection of an infectious virus and virus genome copy numbers in lung homogenates were examined and the results are shown in Fig. 5A and B. All animals were infected with

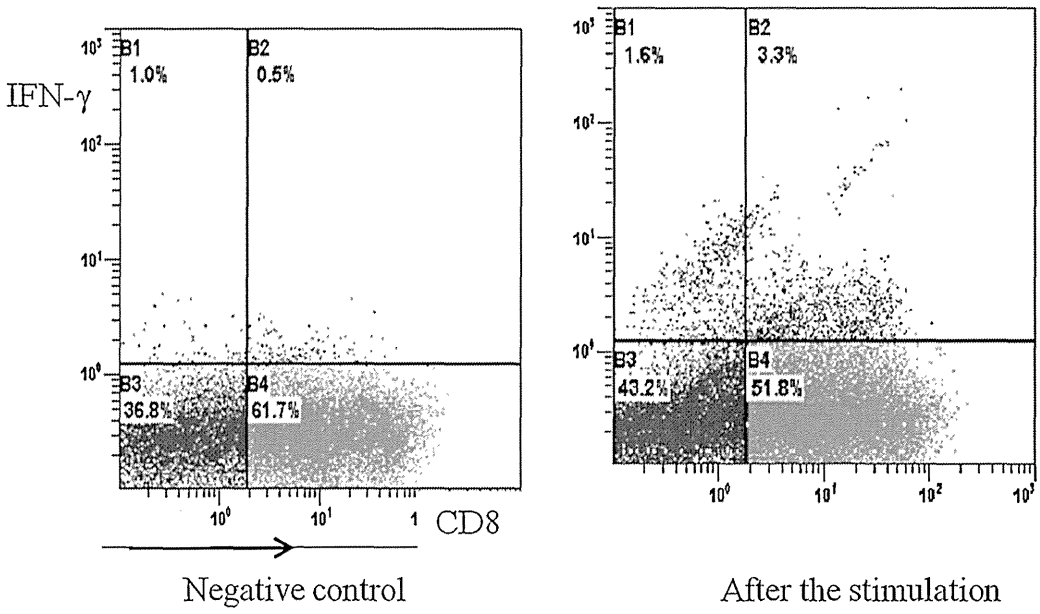

Fig. 3. *Flow cytometer analysis of CD8⁺ IFN-γ⁺ cells in cotton rats.* Splenocytes were obtained four days after the RSV challenge in cotton rats immunized with MVAIK/RSV/F. Splenocytes were stimulated with medium (A) and the RSV-fusion protein peptide (B).

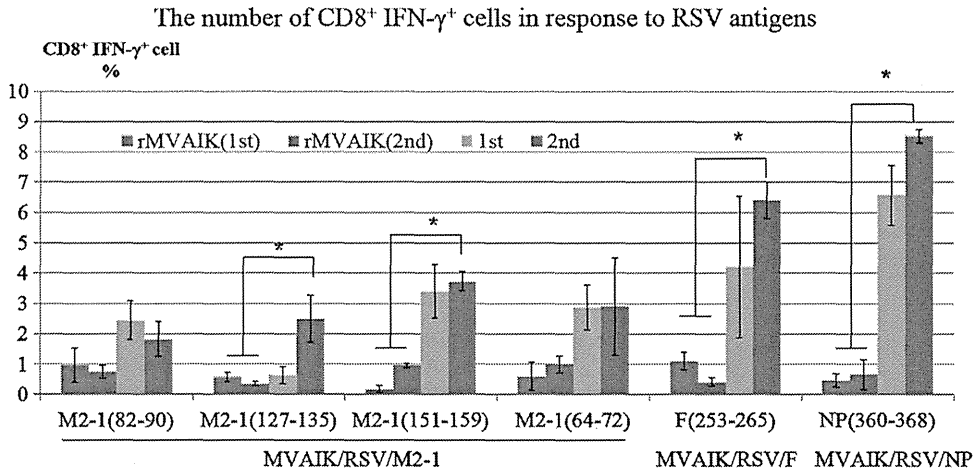

Fig. 4. *The number of CD8⁺ INF-γ⁺ cells stimulated with M2-1, F, and NP peptides.* Splenocytes were obtained after the RSV challenge in the first immunization and reimmunization groups immunized with recombinant viruses. The splenocytes of MVAIK/RSV/M2-1 group were stimulated with M2-1 peptides (82–90, 127–135, 151–159, and 64–72). The MVAIK/RSV/F group was stimulated with the F peptide (253–265), and the MVAIK/RSV/NP group was stimulated with the NP peptide (360–368). The rMVAIK group, which was the control, was stimulated with all peptides. These peptides are listed in Table 1. Significant difference was observed between animals immunized with rMVAIK and the recombinant measles virus (*: $P < 0.05$). Each bar represents the mean of three animals in each group with standard deviations.

1×10^6 PFU of RSV subgroup A (Long strain) though an intranasal route. A total of $10^{5.9}$ PFU of infectious viruses were detected in lung homogenates from animals immunized with rMVAIK (control group) after the challenge, and $10^{4.0}$ PFU of infectious viruses were detected in lung homogenates from animals immunized with MVAIK/RSV/M2-1 after the challenge. A total of $10^{4.4}$ and $10^{4.3}$ PFU of viruses were detected in animals immunized with a single dose of MVAIK/RSV/NP and MVAIK/RSV/F, respectively. Infectious viruses could not be detected after the RSV challenge in cotton rats reimmunized with MVAIK/RSV/M2-1 or MVAIK/RSV/F. Reimmunization with MVAIK/RSV/NP decreased RSV titers from $10^{4.4}$ to $10^{3.0}$ PFU (Fig. 5B).

The results of HE staining and immunostaining using a four clone blend of monoclonal antibodies against RSV P, F, and N proteins and anti-mouse IgG conjugated with HRP are shown in Fig. 6. The infiltration of inflammatory cells and thickness of the alveolar walls were observed in single immunization group after the RSV challenge similar to those of the unimmunized control. The lung tissues exhibited no serious pathological findings after the challenge by the reimmunization than by the first immunization. But, very mild pneumonitis was observed in the lung tissues after the challenge in cotton rats reimmunized with MVAIK/RSV/F than in those reimmunization with the others. To confirm the clearance of RSV-infected cells, lung tissues were examined by immunostaining against RSV P, F, and N proteins. These RSV proteins were detected in the bronchiolar epithelium cells of all animals immunized with a single dose of recombinant viruses when challenged with RSV, similar to the results obtained in the control group. However, no virus antigen was detected around the bronchus in cotton rats reimmunized with recombinant measles virus after the RSV challenge. The detection of the RSV antigen was correlated with the results for the recovery of infectious viruses from lung tissues.

These results suggest that reimmunization with these recombinant viruses may have induced protective immunity.

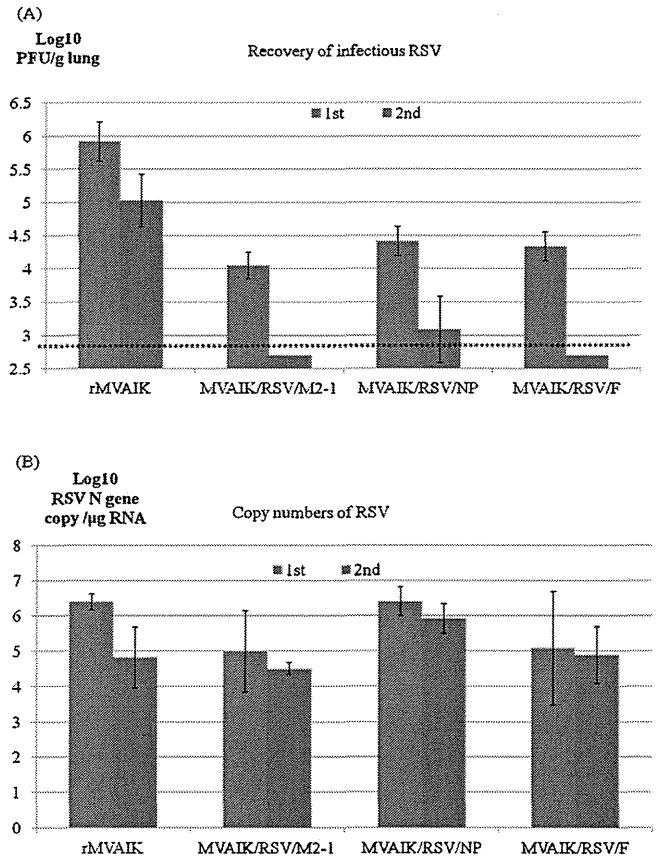

Fig. 5. *Recovery of infectious RSV and genome copy numbers after the RSV challenge.* Three cotton rats were challenged with 1.0×10^6 PFU of RSV Long strain 8 weeks after the first immunization (1st) and 6 weeks after re-immunization (2nd). (A) Virus infectivity was monitored in lung homogenates, and RSV infectivity was shown as PFU in 1 g of lung tissue. (B) The copy number of the RSV genome was shown in the lower panel. One microgram of total RNA from the lung tissue was used for real-time PCR. TaqMan PCR was performed in the RSV-NP gene region. Each bar represents the mean of three animals in each group with standard deviations. B.D.L indicates below the detection limit.

4. Discussion

Most infants have had RSV infections in early life and reinfection provides a strong immunological memory. RSV infections induce immunological stimuli that develop humoral antibodies and T-cell responses in young infants. However, local responses and the duration of immunity were previously shown to be insufficient [17,23,24]. RSV infections are limited to the respiratory tracts without causing a viremic stage, which results in weak immunological responses of a relatively short duration. A RSV vaccine needs to be developed that induces a higher levels of serum antibodies and CTL responses [25,26]. Therefore, measles vaccine-vectored RSV vaccine candidates were developed in the present study. MV vaccines induce life-long immunity and the development of safe, effective, and affordable vaccines, which induce protective immune responses against RSV infections, is expected. CTL activity is also expected to be inducible by measles virus-vector vaccines [27].

Candidates of the recombinant measles viruses expressing RSV M2-1 or NP were developed in the present study. In this study, M2-1 and NP were chosen because these proteins have been shown to increase the number of $CD8^+$ IFN-γ^+ T cells in mice [11,28–30]. Neither of the recombinant measles viruses induced sufficient numbers of $CD8^+$ IFN-γ^+ cells after the first immunization. MVAIK/RSV/NP predictably elicited $CD8^+$ IFN-γ^+ cells after the reimmunization, however MVAIK/RSV/M2-1 unexpectedly induced a poor immune response (Fig. 4). RSV clearance was enhanced by the immunization of cotton rats with MVAIK/RSV/M2-1 or MVAIK/RSV/NP (Fig. 5). These results demonstrated that an increase in the number of $CD8^+$ IFN-γ^+ cells inhibited the expansion of viral infections. The number of $CD8^+$ IFN-γ^+ cells was lower after reimmunization with MVAIK/RSV/M2-1 than with the others, whereas no significant differences were observed in virus clearance.

The M2-1 protein is an antigen that efficiently induces IFN-γ by $CD8^+$ T cells against RSV in mouse models, and the Th1-type immune response was influenced by MHC class I [31]. The M2-1 peptides prepared in the present study were based on previous studies that observed restricted BALB/c mice MHC because the antigen mapping of M2-1 was not reported for the cotton rat. The NP peptide was presented on the MHC of C57BL/6 mice, which led to the production of IFN-γ by splenocytes. The infectious virus titer was reduced by immunization using MVAIK/RSV/M2-1 and led to the absence of pathological findings in the cotton rat model (Fig. 6). This result showed that M2-1 efficiently protected against RSV infections in the cotton rat. Therefore, the synthesized peptides of M2-1 may not have been a match to the MHC of cotton rats in the present study.

MVAIK/RSV/M2-1 induced no detectable neutralizing antibody response against RSV (Fig. 2). Neutralizing antibodies against RSV have been elicited using fusion protein or glycoprotein [32,33]. These proteins are known to induce antibody responses in order to protect against viral infections and many candidate vaccines have been examined [34,35]. In our laboratory, MVAIK/RSV/F and MVAIK/RSV/G, which express the fusion protein and glycoprotein of RSV, have developed and induced neutralizing antibody responses in the cotton rat model. M2-1 may elicit some antibody responses against the M2-1 protein; however, MVAIK/RSV/M2-1 could not develop neutralizing antibody responses against RSV in the present study. MVAIK/RSV/NP induced a weak neutralizing antibody response (Fig. 2). The underlying mechanism has not been elucidated. The antibody responses induced by both recombinant measles viruses were weaker than that following immunization with MVAIK/RSV/F. However, in a previous study, small number of inflammatory cells were noted into the lungs immunized with MVAIK/RSV/F following the RSV challenge. In the present study, MVAIK/RSV/F increased the number of $CD8^+$ IFN-γ^+ cells after reimmunization in the cotton rat (Fig. 4). Therefore, the Th1-type immune response may be weak in animals immunized with MVAIK/RSV/F and cannot inhibit the infiltration of inflammatory cells into the lungs [20]. This is a key issue for evaluating vaccine candidates because the FI-RSV vaccine led to pulmonary eosinophilia and failed to induce Th1 immune responses or CTL activities. Thus, the Th1/Th2 balance is important for safety, together with effectiveness, when considering vaccine candidates [36–38].

In the present study, pulmonary histological findings revealed that MVAIK/RSV/F did not completely inhibit the infiltration of inflammatory cells into the lungs (Fig. 6). Therefore, a stronger Th1-type immune response needs to be induced than that by MVAIK/RSV/F in order to inhibit severe inflammation in the lungs. Cotton rats inoculated with MVAIK/RSV/M2-1 and MVAIK/RSV/NP had higher numbers of $CD8^+$ IFN-γ^+ cells and the absence of an inflammatory response in the lungs after the RSV challenge. These recombinant viruses efficiently induced the clearance of RSV, similar to that induced by MVAIK/RSV/F, and additionally inhibited the infiltration of inflammatory cells into the lungs. Bivalent vaccines containing MVAIK/RSV/F and MVAIK/RSV/M2-1 or MVAIK/RSV/NP are expected to induce effective immune responses.

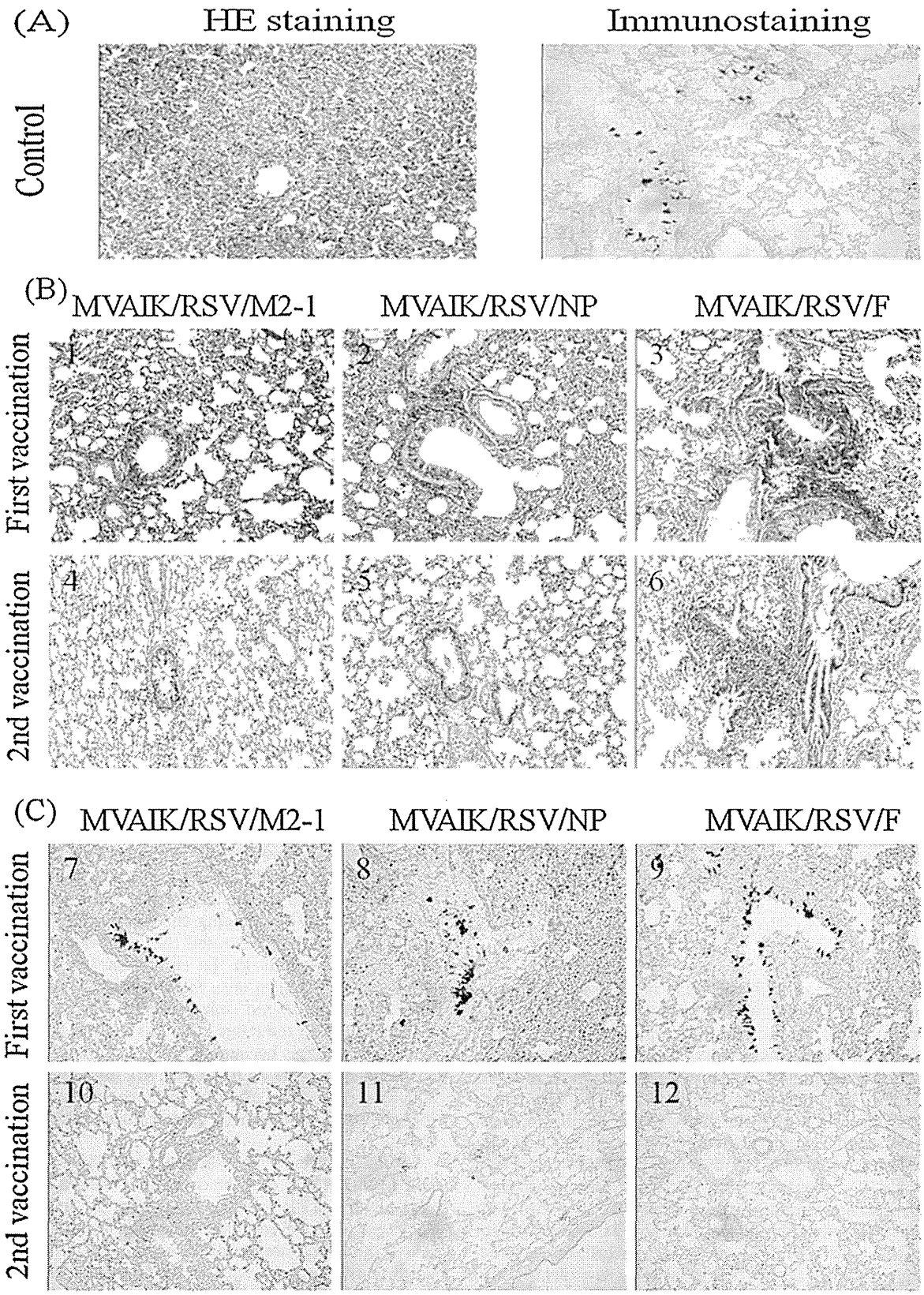

Fig. 6. *Pulmonary histopathology in cotton rats challenged with RSV subgroup A.* Animals were immunized with MVAIK/RSV/M2-1 (panels (B-1), (B-4), (C-7), and (C-10)), MVAIK/RSV/NP (panels (B-2), (B-5), (C-8), and (C-11)), and MVAIK/RSV/F (panels (B-3), (B-6), (C-9), and (C-12)). The results of hematoxylin-eosin staining are shown in panel (A) and panel (B). The results of immunostaining are shown in panel (C). Panel (A) shows the results of lung tissue obtained from the control group infected with RSV. Samples were stained with hematoxylin-eosin or immunostained with a four clone blend of monoclonal antibodies against the RSV P, F, and N proteins and anti-mouse IgG conjugated with HRP.

In conclusion, measles vaccine-vectored RSV vaccine candidates were developed in the present study. Recombinant measles viruses expressed the M2-1 or NP of RSV and increased the number of $CD8^+$ IFN-γ^+ cells after immunization in cotton rats. The combination of bivalent vaccines may elicit antibody responses and $CD8^+$ IFN-γ^+ cells against RSV and control a balanced Th1/Th2 immune response after RSV infection. Therefore, recombinant measles viruses are promising RSV vaccine candidate.

Acknowledgments

The authors thank Dr. Sawada A for his generous supply of MVAIK/RSV/F, and M. Maeda for her help with the cotton rat experiments.

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Journal of Medical Virology 86:658–665 (2014)

Virus Specific Cell-Mediated Immunity May Play a Role in Controlling Reactivated Human Herpesvirus 6B in Patients Under Measles Induced Immunosuppression

Takuji Kumagai,[1]* Tetsushi Yoshikawa,[2] Kimiyasu Shiraki,[3] Mariko Yoshida,[4] Tetsuo Nakayama,[5] Masaru Ihira,[2] and Yoshizo Asano[2a]

[1]*Kumagai Pediatric Clinic, Sapporo, Japan*
[2]*Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan*
[3]*Department of Virology, Graduate School of Medicine, Toyama University, Toyama, Japan*
[4]*Department of Virology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
[5]*Laboratory of Viral Infection, Kitasato Institute for Life Sciences, Tokyo, Japan*

For a better understanding of the cellular immune responses to reactivated HHV 6B the lymphoproliferative response to human herpesvirus 6B (HHV 6B) antigen was measured in three consecutive specimens obtained biweekly from 22 young children and infants suffering from acute measles, and in 19 influenza patients and nine healthy control subjects. HHV 6B DNA in peripheral blood mononuclear cells (PBMCs) was detected in 18 of 22 subjects with measles, but not in the influenza patients or the healthy population. A novel reactivation profile of HHV 6B was found in patients with measles in the milder form of immunosuppression than in patients with organ transplantation. HHV 6B specific lymphoproliferation activities increased correspondingly with reactivation of HHV 6B assessed by detecting HHV 6B DNA in PBMCs in patients with measles, but no significant change in either the antibody response to HHV 6B or DNAemia occurred in serial specimens obtained either from patients with influenza or healthy subjects. This novel form of HHV 6B reactivation without antibody response was observed in patients with measles. The dynamic fluctuations in lymphoproliferative responses in measles may represent the balance between HHV 6B reactivation and its suppression by the host immune system. ***J. Med. Virol. 86:658–665, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: human herpesvirus 6B; cell-mediated immunity; lymphocyte proliferation; measles

INTRODUCTION

Human herpes viruses are distributed ubiquitously due to established latency after primary infection. Reactivation from latency may occur under immunosuppressive treatment such as in patients with hematologic diseases, HIV infection, and in transplant recipients. However, the mode of reactivation differs in each herpes virus group. Severity and duration of immunosuppression contribute to form the clinical profile of reactivation. Recently developed sophisticated techniques such as PCR, RT-PCR, NASBA have made it possible to detect various forms of subclinical reactivation. Reactivation may be clinically apparent such as the Hunt syndrome caused by VZV whereas HSV causes asymptomatic virus shedding. Thus, there are many forms of herpesvirus reactivation reflecting the combination of clinical findings and laboratory observations.

Immune dysfunction associated with acute measles infection in immunocompetent hosts is also known to occur and this measles virus (MV)-induced immune suppression, especially in cell-mediated immunity

Conflicts of interest: none.

[a]Professor (Specially appointed).

The present address of Yoshizo Asano is Zambia Project, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

*Correspondence to: Takuji Kumagai, MD, PhD, Kumagai Pediatric Clinic, W-6, Momijidai, Atsubetsu-Ku, Sapporo, Hokkaido 004-0013, Japan.
E-mail: tkuma@mb.infosnow.ne.jp

Accepted 8 November 2013

DOI 10.1002/jmv.23862
Published online 3 January 2014 in Wiley Online Library (wileyonlinelibrary.com).

(CMI), may increase susceptibility to opportunistic infections. Steps in the underlying mechanisms regarding MV-induced immune dysfunction have been suggested [Avota et al., 2006]. Of interest is whether HHV 6B reactivation is also induced during acute measles and how the host immune system, especially its cellular component, reacts to such reactivated virus. In a previous study, a significant antibody rise to HHV 6B was observed in some but not all of a group of patients with acute measles (18 out of 45) and HHV 6B was isolated in 3 of the 18 subjects [Suga et al., 1992].

The present study was designed to investigate the kinetics of the lymphoproliferative response specific to HHV 6B and to phytohemagglutinin (PHA) based on three consecutive specimens from children in the first 5 weeks of acute measles. Nineteen influenza patients and nine healthy control subjects in whom immunosuppression was not present were also tested. Patients with measles showed a combined profile of HHV 6B DNA synthesis in PBMCs with a lymphoproliferative response specific to HHV 6B. However no antibody response was detected. This study revealed the novel reactivation profile of patients with measles acting as a milder form of immunosuppression than organ transplantation.

SUBJECTS, MATERIALS AND METHODS

Study Subjects

The measles study group consisted of 22 children (11 boys, 11 girls), with a mean age of 46 months (range, 12–93 months) who developed clinical symptoms of acute measles (Table I). A second study group was composed of 19 children (7 boys, 12 girls), with a mean age of 37 months (range, 17–78 months), who developed influenza in January and February 2002.

The third group comprised nine healthy individuals (mean age of 69 months; range, 32–186 months) seen for health checks prior to minor ophthalmologic surgery. Subjects in all three groups had a history of exanthema subitum. Children with a history of immune deficiency, those taking cytotoxic or immunosuppressive drugs, and those who had received immunoglobulin were excluded. The first day of fever was designated as day 0. Three serial peripheral blood samples were collected; the first after 3 ± 2 days of the acute phase of measles or influenza and the second and the third samples at 16 ± 3 days and at 35 ± 4 days, respectively. Three similarly timed specimens were obtained in nine healthy subjects. The diagnosis of acute measles or influenza was based on the clinical picture, rapid antigen test in influenza cases, and later was confirmed by seroconversion and virus isolation (measles cases only).

Informed consent was obtained from the parents of enrolled subjects after the project was thoroughly explained. The study complied with the Human Experimentation Guidelines of the authors' institutions (Fujita Health University School of Medicine and Sapporo Medical University School of Medicine).

HHV 6B Antigen

HHV 6B antigen was prepared as previously reported [Kumagai et al., 2006]. Briefly, cord blood mononuclear cells (CBMCs) inoculated with the Z29 strain of HHV 6B were washed three times with phosphate-buffered saline (PBS) and re-suspended in 1/20th of the original volume and sonicated. After centrifugation, the supernatant was layered onto glycerol–potassium tartrate viscosity-density gradients and centrifuged [Martin and Palmer, 1973]. Virion fraction was used as HHV 6B antigen. A control antigen was prepared in an identical manner using the uninfected CBMCs. Infectivity was eliminated by ultraviolet lamp irradiation. Both virion and control antigens were dialyzed against RPMI 1,640 medium (Grand Island Biological Co., Grand Island, NY). The optimal dilution for the lymphocyte proliferative response was determined and 1/8 dilution was used in the present study.

In Vitro Lymphocyte Proliferation Assay

Lymphocyte proliferation assay was performed as reported previously [Kumagai et al., 1999]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from the subjects by Ficoll–Hypaque (Sigma, St. Louis, MO) gradient centrifugation. PBMCs with or without viral antigen were cultured at a concentration of 1×10^5 cells/well in 200 μl of RPMI 1640 containing 10% autologous serum (obtained at each sampling point). Triplicate cultures were established and incubated at 37°C in 5% CO_2 atmosphere for 6 days, then at the beginning of day 7, 1 μCi of [^{3}H] thymidine was added to each culture and 16 hr later the cultures were harvested onto fiber glass filters using a multiple automated sample harvester. Incorporation of radioactive thymidine into harvested cells was determined by liquid scintillation. The results are expressed as ratios of [^{3}H]thymidine incorporation in antigen stimulated cultures relative to that in control cultures (stimulation index [SI]). A SI greater than or equal to 3.0 was considered to be a positive response. Lymphocyte proliferation assay to PHA was performed in an identical manner except that PHA (10 μg/ml) instead of specific HHV 6B antigen was used as a stimulant and the incubation period was 3 days instead of 7 days.

Determination of Hemagglutination Inhibition (HI) Antibody to Measles

HI antibodies against measles virus were determined as described previously [Miyamura et al., 1997]. Serum samples were diluted at 1:4 with 1/15 M PBS and treated with the same volume of 0.25% kaolin to remove nonspecific factor for the HI test. Supernatant was mixed with 50% green monkey red blood cells (rbcs) to remove the nonspecific rbcs

TABLE I. Clinical and Laboratory Data on Patients With Acute Measles

Patient number	Age year/ month	Sex	Sample number	Measles HI (2^n)	HHV-6IFA		HHV-6 ELISA IgG (2^n)	HHV-6 neutralization (2^n)	Virus isolation	
					IgG (2^n)	IgM (2^n)			Measles	HHV-6
1	7y 2m	M	1st	<3	6	<3	7	4	(+)	(−)
			2nd	5	7	<3	8	5	(−)	(−)
			3rd	3	6	<3	6	5	(−)	(−)
2	5y 4m	M	1st	<3	5	<3	6	4	(+)	(−)
			2nd	5	5	<3	6	5	(−)	(−)
			3rd	6	5	<3	5	4	(−)	(−)
3	2y 0m	M	1st	<3	7	<3	8	6	(+)	(−)
			2nd	7	8	<3	9	6	(−)	(−)
			3rd	7	7	<3	8	5	(−)	(−)
4	4y 0m	M	1st	<3	8	<3	9	4	(+)	(−)
			2nd	5	8	<3	9	6	(−)	(−)
			3rd	7	8	<3	8	6	(−)	(−)
5	4y 9m	M	1st	<3	6	<3	6	5	(+)	(−)
			2nd	7	6	<3	6	5	(−)	(−)
			3rd	8	5	<3	6	5	(−)	(−)
6	3y 8m	M	1st	<3	5	<3	6	4	(+)	(−)
			2nd	7	6	<3	7	5	(−)	(−)
			3rd	8	6	<3	7	5	(−)	(−)
7	5y 0m	M	1st	<3	4	<3	3	4	(+)	(−)
			2nd	7	4	<3	3	4	(−)	(−)
			3rd	7	4	<3	3	4	(−)	(−)
8	5y 11m	F	1st	<3	6	<3	6	4	(+)	(−)
			2nd	8	5	<3	6	4	(−)	(−)
			3rd	9	6	<3	5	4	(−)	(−)
9	3y 4m	F	1st	<3	5	<3	5	4	(+)	(−)
			2nd	7	5	<3	5	4	(−)	(−)
			3rd	8	5	<3	4	5	(−)	(−)
10	3y 10m	F	1st	<3	8	<3	9	6	(+)	(−)
			2nd	5	8	<3	8	4	(−)	(−)
			3rd	7	8	<3	9	6	(−)	(−)
11	3y 3m	F	1st	<3	5	<3	6	4	(+)	(−)
			2nd	7	5	<3	5	4	(−)	(−)
			3rd	7	5	<3	5	4	(−)	(−)
12	3y 3m	F	1st	<3	7	<3	6	4	(+)	(−)
			2nd	8	8	<3	7	5	(−)	(−)
			3rd	7	8	<3	7	4	(−)	(−)
13	1y 8m	F	1st	<3	8	<3	9	6	(+)	(−)
			2nd	7	8	<3	9	6	(−)	(−)
			3rd	7	7	<3	8	6	(−)	(−)
14	3y 7m	M	1st	<3	7	<3	8	5	(+)	(−)
			2nd	7	6	<3	7	4	(−)	(−)
			3rd	7	7	<3	6	5	(−)	(−)
15	4y 6m	F	1st	<3	4	<3	5	4	(+)	(−)
			2nd	6	4	<3	5	4	(−)	(−)
			3rd	6	3	<3	5	4	(−)	(−)
16	7y 9m	M	1st	<3	3	<3	4	4	(+)	(−)
			2nd	6	3	<3	4	5	(−)	(−)
			3rd	6	3	<3	4	4	(−)	(−)
17	2y 3m	M	1st	<3	8	<3	8	7	(+)	(−)
			2nd	7	8	<3	8	6	(−)	(−)
			3rd	7	8	<3	7	6	(−)	(−)
18	2y 3m	F	1st	<3	8	<3	7	5	(+)	(−)
			2nd	7	8	<3	7	6	(−)	(−)
			3rd	8	8	<3	7	6	(−)	(−)
19	1y 1m	M	1st	<3	6	<3	n.d.	n.d.	(+)	(−)
			2nd	7	6	<3	n.d.	n.d.	(−)	(−)
			3rd	7	5	<3	n.d.	n.d.	(−)	(−)
20	2y 1m	F	1st	<3	6	<3	n.d.	n.d.	(+)	(−)
			2nd	7	6	<3	n.d.	n.d.	(−)	(−)
			3rd	7	6	<3	n.d.	n.d.	(−)	(−)
21	1y 0m	F	1st	<3	6	<3	n.d.	n.d.	(+)	(−)
			2nd	6	6	<3	n.d.	n.d.	(−)	(−)
			3rd	6	5	<3	n.d.	n.d.	(−)	(−)
22	6y 0m	F	1st	<3	4	<3	n.d.	n.d.	(+)	(−)
			2nd	<3	4	<3	n.d.	n.d.	(−)	(−)
			3rd	6	3	<3	n.d.	n.d.	(−)	(−)

HHV, human herpesvirus; IFA, immunofluorescent antibody; ELISA, enzyme linked immunosorbent assay; HI, hemagglutination inhibition; n.d., not done because of sample volume constraints.

agglutination; at this step, serum samples were diluted 1:8. Serum samples were diluted serially and four units of hemagglutinin antigen was added. After the addition of 0.5% of green monkey rbcs, the reciprocal of the dilution that inhibited hemagglutination completely, was considered HI antibody titer.

Determination of Hemagglutination Inhibition (HI) Antibody to Influenza

The HI test was performed by a standard microtiter assay with human erythrocytes after removing nonspecific inhibitors with receptor-destroying enzyme and cold agglutinins by hemadsorption at 4°C. For each antigen, all specimens were tested on the same day using identical reagents.

Determination of Immunofluorescent Antibodies (IFA) to HHV 6B Antigen

Antibody titers to HHV 6B were measured with an indirect-immunofluorescence assay as described previously [Yoshikawa et al., 1989]. A representative strain of HHV 6 species B (FG-1), which was isolated from peripheral blood mononuclear cells obtained from an exanthema subitum patient, was used as the standard antigen. The antibody titer was defined as the reciprocal of the plasma dilution that showed specific fluorescence.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA antibody titers were determined as described previously [Asano et al., 1990]. Briefly, the cell lysate of the CBMCs infected with HHV 6B (S29 strain) was used as antigen. Wells of a microplate were coated with antigen followed by addition of diluted serum samples. Then, secondary antibody conjugated with alkaline phosphatase was added and enzyme reaction was developed with substrate. The results (net absorbance) were expressed as the reciprocal of the plasma dilution that showed an absorbance reading above cutoff value. Studied samples with net absorbance readings of $\geq$3 SD above the mean absorbance of the negative reference sera were considered reactive for anti-HHV 6B antibody.

Determination of Neutralization Antibodies to HHV 6B

Virus stocks were prepared from CBMCs infected with the Z29 strain of HHV 6B, and a dot blot assay, described elsewhere, was employed to determine the titers of neutralization antibody to HHV 6B [Tsukazaki et al., 1998]. Briefly, 25 µl of serial twofold serum dilutions or medium without sera (control) were prepared on each well of a 96-well microtiter U-bottom plate and mixed with equal volumes of HHV 6B virus stock, then incubated for 1 hr at 37°C in a CO_2 incubator. Fifty microliter of CBMCs was added to each well, centrifuged and washed once with medium, the plate was incubated for 7 days at 37°C in a CO_2 incubator. To monitor the virus growth in each well, a dot blot antigen detection was performed as described previously [Tsukazaki et al., 1998]. In brief, after 7 days of incubation the plate was centrifuged and the cells were washed with PBS, and lysed with lysis buffer. The cell lysates were spotted onto a nylon membrane and viral antigens were detected using monoclonal antibodies specific to HHV 6B.

Virus Isolation

Isolation and identification of HHV 6B was conducted by co-cultivating PBMCs from subjects with CBMCs, as described elsewhere [Asano et al., 1989]. The cultures were maintained at 37°C in a CO_2 incubator. Virus isolation was considered positive if large cell formation of the cultured cells and specific immunofluorescence staining of the cultured cells with the antibodies to HHV 6B or 7 were observed. Also, MV was isolated by co-cultivation of PBMCs with B95a cells [Kobune et al., 1990]. Cultures which developed CPE were examined by immunofluorescence with fluorescein-conjugated mouse anti-MV monoclonal antibody for identification.

Real-Time PCR (Polymerase Chain Reaction) for HHV 6B

DNA samples were extracted from PBMCs using a QIAamp Blood Kit (QIAGEN, Chatsworth, CA) and stored at –20°C. Real-time PCR was used to quantitate the copy numbers of HHV 6B DNA. The real-time PCR methods used to assess HHV 6B levels have been described elsewhere [Tanaka et al., 2000]. PCR reactions were performed using the TaqMan PCR Kit (PE Applied Biosystems, Foster City, CA). For each viral DNA assessment, standard curves were constructed using the CT values obtained from serial dilution of plasmid DNA containing the target sequence. CT values for each sample were plotted on a standard curve, allowing automatic calculation of DNA copy numbers using Sequence Detector v1.6 software (PE Applied Biosystems). Each sample was tested in duplicate; the mean of the two values was used to determine the sample copy number. Performance of real-time PCR with primers and probes provided with the Taqman-beta actin reagent kit (PE Applied Biosystems) allowed us to calculate the copy numbers of viral DNA per cell. Viral DNA load was calculated as copy number per 1 million cells.

Statistics

In this study, measurements were made repeatedly (three times) in each subject, such as the early, intermediate and late stages of the infection. Thus, one-way repeated-measures analysis of variance

(ANOVA) should be employed on the same assumption as for a *t*-test, namely, that the distribution of differences in the population approximates a normal distribution. However, due to skewed and inhomogeneity of variance for antibody titers and lymphoproliferative responses to HHV 6B antigen and to PHA among patients with measles and influenza, the *P*-value was calculated using the non-parametric test analogous to repeated measures one-way ANOVA (Friedman's test). If Friedman's test detects a statistically significant difference among the means of the three populations, multiple comparison post-test was applied to detect which population showed the highest activity. Non-parametric multiple comparison post-test is called Dunn's test which is a counterpart of Dunnett's test for parametric comparison [Motulsky, 1995]. Regarding data of the nine healthy subjects, Bartlett test was used to confirm normal distribution of variance in the lymphoproliferative responses to HHV 6B antigen and for PHA, two-way ANOVA was performed. All statistical analyses were performed using StatFlex 6.0 (Artech, Osaka, Japan).

RESULTS

Clinical Characteristics of Subjects and Virological Evaluation of HHV 6B Reactivation in MV Infections, Influenza and Healthy Individuals

Symptoms shown by subjects with acute measles were all typical such as high fever followed by the appearance of a skin rash. In addition to the clinical picture, MV infection was verified by an increase in HI antibody titer, from negative to a high value (Table I).

In contrast, no significant HHV 6B antibody elevation occurred in any subject except for case 4 in whom neutralization antibody rose from 2^4 to 2^6. In addition, MV was isolated from the acute phase peripheral blood specimens, further substantiating the diagnosis of acute MV infection, whereas HHV 6B was not isolated in any sample.

Influenza patients showed typical clinical symptoms including fever, cough, and rhinorrhea, but none developed pneumonia. HI antibody titer directed against H1N1 (New Caledonia) was significantly elevated in 3 of 19 whereas a significant HI antibody rise against H3N2 (Panama) was observed in the remaining 16 subjects. All subjects in this group had an IFA and neutralization antibody of more than 2^4 directed against HHV 6B. However, no significant antibody boost occurred in the serial serum samples.

All healthy children had positive IFA and neutralization antibody directed against HHV 6B of more than 2^2. Two cases in this group developed elevated neutralization antibody during the observation period, which was from 2^2 to 2^5 in both cases.

HHV 6B was not isolated from any of the specimens obtained from influenza patients and healthy children.

Real-Time PCR Detection of HHV 6B DNA in PBMCs

HHV 6B DNA was detected in 18 of 22 subjects with measles and copy numbers in PBMCs are shown in Figure 1. None was detected in plasma specimens. In 15 of 18 subjects with HHV 6B DNAemia, more than 10 copies/μg of viral DNA were detected. The kinetics of HHV 6B DNA copy numbers in PBMCs varied, however, dynamic fluctuations in copy numbers were observed in 17 subjects. In 10 patients, HHV 6B DNA was negative in the first specimen, becoming positive thereafter. Thirteen subjects had positive HHV 6B DNA at more than one time point.

None of the children with influenza and none of the healthy population showed positive HHV 6B DNA in their serial specimens.

In Vitro Lymphoproliferative Responses Specific to HHV 6B Antigen During Acute Measles, Influenza and in Healthy Subjects

Although the time kinetics of the HHV 6B specific lymphoproliferative response varied in measles patients, in most cases it was low in the acute phase and subsequently increased (Friedman's test, $P = 0.0251$), and then waned (Fig. 2A, upper). Dunn's test as the post-test showed that the peak response in lymphocyte proliferation assay was observed in the second specimens ($P < 0.01$) and no significant difference was observed between the first and the third samples. In contrast, in influenza patients, there was no significant difference in lymphoproliferative responses specific to HHV 6B between the serial 3 samples (Friedman's test, $P = 0.2290$, Fig. 2B, upper). Also, healthy children did not show any fluctuations in activity of specific lymphocytes between the serial specimens (ANOVA, $P = 0.0578$, Fig. 2C, upper).

In Vitro Lymphoproliferative Responses to PHA

In terms of lymphoproliferative responses to PHA in measles patients, lymphocyte activities varied significantly between the 3 specimens (Friedman's test, $P < 0.0001$). The second and third samples showed significantly higher responses compared with the first sample (Dunn's test, $P < 0.0012$ and 0.001, respectively), and the peak lymphoproliferative response to PHA was observed in the third samples (Fig. 2A, lower). Thus, the PHA specific lymphocyte proliferation responses exhibited significantly different time course characteristics from that to HHV 6B antigen.

Regarding lymphocyte responses to PHA in influenza patients and the healthy population, no significant differences were observed between sampling points (Friedman's test, $P = 0.2414$ and ANOVA, $P = 0.0578$, respectively; Fig. 2B and C, lower).

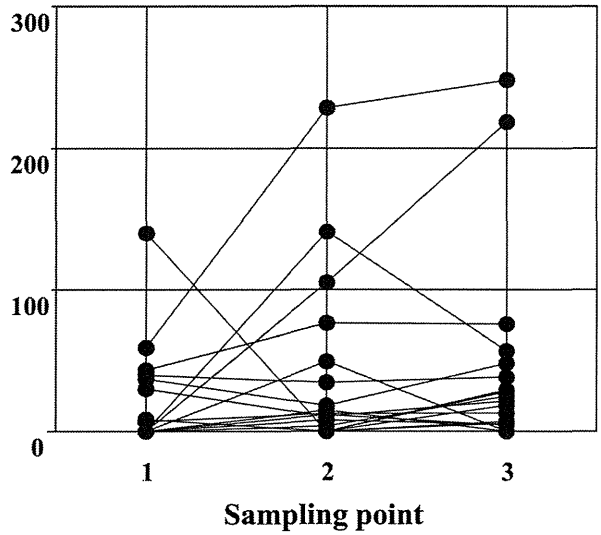

Fig. 1. Abscissa represents the time point of collecting blood samples indicated in the text and the ordinate the HHV 6B DNA copy number/μg DNA detected in PBMCs of measles patients. Closed circles represent the HHV 6B DNA copy numbers in each sampling point.

DISCUSSION

HHV 6B specific CMI can be elicited in infants and young children during the course of exanthema subitum [Kumagai et al., 2006]; however, its role after primary infection especially in later life is unknown. In the acute phase of exanthema subitum, NK cells seem to be crucial to resolving the primary HHV 6B infection [Takahashi et al., 1992; Kumagai et al., 2006], but the role of HHV 6B specific lymphocytes in the recovery from primary infection remains unknown. One possibility is that they might be involved in suppressing internal reactivation in later life.

MV infection initially induces anergy, reduced reaction to the tuberculin test, and temporary immunosuppression also occurs during the course of the illness. Measles virus-induced immunosuppression is characterized by (1) lymphopenia possibly due to loss of precursors, infection-dependent loss and loss by apoptosis, (2) prolonged cytokine imbalance consistent with suppressed cellular immunity, and (3) silencing of peripheral lymphocytes by viral proteins [Avota et al., 2010]. In this context, it has been shown that HHV 6B reactivation occurred during acute measles [Suga et al., 1990, 1992]. Consequently, time kinetic studies of HHV 6B specific CMI during the course of acute measles might illuminate the role of CMI long after recovery from the primary infection and may present the novel pattern of immunosuppression by measles comparing with more rigorous immunosuppression such as associated with organ transplantation. Measles causes immunosuppression but it may be milder producing a different profile of reactivation.

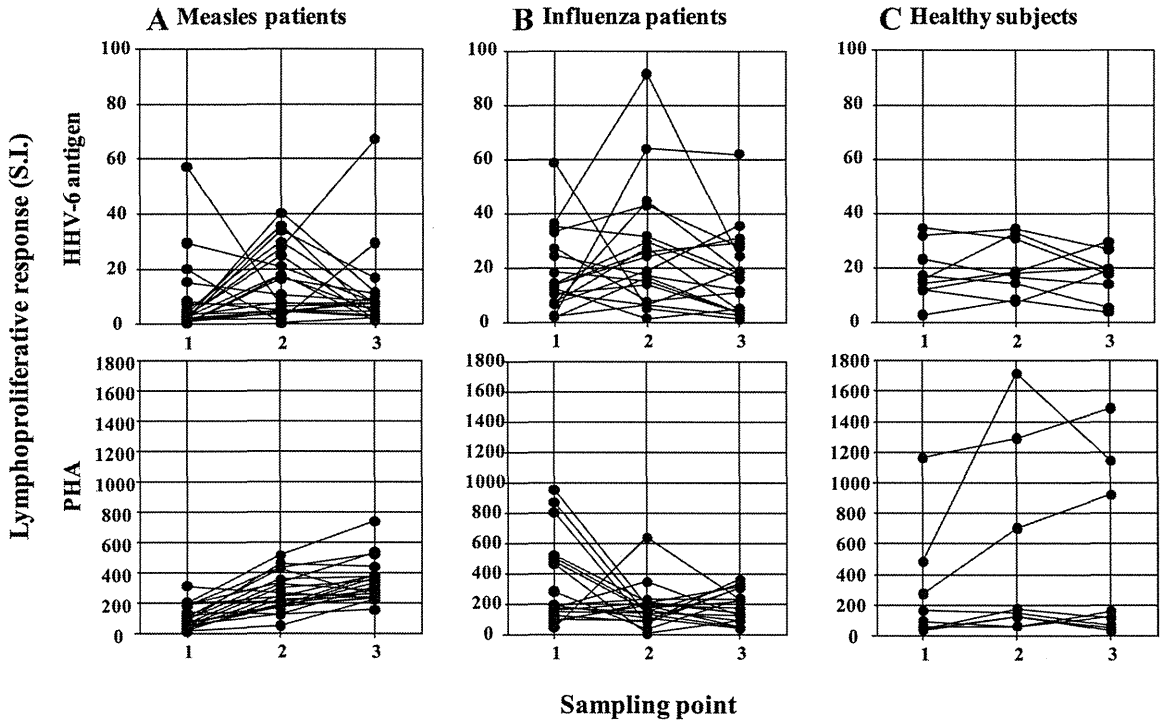

Fig. 2. The upper row shows the development of lymphoproliferative responses specific to HHV 6B antigen in subjects with acute measles (**A**), influenza (**B**), and in the healthy population (**C**). The lower row shows the kinetics of the lymphoproliferative responses to PHA in the same groups. Abscissa represents the time point of collecting blood samples indicated in the text and the ordinate the stimulation index (SI). Closed circles represent SI of the lymphoproliferative responses.

MV infection allows replication of HHV 6B in the PBMCs of subjects that stimulates specific lymphocyte responses. Mobilized CMI may have suppressed viral reactivation under limited level characterizing the combination of DNA synthesis with possibly a production of immediate-early and/or early viral proteins but without specific antibody responses or virion formation. This novel type of reactivation might be named "abortive" reactivation.

In the present study HHV 6B specific lymphocyte proliferation activity was low in the acute phase and increased subsequently, peaking around day 16 and declining 5 weeks after the onset. In contrast, lymphocyte proliferation activities to PHA increased progressively from the acute to the convalescent phase of measles peaking in the third samples, supporting the view that lower lymphocyte proliferation activities to PHA in the acute stage of measles may reflect depressed CMI caused by MV infection while the increased activities of lymphoproliferative responses in the second and the third samples imply recovery of CMI from the influence of the MV infection. Taken together, even under the depressed immune function resulting from measles virus infection, HHV 6B specific CMI developed in a characteristic pattern composed of elevation and waning resembling that observed in acute varicella [Kumagai et al., 1980]. This pattern might reflect the fact that HHV 6B reactivation during the course of acute measles, and the time course characteristics represent HHV 6B specific T cell mobilization to antigenic re-exposure. To support interpretation of the results in measles patients, the kinetics of responses in lymphocyte proliferation assay to both HHV 6B and PHA were observed in a group of influenza patients and of a healthy population as a control group. Lymphoproliferative responses in both groups as control populations did not show any statistically significant changes in activities during the observation period, substantiating the view that the changes in lymphocyte activities in measles patients are specifically elicited by and directed against reactivated HHV 6B.

Regarding this specific point, one might argue that the present study gave no convincing evidence of HHV 6B reactivation, as there was no increase in antibody titer, and the virus could not be isolated. Also, HHV 6B DNA in PBMCs of healthy subjects without signs of HHV 6B reactivation has been documented [Cuende et al., 1994; Alvarez-Lafuente et al., 2002; Caserta et al., 2004]. Indeed, until now, HHV 6B reactivation was known to be associated with specific antibody elevation and positive virus isolation. However, even in a severe immune suppression induced by immunosuppressive therapy after organ transplantation, only some of the recipients show such a "typical" or "complete" type reactivation [Ohashi et al., 2008]. Even in the previous study, only a few subjects (3/42) revealed positive virus isolation and only two showed elevated antibody titers. Thus, in measles induced immunosuppression, there might be an "abortive" or "incomplete" type subclinical reactivation which is characterized by the presence of specific CMI response and DNA synthesis in PBMCs with lack of virus isolation and antibody rise. HHV 6B is reactivated in the latently infected macrophages and lymphocytes. Viral immediate early and early antigens synthesized to stimulate T-cell response and DNA is synthesized to be detected as DNA in the mononuclear cells. In early reactivation in measles subjects, sensitized T cells acting against immediate early antigens may suppress further replication cycles of HHV 6B and virion formation might be suppressed rendering any increase in the neutralizing antibody titer undetectable. In this regard, antibody determination to immediate early (IE) and early antigen (EA) was carried out additionally (data not shown) [Mori et al., 2000], showing that some patients responded to IE and EA and others did not, thus, the results could not explain this discrepancy. Differences of the major recognizing antigens between lymphocytes and antibodies against immediate early, early, and late glycoprotein antigens may present but not detected by this assay using sera. Furthermore, in our previous study, the CMI response was shown to be more sensitive than the humoral immune response in detecting host immune function in the subjects with varicella [Kumagai et al., 1999]. One possible interpretation is based upon the data composed of three consecutive specimens obtained at different stages of acute measles virus infection. Accordingly, the dynamic fluctuations in quantity of viral DNA in 17 out of 22 in subjects in a short period of time in combination with a statistically significant wax and wane pattern of elicitation of virus specific CMI response strongly support the view presented above. The mode of reactivation of various herpes viruses differ from each other. In HSV infections, even clinically apparent recurrence occurred without significant changes in the levels of virus-specific antibodies [Zweerink and Stanton, 1981; Zweerink and Corey, 1982; Kahlon et al., 1986]. In fact, measles impairs the immune response, but the duration of immunosuppression is shorter than that in organ transplant patients. Measles does not induce HSV or CMV diseases. Thus, this novel form of subclinical reactivation response might be unique to measles.

In addition, the influenza population data raises another interesting consideration. The cause of reactivation of HHV 6B in subjects with acute measles remains unknown. MV is known to suppress cell-mediated immunity, but it also causes marked imbalance of cytokine profiles during the course of the disease suggesting that cytokine excess might be involved in deranging the immune system. Of interest is whether the same level of HHV 6B reactivation in association with subsequent elevated activities of HHV 6B specific CMI occur in subjects with other viral infections that are not known to cause immunosuppression, but do invoke hypercytokinemia, influenza being an example. The present data suggest

that the level of hypercytokinemia induced by influenza virus infection was insufficient to provoke HHV 6B reactivation and did not affect mobilization of HHV 6B specific lymphocytes. However, such a conclusion is beyond the scope of this article because cytokine profiling was not part of the protocol mainly because of sample volume constraints.

The present study is limited by its small sample size. However, the observed temporal characteristics of HHV 6B specific lymphocyte proliferation responses as well as those to PHA were statistically robust, and this distinctive and unique approach to compare specific lymphoproliferative activities in three consecutive specimens minimized this limitation.

In conclusion, the dynamic fluctuations in specific lymphocyte proliferation activities observed in this study seem to reflect the process of internal re-exposure of the CMI system to HHV 6B antigen, involving both reactivation and suppression. The present data expands understanding of the HHV 6B-host interaction.

ACKNOWLEDGMENTS

We thank Toyo Okui, DVM, PhD, for her excellent research assistance; Nobuo Nagata, MD, PhD, for his sample collection; Yoshinobu Okuno, MD, PhD, for his HI antibody determination for influenza; Masao Yamada, MD, PhD, for their determination of neutralizing antibody for HHV 6B; Kiyoshi Ichihara, MD, PhD, for his statistical input; and Hiroyuki Tsutsumi, MD, for his contribution for the ethical issue of this study. We are particularly grateful to Prof. Yasuko Mori (Division of Clinical Virology, Center for Infectious Diseases, Kobe University Graduate School of Medicine) for thoughtful discussion regarding antibody responses of measles patients to IE and EA antigens of HHV 6B. We are indebted to Professor Peter M. Olley (Professor Emeritus of Pediatrics, University of Alberta at Edmonton) for his invaluable help in revising the manuscript. We also thank Professor Pearay L. Ogra (Professor Emeritus, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York: John Sealy Distinguished Chair, Professor and Chairman Emeritus of Pediatrics, University of Texas Medical Branch at Galveston) for his thoughtful review of the manuscript.

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VIRAL IMMUNOLOGY
Volume 27, Number 8, 2014

Pp. 368–374
DOI: 10.1089/vim.2014.0010

Original Research Articles

Humoral Immune Response to Influenza A(H1N1)pdm2009 in Patients with Natural Infection and in Vaccine Recipients in the 2009 Pandemic

Takuji Kumagai[1,*] Tetsuo Nakayama,[2,*] Yoshinobu Okuno,[3] Tetsuo Kase,[4] Naoko Nishimura,[5] Takao Ozaki,[5] Akiko Miyata,[6] Eitaro Suzuki,[6] Teruo Okafuji,[6] Takao Okafuji,[6] Hitoshi Ochiai,[6] Nobuo Nagata,[1] Hiroyuki Tsutsumi,[7] Masatoshi Okamatsu,[8] Yoshihiro Sakoda,[8] Hiroshi Kida,[8] and Toshiaki Ihara[9]

Abstract

The 2009 pandemic H1N1 mainly affected adolescents and children, and most of the elderly in Japan escaped clinical illness. To clarify the role of humoral immunity in the infection, the time kinetics of hemagglutination inhibition (HI), neutralization (NT), and IgG subclass antibody response directed against influenza A(H1N1)pdm2009 were analyzed in three consecutive specimens obtained from 51 young adults and children (group 1) who contracted pandemic influenza and from 74 pediatric clinic employees (group 2) inoculated with pandemic monovalent vaccine. In group 1 patients, 6 and 30 patients had lower HI and NT antibody in the acute phase respectively. Thereafter, HI and NT antibody titers increased fourfold or more in 50 patients with peak response in the third specimens obtained four weeks after the onset. IgG1 in 45 patients, IgG3 in 18 patients, and IgG4 in 29 patients showed elevated responses. Forty (54%) and 70 (95%) subjects in group 2 had positive HI and NT antibodies in the prevaccination samples, with increased antibody responses in the follow-up peaking in the second specimens. Forty of those vaccinated had increased IgG1 responses peaking in the third specimens, whereas elevated IgG3 was observed in 22 recipients with the highest level in the second samples. IgG4 did not show any increase in subjects in group 2. A few participants showed an IgG2 response in both groups. An immunologically naive population contracted influenza with apparent clinical symptoms. However, already primed subjects through subclinical infection elicited the unique pattern of IgG subclass responses by vaccination, which differed from those of naive populations.

Introduction

In 2009, a newly emerged influenza A(H1N1)pdm2009 virus caused a worldwide pandemic. In spite of pessimistic concerns that there was no herd immunity, H1N1 mainly affected adolescents and children, while the majority of the elderly escaped clinical illness (4,5,9,12,16,19,24). Hardelid *et al.* pointed out that the same age distribution was observed in other pandemics such as those in 1918, 1957, and 1968 (12). In addition, many middle-aged and elderly people acquired antibodies directed against 2009pdm influenza virus without any clinical symptoms (3,4). In Japan, we also observed a similar distribution of subjects naturally infected in 2009. However, the cause of this skewed age distribution of the new pandemic influenza remains obscure.

Although many authors describe an important role of specific cell-mediated immunity (CMI) in protection (10), actual studies of specific CMI in humans is quite difficult in

We dedicate this work to Dr. Hitoshi Kamiya, Emeritus President of the Japanese Society for Vaccinology, who passed away in 2011. He established this research team.

[1]Pediatric Allergy and Infectious Diseases Society of Sapporo, Kumagai Pediatric Clinic, Sapporo, Japan.
[2]Laboratory of Viral Infection I, Kitasato Institute for Life Sciences, Tokyo, Japan.
[3]Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan.
[4]The Osaka Institute of Public Health, Osaka, Japan.
[5]Department of Pediatrics, Konan Kosei Hospital, Konan, Aichi, Japan.
[6]The Society for Ambulatory and General Pediatrics of Japan, Tokyo, Japan.
[7]Department of Pediatrics, Sapporo Medical University, Sapporo, Japan.
[8]Department of Microbiology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan.
[9]Department of Pediatrics, National Hospital Organization Mie National Hospital, Tsu, Mie, Japan.
*These authors contributed equally to this work.

the clinical setting mainly because of sample volume constraints, especially in those with a primary immune response to influenza who are generally small children and infants. Also, it is virtually impossible to handle short-lived samples such as lymphocytes in the clinic during the heavy workload created by a pandemic. However, it is established that induced subclass IgG composition specific to antigen reflects a balance of Th1/Th2 activation in animal models (14), suggesting that it is worthwhile trying to trace the imprint of Th1/Th2 activities occurring in subjects with pandemic influenza or post-vaccination by studying IgG subclass responses to pandemic influenza.

In linking data obtained in humans with that from mice, we need to consider the confusing historical background regarding IgG subclass nomenclature. Human IgG subclasses have been identified according to the time of their discovery, but for the mouse, IgG subclasses reflect their electrophoretic mobility (22). Functional difference between mouse IgG subclasses and their similarly named human fractions are not yet fully documented. In mice, these responses correlate with IgG2a, IgG2b, and IgG3, regulated by the production of IFN-γ (21), while in humans such interrelationships are not yet well defined. IgG1 is the most abundant subtype, making up more than 50% of total IgG, and IgG4 is the smallest component. Human IgG1 reflects not only Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depend on IL-4 and IL-13, which are considered the Th2 response (2,18).

Primary antibody responses require T-cell help through functionally different Th1 and Th2 cytokines secreted by antigen-specific CD4+ T-lymphocytes. Therefore, the analysis for IgG subclass responses after infection or vaccination may provide additional information in terms of CD4+ T-cell effector functions, which might be supposed to reflect the balance of Th1 and Th2 activation provided by the antigenic stimulus of the novel influenza pandemic. Thus, our aim in the present study was twofold: first, to clarify the role of humoral immunity in the infection of 2009 pandemic; second, to evaluate the induced subclass IgG composition. To achieve these goals, we determined the time kinetics of hemagglutination inhibition (HI), neutralization (NT), and IgG subclass antibody response directed against influenza A/California/07/2009(H1N1) in patients with natural infection and subjects immunized with pandemic monovalent vaccine.

Materials and Methods

Study subjects

The study groups consisted of 51 healthy young adults and children (24 females) with a mean age of 7.6 years (range 0.5–25 years), who contracted pandemic influenza caused by influenza A(H1N1)pdm2009, and 74 pediatric clinic employees (63 females) with a mean age of 38.5 years (range 19–72 years) inoculated with one dose of pandemic vaccine. The vaccine formulation used in this study was monovalent HA split product of influenza A(H1N1)pdm09 virus made by Japanese vaccine manufacturers. Diagnosis of influenza was made by rapid test (ImmunoAceFlu®; Tauns, Izunokuni, Japan) followed by virus isolation, polymerase chain reaction (PCR), and seroconversion with HI and NT antibody determination. A detailed history was taken from pandemic vaccine recipients as to symptoms suggesting influenza such as fever, cough, rhinorrhea, headache, diarrhea, muscle pain, and arthralgia. None had had any of these symptoms during the past year. Three consecutive peripheral blood specimens (acute phases, 2 weeks and 4 weeks after the onset of illness in patients with influenza and both prior to and 2 and 4 weeks after vaccination in recipients) were obtained. Blood sampling in patients with natural infection was based on the first day of fever designated as day 0, and the first specimens were collected after 2 ± 2 days of the acute illness. Patients were recruited from September 16 through October 20 followed by an additional two samplings. Regarding pandemic vaccine recipients, the first specimens were obtained on the day of immunization, which extended from October 26 through November 21. Two late samplings were included. This study was approved by the ethical committee of Sapporo Medical University and by the separate institutions of the authors. Informed consent was obtained from the subjects after full explanation, and consent was also obtained from all children aged 6 years or more.

Influenza virus and antigen preparation used for antibody assay

A/California/07/2009 virus purified whole virion was prepared by means of sucrose density gradient centrifugation for HI test. HA split vaccine bulk material of influenza A(H1N1)pdm2009 was used to determine IgG subclass antibody titer, which was adjusted to 333 ng/mL of HA protein in phosphate-buffered saline (PBS) as antigen.

Determination of HI and NT antibody specific to influenza A/California/07/2009 virus

The serum samples were stored at $-35°C$ until testing. The HI test was performed by a standard microtiter assay with human type O erythrocytes after removal of nonspecific inhibitors with receptor-destroying enzyme and of cold agglutinins by hemadsorption at 4°C. All specimens were tested on the same day using identical reagents.

Determination of NT antibody titers was carried out by the microneutralization (MN) assay (17). In brief, serially diluted sera (1:10 to 1:1280) in Dulbecco's minimal essential medium (DMEM) were prepared in a 96-well microplate. An equal volume of virus fluid (50 FFU/25 μL) was added and incubated at 37°C for 60 min in a 5% CO_2 incubator. As a positive control, viruses were mixed with an equal volume of DMEM without serum. After incubation, 50 μL of the NT mixture was inoculated onto a monolayer of MDCK cells in a 96-well microplate. After incubation for 16–20 h, cells were fixed with absolute ethanol and reacted with mouse monoclonal antibody against influenza A virus nucleoprotein (NP; clone C43) (17), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (A2304; Sigma). Cells expressing viral antigens were visualized by 3,3′-diaminobenzidine tetrahydrochloride hydrate (D5637; Sigma). The MN titer was indicated by the reciprocal value of the maximum dilution at which the number of foci showed 50% reduction compared with the positive control.

In both the HI and NT tests, positive cutoff was set at 1:10. Thus, an antibody level $<1:10$ was designated as negative. The criterion for seroconversion was defined as elevation of antibody level by fourfold or more. To investigate antibody

responses in detail, we classified them into three categories: no elevation; antibody level unchanged or less than the first specimen; twofold increase and fourfold ore more elevation.

Determination of IgG subclass antibodies

We performed quantitative enzyme immunoassay (EIA) to detect IgG subclass antibodies against influenza A/California/07/2009 strain. Wells of a 96-well plate were coated with virus antigen, 33 ng/100 μL of split vaccine materials calibrated to the amount of HA protein for 16 h at room temperature. After four washes with PBS containing 0.05%Tween 20, wells were coated with Blocking One (Nacalai Tesque) for 45 min at room temperature. Serial twofold dilutions of serum samples were incubated in 100 μL/well for 60 min at room temperature, starting at 1:200 for IgG1, IgG2, and IgG4, and at 1:20 for IgG3. Secondary antibodies were added at 1:3,000 dilution for anti-human IgG1, at 1:1,000 dilution for IgG2, at 1:2,000 dilution for IgG3, and 1:2,000 dilution for IgG4 (Invitrogen). The plate was incubated for 60 min at room temperature. The wells were washed, and 100 μL of enzyme substrate was added, which was prepared with 40 mg of O-phenylenediamine (Wako Junyaku) in 100 mL of Na-citrate buffer, with 20 μL of 30% H_2O_2 added just before use. After 20 min incubation, 50 μL of 4N-H_2SO_4 was added, and optical density was measured at 450/630 nm. EIA antibody titers were expressed as the reciprocal dilution of 100×2^n that gave twofold value of optical density in the negative control well for each serum sample, derived from regression curve analysis with three dilution point plotting.

Statistical analysis

Consistency in HI, NT, and IgG subclass antibody directed against A/California/07/2009 virus at three points after onset of influenza and of pre- and post-vaccination was examined by using a nonparametric repeated-measures analysis of variance (Friedman's test) followed by Dunn's test as post-test employing StatFlex software v6. For calculation of geometric mean titer (GMT) estimates, a titer of < 10 was assigned a value of 5, and a titer of ≥ 8 in HI, NT test, and that of 9 in IgG subclass determination were assigned a value of 9 and 10 respectively.

Results

Time course characteristics of the antibody response specific to pandemic H1N1 2009 in naturally infected subjects was profiled (Fig. 1). GMTs determined in the first, second and third specimens with 95% confidence intervals (Cis) were 5.4 [5.1, 5.8], 12.4 [8.6, 17.8], 120.4 [94.1, 154.1] in HI, and 8.7 [7.4, 10.2], 44.7 [28.1, 71.1], 407.9 [312.6, 532.1] in NT, respectively. Statistically significant antibody elevations were observed (Friedman's test, $p < 0.0001$), and Dunn's test confirmed that peak antibody responses occurred in the third specimens ($p < 0.001$), implying that a primary immune response accompanied natural infection. More specifically, 6 of 51 patients had low-level HI, and 30 of the 51 low-level NT antibody in the first specimens, and 50 showed a fourfold increase or more in HI and NT antibody titers in the follow-up. The remaining patient had HI antibody of 1:10 in the first specimen followed by elevation to 1:20 in the second specimen. They also had NT antibody of 1:40 in the first specimen and did not show any increase. Virus isolation and PCR were positive in this patient.

IgG subclass antibody GMTs in three consecutive specimens obtained from patients were: for IgG1, 273.1 [198.0, 376.6], 737.3 [475.5, 1143.0], 1788.2 [1270.0, 2517.8]; for IgG3, 33.4 [22.4, 49.8], 39.8 [27.1, 58.3], 50.2 [33.7, 74.9]; and for IgG4, 476.6 [322.1, 705.2], 914.2 [548.8, 1522.9], 1780.1 [927.3, 3417.2]. IgG1, IgG3, and IgG4 antibody levels rose significantly ($p < 0.0001$ for IgG1; $p = 0.0045$ for IgG3; $p < 0.0001$ for IgG4) and peaked in the third specimen ($p < 0.05$ for IgG1; $p < 0.002$ for IgG3; $p < 0.001$ for IgG4). Only three cases developed IgG2 antibody at a very low level. Among 51 patients, initial specimens had some level of IgG1 (16 patients), IgG3 (15), and IgG4 (25) antibody activity, and 9, 2, and 21 of the above-mentioned cases exhibited a subsequent rise of fourfold or more. Twofold elevations were also observed in two cases for IgG1, three for IgG3, and four for IgG4. Five cases for IgG1 and 10 for IgG3 did not show any increase. IgG1 in 34 cases, IgG3 in 13 cases, and IgG4 in four cases were negative in the first samples but eventually manifested a fourfold or more rise in 32, 12, and 4 patients. The remaining two patients for IgG1 and one for IgG3 showed a twofold increase. No elevated response for IgG1, IgG2, IgG3, or IgG4 was observed in 1, 48, 23, and 22 subjects, respectively, throughout natural infection.

GMTs in HI and NT in pre- and post-vaccination serial specimens were 11.3 [8.9, 14.4], 70.1 [50.8, 96.9], 62.8 [45.4, 86.7], and 38.1 [28.2, 51.5], 329.0 [231.6, 467.4], 304.8 [215.6, 430.9], respectively (Fig. 2). Post-vaccination antibody titers were significantly higher than prevaccination ($p < 0.0001$) titers for both HI and NT. Antibody titers peaked in the second specimens, then declined in the third ($p < 0.001$). Of those vaccinated, 11 of 74 exhibited prevaccination HI antibody of ≥ 1:40, three of whom subsequently manifested fourfold or more increases. Four participants showed only a twofold rise thereafter, and the remaining four showed no elevation. Twenty-nine had prevaccination HI antibody of 1:10 to 1:20, and subsequent antibody increases were observed in all but two cases. Among these, 24 had fourfold or more increases, and three showed a twofold increase. Thirty-four had no HI antibody in prevaccination samples, and 23 of those showed fourfold elevation or more, 11 a twofold HI antibody rise, and two had no HI antibody response. Regarding NT antibody, 42 had a prevaccination titer of ≥ 1:40, and 29 of those showed fourfold or more increases thereafter. The remaining nine revealed a twofold rise, and four did not exhibit any increased antibody response. Twenty-eight had positive NT antibodies of 1:10 to 1:20 in prevaccination samples, and 22 of those developed antibody rises of fourfold or more, four showed twofold increases, and two revealed no elevation. The remaining four did not have NT antibody before vaccination but developed a fourfold or more antibody response.

Regarding IgG subclass antibody developments in vaccine recipients, GMTs of IgG1 in consecutive three specimens were 1085.4 [827.4, 1423.7], 1970.0 [1541.7, 2517.3], 2020.2 [1587.3, 2571.1]; those of IgG3 were 88.4 [56.5, 138.5], 134.2 [88.5, 203.5], 128.8 [81.2, 204.6]; and those of IgG4 were 172.3 [129.0, 230.1], 172.2 [128.9, 230.0], 171.5 [127.7, 230.4]. IgG2 was found in only six subjects. IgG1 and IgG3 antibodies levels were significantly elevated ($p < 0.0001$ for IgG1; $p = 0.0001$ for IgG3), and the peak IgG1 responses occurred in the third samples ($p < 0.05$), whereas the levels of IgG3 titers

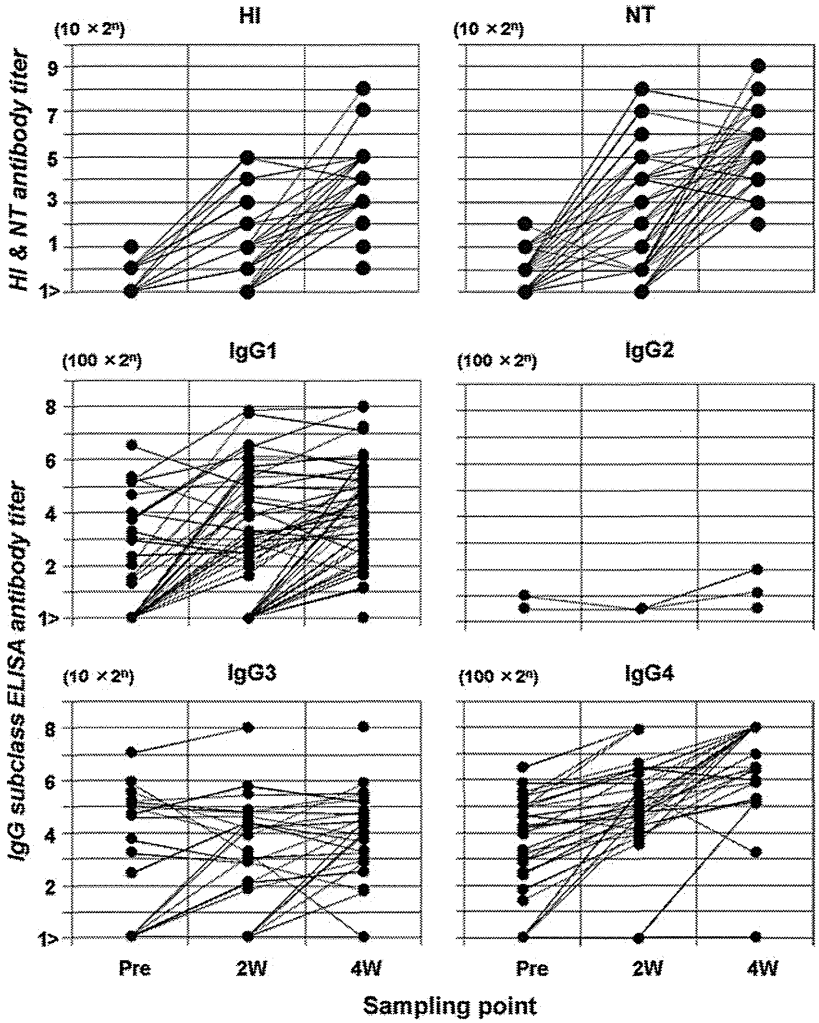

FIG. 1. Hemagglutination inhibition, neutralization, and IgG subclass antibody titers specific to A/California/07/2009(H1N1) in group 1 patients with natural infection. Black dot shows antibody titer. Abscissa in sampling point after onset of illness. Ordinate in serum dilution.

peaked in the second samples ($p < 0.001$). IgG1 was positive in prevaccination specimens in 70 subjects, and 19 and 17 of those showed fourfold or more and twofold increases thereafter, respectively. Four recipients had no IgG1 antibody in the first specimens and showed a fourfold or more rise in the follow-up. No IgG1 antibody rise was observed in 34 patients who had positive antibody prevaccination. In terms of IgG3 in vaccine recipients, 49 had positive prevaccination samples, and 5 and 10 of those showed fourfold or more and twofold rises thereafter. Seven recipients had no IgG3 in the first specimens and acquired IgG3 fourfold or more. Thirty-four who had positive IgG3 in the first samples did not show any elevation thereafter. Eighteen recipients did not have IgG3 in any of three serial specimens. Only 13 recipients had positive IgG4 in prevaccination samples and showed no elevation in the follow-up. Sixty-one subjects did not have any IgG4 antibody in serial three specimens.

Discussion

Our main purpose in the present study was to determine the time kinetics of the development of HI and NT antibody to influenza A(H1N1)pdm2009, in order to clarify the role of the humoral immune response to newly appearing influenza subtypes. We observed a fourfold increase in HI antibody titers with the peak titer appearing 4 weeks after infection in almost all patients with natural infection, suggesting that a primary immune response was elicited during the course of pandemic influenza. A fourfold rise in antibody response does not necessarily imply a primary immune response if it occurred after contracting seasonal influenza. However, influenza A(H1N1)pdm2009 was a newly emerged reassortant subtype influenza virus, which did not strongly cross-react to any other subtypes, including even A/USSR/90/77 (H1N1) (1). Thus, it is likely that a fourfold elevation in antibody response to this particular strain strongly suggests a primary infection. A distinctive and unique point of the present study was the detailed time kinetic observation of antibody development using three consecutive specimens obtained from subjects with natural infection and those who were vaccinated.

Regarding antibody development in pandemic vaccine recipients, 40 (54%) and 70 (95%) of 74 subjects had positive HI and NT antibodies, respectively, in prevaccination specimens. These antibodies were assumed to be obtained through asymptomatic infection, which is supported by the