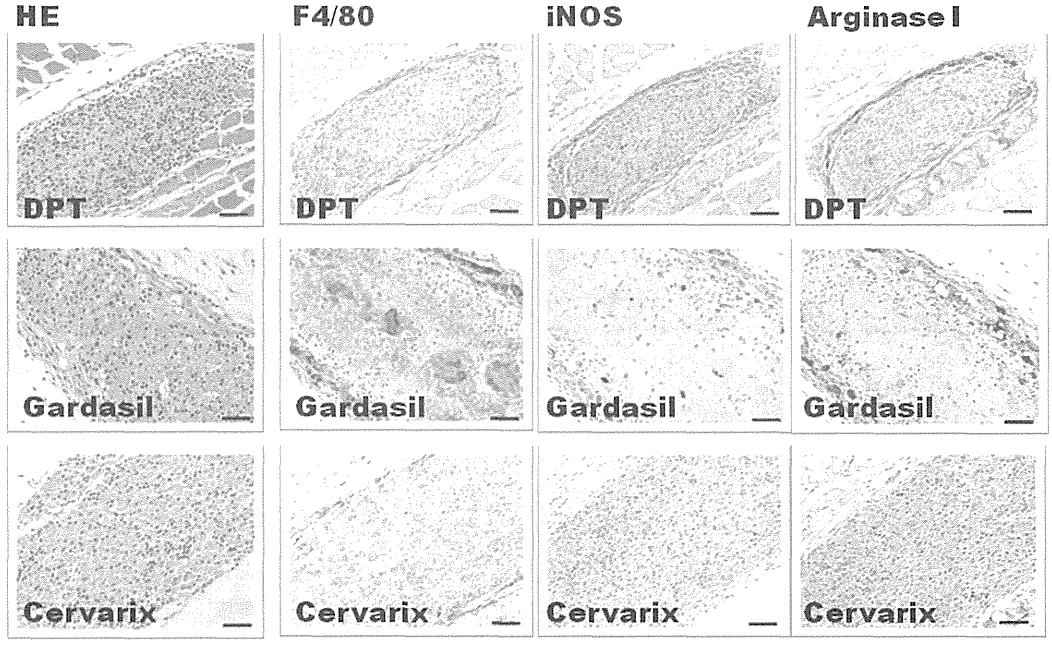

Fig. 3. Characteristics of inflammatory cells in the inflammatory nodules, inflammatory nodules were observed after the inoculation with aluminum-adjuvanted vaccines. The results of HE staining and immuno-staining with F4/80, iNOS, and arginase I are shown after the inoculation with DPT, HPV Gardasil, and HPV Cervarix.

Histological findings following IM of Cervarix, which contains MPL, appeared to be different from those in muscle tissues injected with DPT, PCV7, and Gardasil, in which aluminum is used as an adjuvant at different concentrations. Macrophages were stained with F4/80, iNOS, and arginase I, and the results are shown in Fig. 3. The inflammatory nodules were investigated 1 month after IM. Migrated cells located at the marginal lesions of inflammatory nodules in muscle tissue injected with DPT and Gardasil were stained with F4/80, iNOS, and arginase I. However, those infiltrated after IM of Cervarix were positive for F4/80 and weakly positive for arginase I, but not for iNOS.

3.2. Comparison of histological findings through SC and IM

No histopathological differences were observed following IM of non-adjuvanted vaccines. Aluminum-adjuvanted vaccines induced similar histological changes, except for Cervarix. The DPT vaccine is a basic vaccine for young infants and is administered through SC, which is recommended by the immunization law in Japan. Histological changes were compared 1, 3, 6, 9, and 12 months after IM or SC of three doses with a four-week interval in three mice for each group. Histological changes 1 month after the three injections were similar to those 1 month after the single dose, as shown in Fig. 1, and those observed 3, 6, and 9 months after immunization are shown in Fig. 4. Inflammatory nodules were relatively larger 3 months after IM than those after SC, but became smaller 6 months after IM. Histological findings of nodules after IM were similar to those after SC, and their sizes were the same until 9 months after the injection.

Histopathological changes were compared 1 year after SC and IM of three doses of DPT and the results are shown in Fig. 5. Three mice were examined: inflammatory nodules remained in two mice after SC but not in one mouse. Although IM initially induced large inflammatory nodules, this appeared to be absorbed earlier than SC, and inflammatory nodule remained in one among three mice through IM administration.

3.3. Production of cytokines at the injection site and serum cytokine profiles

All effective vaccines have stimulatory signals that activate the innate immune response to induce acquired immunity through the production of cytokines or chemokines. These vaccines are associated with the occurrence of the local reactions, indurations, swelling, redness, and erythema, and/or occasionally with the systemic adverse events such as fever. Cytokine production was investigated in the early phase following immunization with DPT, Hib, PCV7, JEV, and two HPV vaccines (Cerverix and Gardasil). The quadriceps muscles were dissected from both injected and non-injected sites, and were homogenized. IL-1β, IL-2, IL-4, IL-6, IL-10, Eotaxin, G-CSF, KC, MCP-1, and TNF-α were assayed. Cytokine production was expressed as the ratio of the cytokine concentration in the injected site to that in the non-injected site, and the results of local production are shown in Fig. 6. Changes in TNF-α levels are shown, and ranged between 0.5 and 1.5 fold of those in the injected sites. No significant difference was observed in the cytokine ratios of the injected and non-injected sites for IL-2, IL-4, IL-10, and Eotaxin, which was similar to that of TNF-α. IL-1β levels were 9.8 times higher (injected site 254.26: control 30.57 pg/ml) after 3 h and increased to 47.7 times (injected site1049.46: control 28.33 pg/ml) 48 h after the inoculation with Cervarix. IL-1β levels were from 2.33 to 2.80 times higher (41.45–84.78 pg/ml) following the inoculation of Gardasil, but were approximately 2-3 times or lower following inoculation with the other vaccines. G-CSF levels were 276 times higher (injected site 96.71: control 0.58 pg/ml) 48 h after the inoculation with Cervarix, and were 23 times higher (injected site 8.78: control 0.59 pg/ml) 24 h after the inoculation with Gardasil. These levels were 13.26 times higher 6 h after DPT and 5.73 times higher 3 h after Hib. IL-6 levels were 139 times higher (injected site 127.26: control 1.09 pg/ml) 48 h after the injection with Cervarix, while the other vaccines increased IL-6 levels by 2-3 times (1.55–4.14 pg/ml). Cervarix induced higher levels of IL-1β, IL-6, and G-CSF, but not significant. It also induced higher MIP-1 levels at 3 h, and these were decreased at 6 h. Higher

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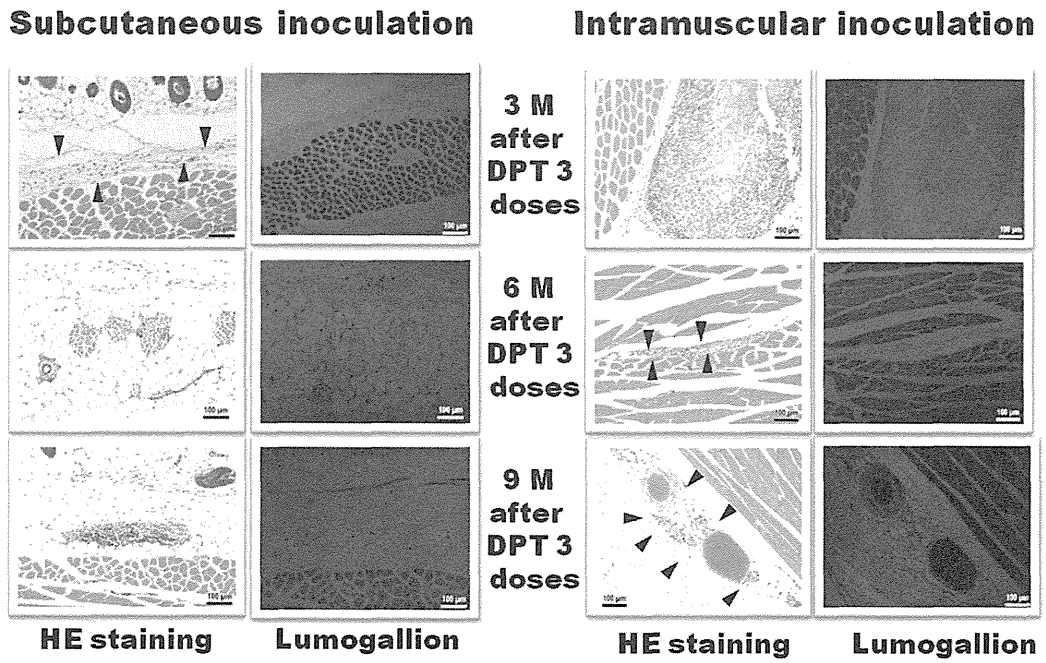

Fig. 4. Comparison of histological changes after the immunization with three doses of DPT. Injected tissues were obtained 3, 6, and 9 months after the immunization with three doses, and were examined with HE and Lumogallion staining.

levels of these cytokines were induced at 24 and 48 h, and Gardasil induced a similar MIP-1 production pattern (data not shown).

The results of serum cytokine kinetics are shown in Fig. 7. Five mice were assayed and the mean concentrations of five sera are shown. Peaks of IL-1β levels were detected 6 h after the injection of all vaccines, except Hib, which induced peak IL-1β levels 24 h after the injection. Higher IL-1β levels were detected after the injections with PCV7, JEV, and Cervarix. Hib induced a peak in serum G-CSF levels 3 h after the injection, DPT and Cervarix at 6 h, and PCV7 at 24 h. A significant higher level of IL-6 was observed 3 h after the injection with Cervarix, in comparison with those after the other vaccines, but decreased to the similar level 6 h after the injection with PCV7. All vaccines induced TNF-α and their peak levels were observed within 24 h of the injection with no significant difference.

Cervarix induced extremely high IL-1β, IL-6, and G-CSF levels at the injection site, but, however, systemic serum cytokine levels were similar to the others.

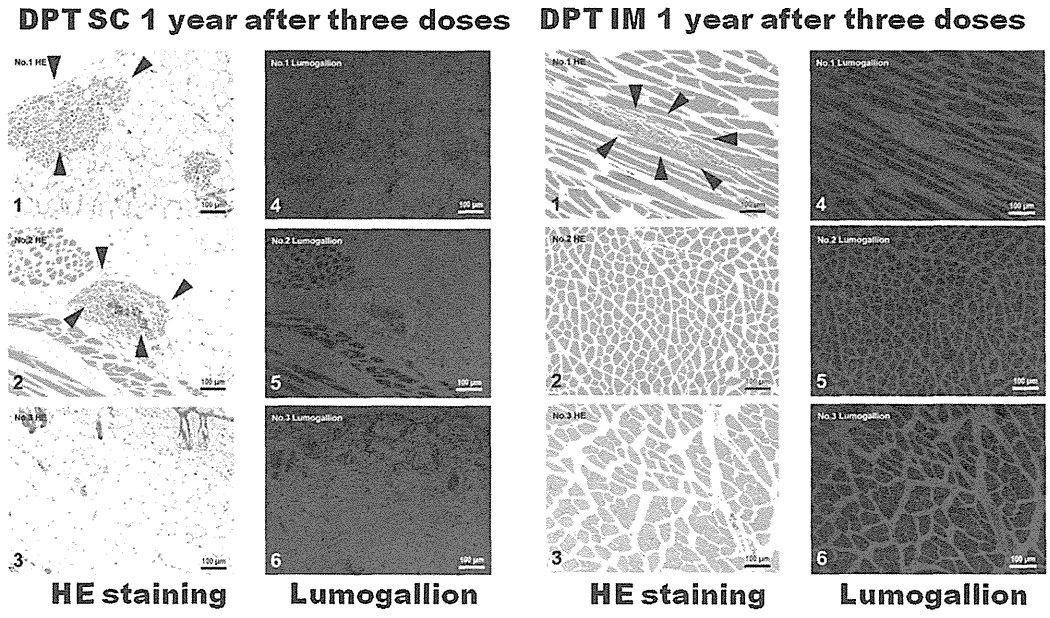

Fig. 5. Comparison of histological changes one year after the immunization with three doses of DPT. Injected tissues were examined with HE and Lumogallion staining.

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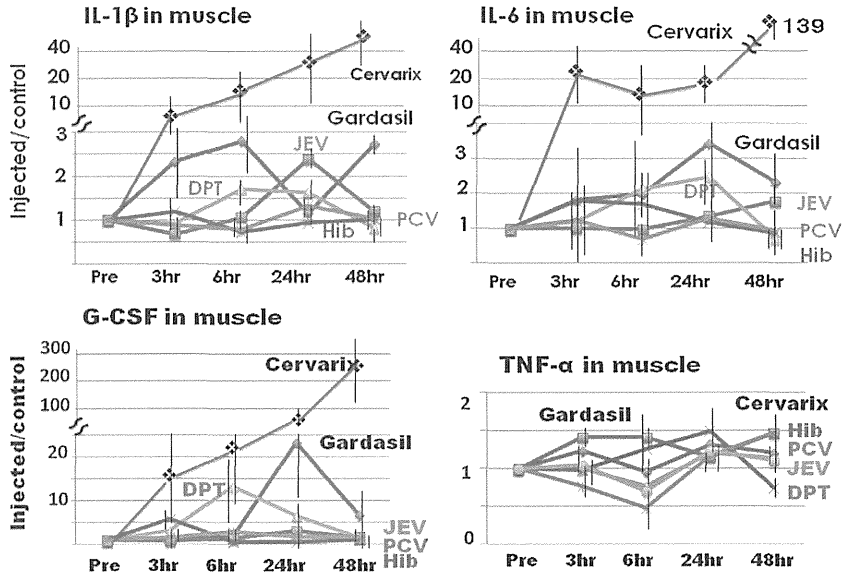

Fig. 6. Cytokine production in muscle tissues injected with DPT, Hib, PCV, JEV, Cervarix, and Gardacil. The quadriceps muscles were removed, homogenized, and subjected to BioPlex cytokine panels 3, 6, 24, and 48 h after the injection. Cytokine production was shown as the ratio of the cytokine concentration in the injected site to that in the opposite site. Each bar represents the mean ratio with ±1.0 SD of three mice.

3.4. Histopathological findings in the early phase following the injection

Cervarix induced higher levels of cytokines at the injection site than those by the other vaccines. Histopathological findings were examined after IM of Cervarix, Gardasil, and JEV. JEV induced no inflammatory responses 1 month after IM, as is shown in Fig. 1, and no pathological changes in the very early phase (data not shown). HE and Lumogallion staining of the series of IM of Cervarix are shown in Fig. 8. Inflammatory nodules were observed 3 h after IM and the predominant infiltrating cells were polymorph nuclear neutrophils (PMNCs) with some eosinophils. Migrating cells engulfing aluminum were observed in the draining inguinal lymph node 48 h after the injection. These results were similar to those observed after the injection with Gardacil.

4. Discussion

The number of reported cases of muscle contracture increased in the 1960s. The histopathological findings of muscle contracture revealed the infiltration of inflammatory cells, muscle cellular necrosis, fibrosis, and scar tissue formation, similar to that in an experimental animal model, and the Investigation Committee on Muscle Contracture suggested that the histopathological changes induced in the muscle tissues of experimental animals by all medicinal preparations through IM should be

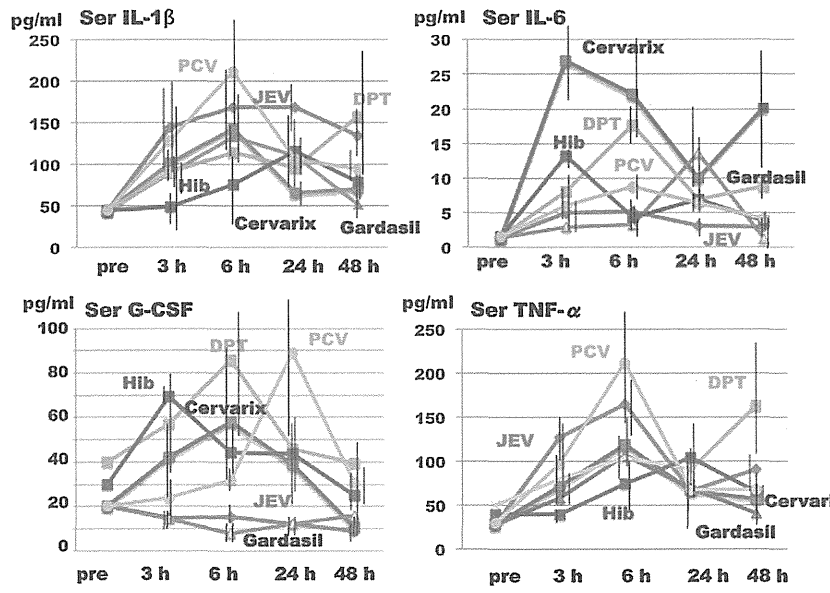

Fig. 7. Kinetics of serum cytokine production after the injection with DPT, Hib, PCV, JEV, Cervarix, and Gardacil. Serum samples were obtained 3, 6, 24, and 48 h after the injection. Each bar represents the mean ratio with ±1.0 SD of there mice.

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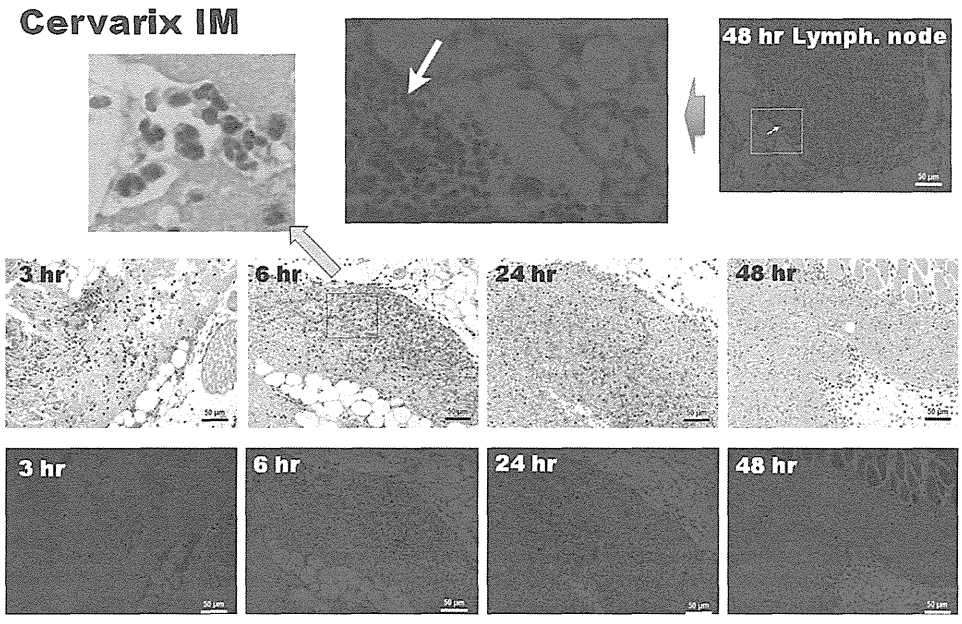

Fig. 8. Histopathological changes in muscular tissues 3, 6, 24 and 48 h after the injection with HPV Cervarix. The middle panels show the HE staining and lower panels show Lumogallion staining. Upper magnified HE staining shows migratory cells in muscle tissues 6 h after immunization. Aluminum positive cells were detected 48 h after the injection with Cervarix. Right upper panels show Lumogallion staining of left inguinal draining lymph node.

examined to assess muscle tissue damage [2]. The objective of the present study was to examine the safety of vaccines through IM. Petousis-Harris et al. [23] recommended vaccine administration techniques to reduce reactogenicity in a specified site of IM, the length of the needle, and injection angle. A significantly higher rate of local reactions was observed following the fifth DTaP vaccination in the arm than in the thigh [6–8]. Kataoka et al. [24] compared the local reactivities of DPTs produced by foreign and Japanese companies that had different local responses in mice. The Japanese DPT caused a milder inflammatory response than that of the DPT produced by foreign vaccine companies. They suggested the possibility of the residual toxicity of DPT components rather than the different concentrations of the aluminum adjuvant. The incidence of local reactogenicity was shown to depend on the vaccine preparation with or without aluminum adjuvant: however, no consistent relationship was observed between the quantity of aluminum and extensive local reactions [25]. Although aluminum has been used as an adjuvant in many kinds of vaccines, no comparative study has examined local reactions after immunizations with different vaccine preparations.

All recommended inactivated vaccines in Japan were tested in the present study. Non-adjuvanted vaccines (influenza, JEV, and Hib vaccines) induced no pathological local reaction, except for small local inflammatory lesions with the infiltration of inflammatory cells without muscle degeneration. Aluminum-adjuvanted vaccines induced inflammatory nodules. DPT, PCV, and Gardasil induced similar changes with central necrosis and the peripheral infiltration of inflammatory cells. Infiltrating cells were similar 1 month after IM with DPT, PCV, and Gardasil, and were positive for F4/80, iNOS, and Arginase I, however, Cervarix induced a different type of macrophage that was positive for F4/80, weakly positive for Arginase I, but negative for iNOS. This suggests a lack of M1 macrophages at the injection site following injection of Cervarix. Diversity and differentiation plasticity are characteristics of macrophages, which undergo their dynamic changes during different stages of inflammation. In response to the IFNs, chemokines and cytokines signaling through MPL-TLR 4 engagement, macrophages are polarized to M1 phenotype and toward the M2 phenotype by IL-4 and IL-13 [26]. Functional skewing of macrophages occurs under physiological and pathological conditions and the complex mechanisms have been under investigation through the signaling pathways, transcriptional networks, and microenvironments [27].

Inflammatory nodules observed 1 month after IM of Cervarix were identified as macrophagic nodules. The different characteristics of inflammatory nodules may be caused by a combined adjuvant, and may be related to the strong antibody response following the immunization with Cervarix [28]. Aluminum has been used as an adjuvant for a long time because it prolongs the retention of adsorbed antigens at the injection site (depot effect) [29]. Long-term observations following IM or SC of DPT revealed that aluminum was retained until 9-12 months at the injected sites. It is well known that DPT induced inflammatory nodules, which persisted for several months without any pathological signs at the opposite site in Cynomolgus monkey [30]. Although a higher volume was used in this study, antigen retention was not required for the potentiation of immunity by aluminum adjuvant, and recent findings on innate immunity have revealed that aluminum adjuvant initiate primary immune-stimulation of the antibody response [31]. Innate immunity consists of two different patterns, PAMPs and DAMPs. PAMPs recognize microbial components or the products of bacteria and viruses, and other endogenous products released from damaged cells (damage or danger signals) stimulate DAMPs, which activate inflammasomes. NALP3 inflammasomes recruit caspase, which converts IL-1β and IL-18 from pro-inflammatory cytokines [32]. The stimulation of inflammasomes is essential for the key mechanism but it is controversial whether NALP3 has an essential role or not for aluminum adjuvant [10,32–34]. These inflammatory cytokines enhanced the expression of the co-stimulatory molecule, which was recognized by CD4 helper cells together

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with MHC II [12–14]. Recently, the initiating event is considered to be cellular damage, releasing cellular components, host DNA, and intra-nuclear contents, also known as neutrophil extracellular traps (NETs) [35,36]. They are recognized as DAMPs and stimulate inflammasomes to produce the inflammatory cytokines, IL-1β, IL-18 and TNF-α.

In the present study, histopathological changes and cytokine production were investigated in the very early phase following IM of aluminum-adjuvanted and non-adjuvanted vaccines. PMNCs and eosinophils migrated to the injected site within 3 or 6 h, and macrophages were dominant 1 month after IM. These results were similar to those reported by Lu and HogenEsch [36]. M2 macrophages were dominant 1 month after IM of Cervarix. Cervarix contained a combination of 100 μg/ml MPL and 1 g/ml aluminum. MPL induced the migration of neutrophils, monocytes/macrophages, and activated natural killer cells, whereas, aluminum induced the migration of neutrophils and eosinophils, depending on the Th1 and Th2 adjuvants [37]. MF59 was used as an oil emulsion adjuvant for the influenza vaccine, and the immune-stimulating mechanism underlying the new adjuvants MF59 and MPL was extensively investigated in comparison with aluminum [38,39]. However, inflammatory responses stimulated with single component of MPL or aluminum were not evaluated in the present study, and the results represented total effects of AS04. Neutrophils were recruited following IM of Cervarix, which contains a combination of MPL and aluminum, and induced markedly high levels of the inflammatory cytokines, G-CSF, IL-6, KC, and MCP-1, but not TNF-α. G-CSF levels were 276 times higher at 48 h after the injection with Cervarix, and 23 times higher 24 h after the injection of Gardasil. G-CSF levels were 13.26 times higher 6 h after DPT, and 5.73 times higher 3 h after Hib. All tissues, macrophages, endothelial cells, fibroblasts, and mesenchymal cells, are potent producers of G-CSF during infection, which modulates inflammatory responses [40]. G-CSF stimulates myeloid progenitor cells to generate neutrophils and promotes their differentiation into mature neutrophils, and enhances the production of IL-1β, IL-6 and TNF-α from M1 macrophages [26,27,40]. The key functions of neutrophils are bactericidal killing, phagocytosis, and superoxide production, and they also play crucial roles in the initial stage of inflammation [40]. G-CSF was induced at the injected sites, which resulted in the recruitment of neutrophils, followed by NETs. This danger signal stimulates DAMPs to modify acquired immunity, and damage caused by NETs is followed by an inflammatory response by M1 macrophages, which is necessary for limiting the inflammatory areas and cleaning cellular debris. This acute phase inflammation is followed by appearance of M2 macrophages for resolution phase [27,28].

In 2013, the patients with chronic pain were reported following immunization with HPV, and some had unexplained neurological illness. HPV vaccination is temporarily suspended until the causal relationship would be clarified. The present study provides the experimental evidence that inflammatory response was limited at the injected site, not in systemic. Although the nodules persisted for several months, inflammatory responses did not persist over 6-12 months.

In conclusion, all vaccine preparations did not induce the muscle contracture observed through multiple IMs of antibiotics with or without anti-pyretics in an experimental mouse model. Non-adjuvanted vaccines did not induce inflammatory reactions, however, aluminum-adjuvanted vaccines induced inflammatory nodules.

Conflict of interest

All authors have no conflict of interest regarding this study.

Acknowledgments

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

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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

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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).

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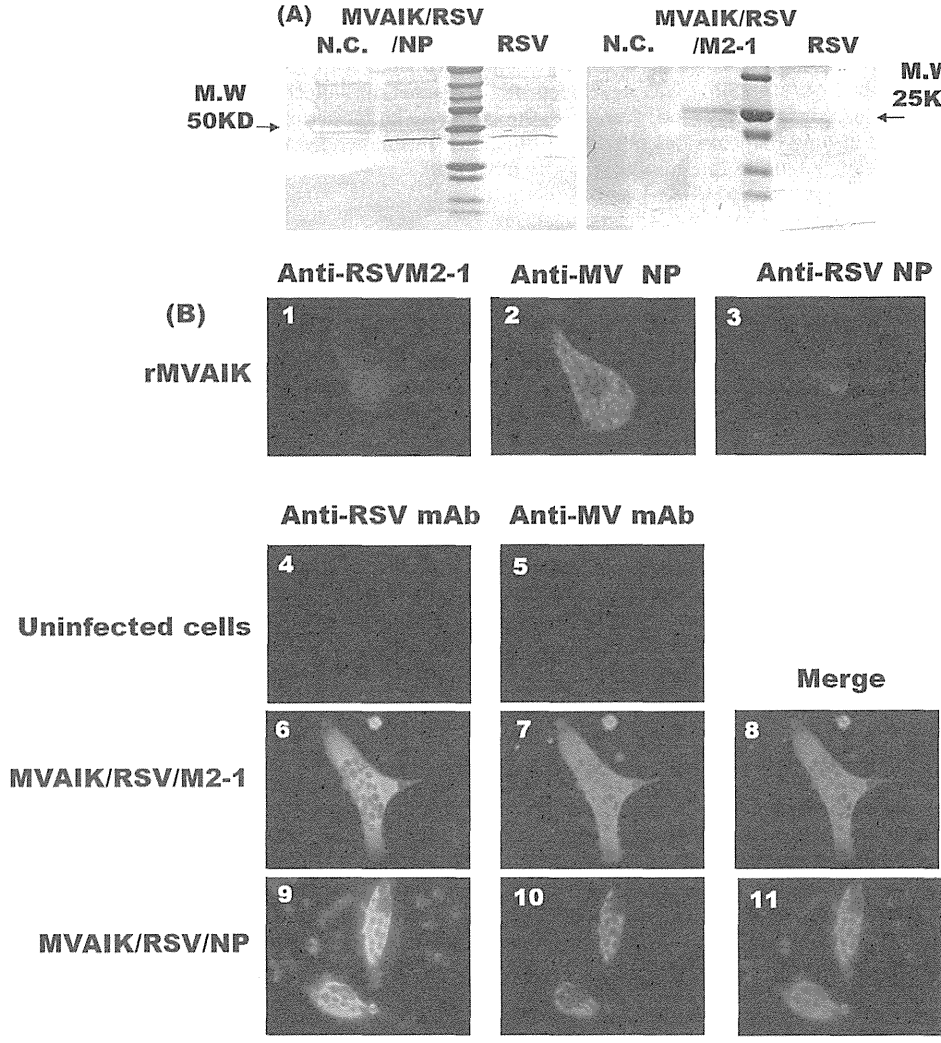

Fig. 1. *Expression of MV-N, RSV-M2-1, and N proteins.* (A): B95a cells were infected with MVAIK/RSV/M2-1 or MVAIK/RSV/NP, and were also mock infected (N.C.). The positive control was prepared in HEp-2 cells infected with the RSV Long strain. The results of western blotting were obtained using a monoclonal 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

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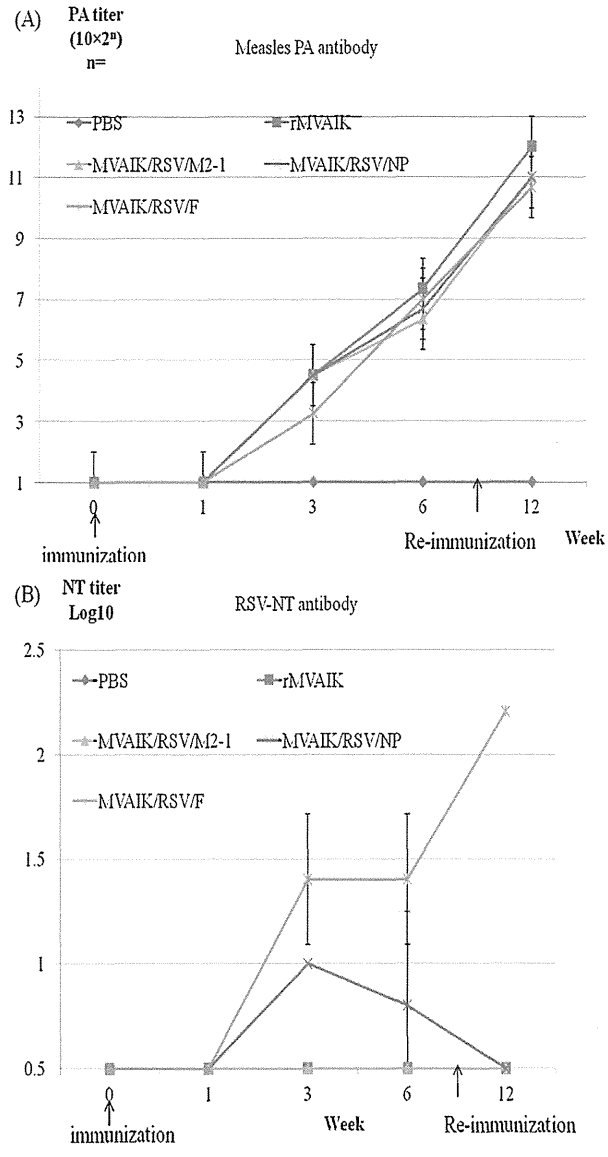

Fig. 2. *Serological responses of PA antibodies against MV and neutralizing antibody responses against RSV.* Serum samples were obtained before immunization and 1, 3, 6, and 12 weeks after immunization with mock (PBS), rMVAIK, MVAIK/RSV/M2-1, MVAIK/RSV/NP, and MVAIK/RSV/F. PA antibodies were expressed 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.

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.

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

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

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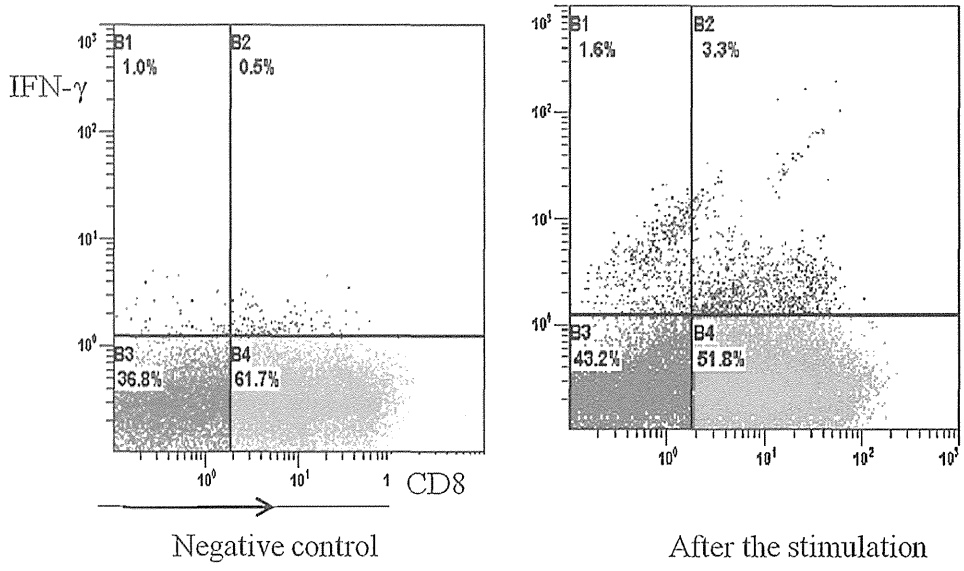

Fig. 3. *Flow cytometer analysis of CD8⁺ IFN-γ⁺ cells in cotton rats.* Splenocytes were obtained four days after the RSV challenge in cotton rats immunized with MVAIK/RSV/F. Splenocytes were stimulated with medium (A) and the RSV-fusion protein peptide (B).

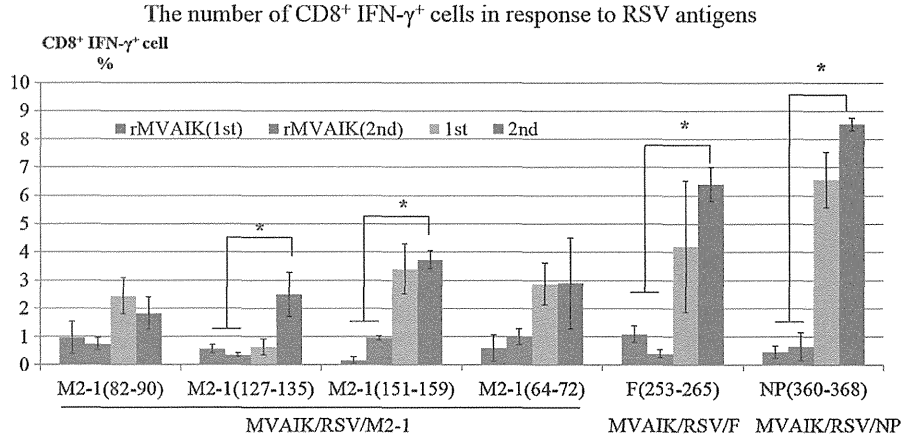

Fig. 4. *The number of CD8⁺ INF-γ⁺ cells stimulated with M2-1, F, and NP peptides.* Splenocytes were obtained after the RSV challenge in the first immunization and reimmunization groups immunized with recombinant viruses. The splenocytes of MVAIK/RSV/M2-1 group were stimulated with M2-1 peptides (82–90, 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.

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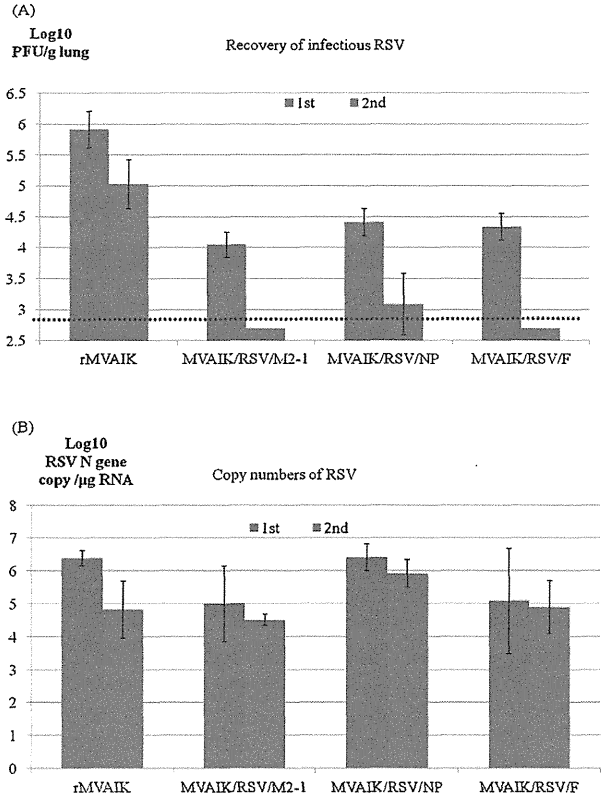

Fig. 5. *Recovery of infectious RSV and genome copy numbers after the RSV challenge.* Three cotton rats were challenged with 1.0×10^6 PFU of RSV Long strain 8 weeks after the first immunization (1st) and 6 weeks after re-immunization (2nd). (A) Virus infectivity was monitored in lung homogenates, and 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.

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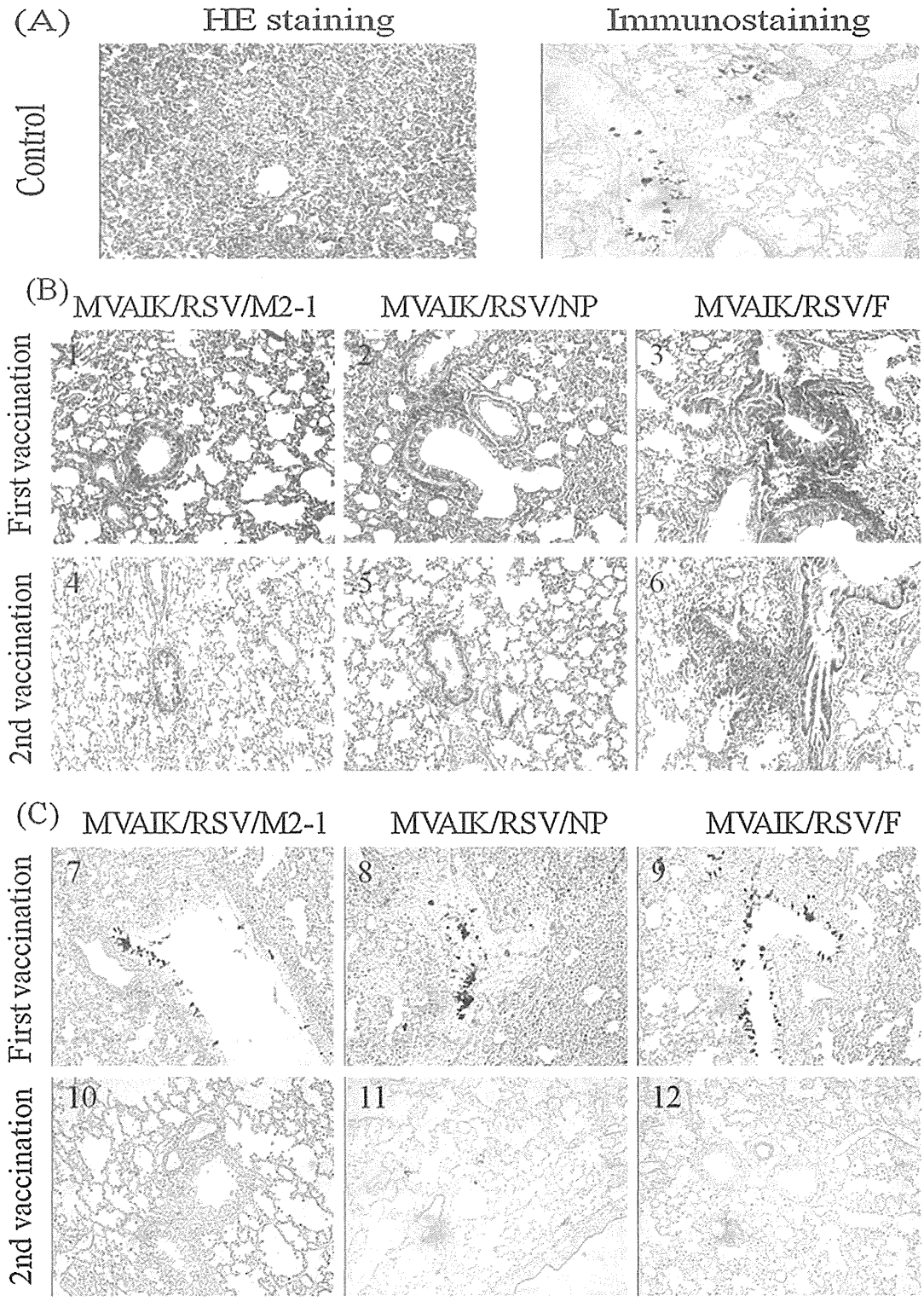

Fig. 6. *Pulmonary histopathology in cotton rats challenged with RSV subgroup A.* Animals were immunized with MVAIK/RSV/M2-1 (panels (B-1), (B-4), (C-7), and (C-10)), MVAIK/RSV/NP (panels (B-2), (B-5), (C-8), and (C-11)), and MVAIK/RSV/F (panels (B-3), (B-6), (C-9), and (C-12)). The results of hematoxylin-eosin 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.

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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.

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

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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.

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(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 ≥ 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).

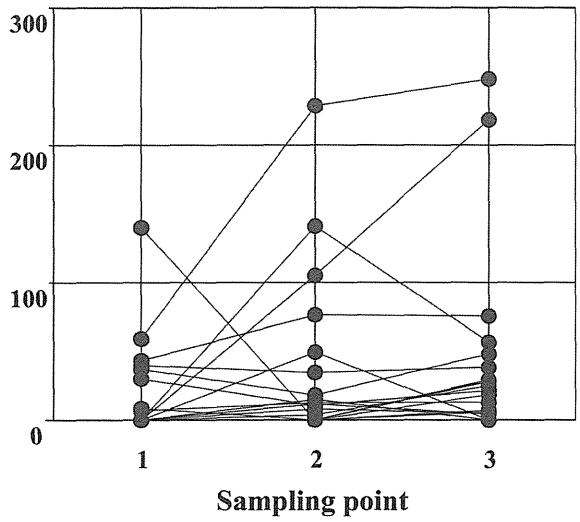

Fig. 1. Abscissa represents the time point of collecting blood samples indicated in the text and the ordinate the HHV 6B DNA copy number/μg DNA detected in PBMCs of measles patients. Closed circles represent the HHV 6B DNA copy numbers in each sampling point.

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.

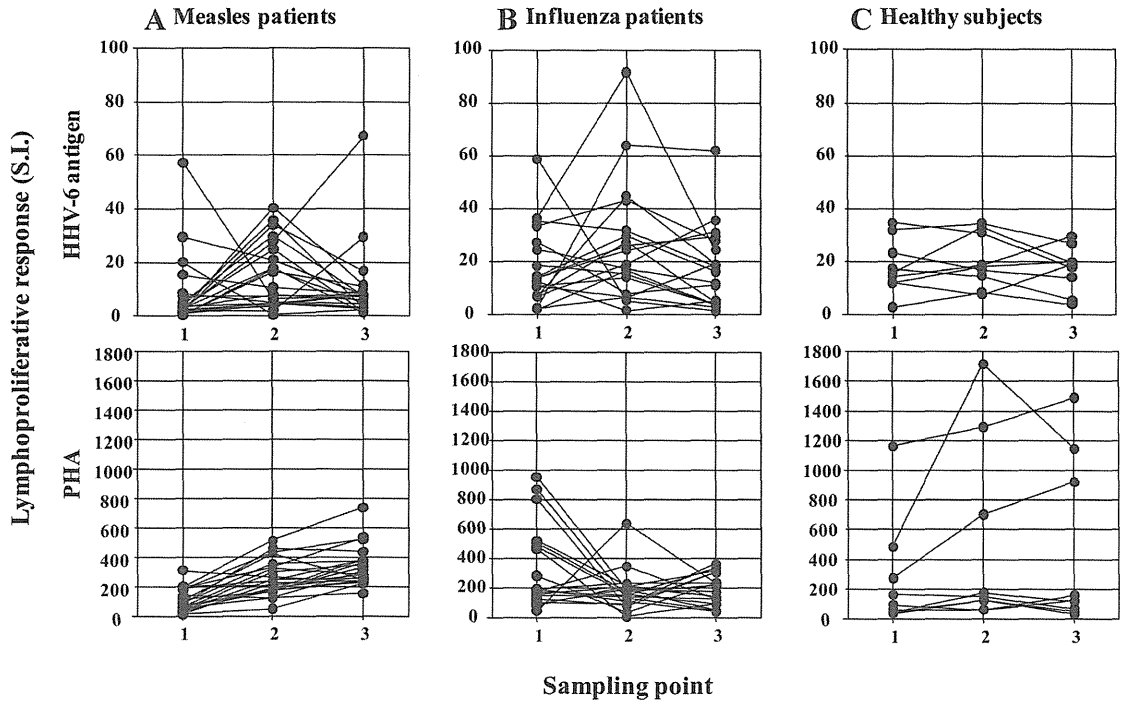

Fig. 2. The upper row shows the development of lymphoproliferative responses specific to HHV 6B antigen in subjects with acute measles (**A**), influenza (**B**), and in the healthy population (**C**). The lower row shows the kinetics of the lymphoproliferative responses to PHA in the same groups. Abscissa 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.