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AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats

Akihito Sawada[a], Katsuhiro Komase[b], Tetsuo Nakayama[a,*]

[a] Laboratory of Viral Infection I, Kitasato Institute for Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan
[b] Department of Virology III, National Institute of Infectious Diseases, Musashimurayama, Tokyo 208-0011, Japan

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ABSTRACT

Respiratory syncytial virus (RSV) is the most common cause of respiratory infection in infants, and no vaccine is available. In this report, recombinant AIK-C measles vaccines, expressing the RSV G or F protein of subgroup A (MVAIK/RSV/G or F), were investigated as a RSV vaccine candidate. MVAIK/RSV/G or F had the original *ts* phenotype and expressed RSV/G or F protein. Cross-reactive neutralizing antibodies against RSV subgroups A and B were detected in cotton rats immunized intramuscularly with MVAIK/RSV/F but not MVAIK/RSV/G. In cotton rats infected with RSV, RSV was recovered and lung histopathological finding was compatible with interstitial pneumonia, demonstrating thickening of alveolar walls and infiltration of mononuclear cells. When cotton rats immunized with MVAIK/RSV/F were challenged with homologous RSV subgroup A, no infectious RSV was recovered and very mild inflammation was noted without RSV antigen expression. When they were challenged with subgroup B, protective efficacy decreased. When cotton rats immunized with MVAIK/RSV/G were challenged with RSV subgroup A, low levels of infectious virus were recovered from lung. When challenged with subgroup B, no protective effects was demonstrated, demonstrating large amounts of RSV antigen in bronchial-epithelial cells. MVAIK/RSV/F is promising candidate and protective effects should be confirmed in monkey model.

1. Introduction

Human respiratory syncytial virus (RSV) is a member of the family *Paramyxoviridae* in the order *Mononegavirales*. The *Paramyxoviridae* consist of two subfamilies, *Paramyxovirinae* and *Pneumovirinae* [1]. Classified into the genus *Pneumovirus*, RSV is characterized by a non-segmented, negative sense, single-stranded RNA genome, and has approximately 15,200 nucleotides. All members of the paramyxovirus family are similar in structure and characteristics [2]. Viral particles of RSV are surrounded by a lipid bilayer with two viral glycoproteins, G and F [1], involved in the attachment to, fusion with, and entry into cells during infection. G protein is not always required for infection and cell fusion and the expression of F protein alone leads to cell fusion [3]. RSV was first isolated in 1956 and two antigenically different subgroups, A and B, co-circulate [4]. RSV is the most common cause of lower respiratory infections in infants and young children worldwide, and is responsible for a variety of illnesses, including 20–25% of pneumonia cases and 45–50% of bronchiolitis cases among hospitalized children [5]. The peak of serious RSV infections is at 2–6 months of age and most children experience an RSV infection by two years of age [6]. The infection causes serious illnesses especially in babies born prematurely and having chronic lung diseases, or congenital heart diseases. RSV also causes lower respiratory tract infections in the elderly, and in immunocompromised hosts [7]. The global annual morbidity and mortality for RSV are estimated to be 64 million and 160,000 deaths, respectively [8].

A recent study of the immune response to RSV showed the importance of innate immunity in regulating adaptive immune responses [9]. Adaptive immunity is generally considered effective due to neutralizing antibodies (NT) and cellular immune responses for the clearance of viruses are influenced by innate inflammatory responses. Secretory and NT antibodies were generated after repeated infections with RSV, although the responses were weak in young infants [10]. The presence of IgG antibodies in the lung has been shown to reduce viral load [11]. Even a natural infection did not provide long-term protective immunity against reinfection in young infants, and a humanized monoclonal antibody against the F protein is available as a prophylaxis against RSV, or for reducing serious diseases in high-risk infants during epidemics [12]. However, the high medical costs for monthly administration mean that there is a great need to develop an RSV vaccine [13]. There are several obstacles to developing a RSV vaccine. An aluminium-precipitated formalin-inactivated RSV vaccine (FI-RSV) was developed in the 1960's, but did not prevent infections [14]. In fact, symptoms were exacerbated among recipients subsequently

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

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infected with RSV. FI-RSV generated only binding antibodies without neutralizing activity because of the denatured F protein, and did not induce cytotoxic T cell lymphocytes (CTL) activity [15]. Several strategies have been adopted to develop subunit vaccines, live attenuated vaccines through conventional methods of cloning or selecting *ts* mutants, genetically modified-strain by reverse genetics, and vaccinia virus vector-based recombinant vaccines [16–18].

Recently, a method for direct manipulation of the genomic RNA of *Mononegavirales* has been established, known as the infectious cDNA clone system [19]. The transcription and replication of minigenome RNA are driven by viral proteins, which are co-expressed by plasmids or helper viruses. Using this system, the infectious recombinant viruses can be retrieved from the authentic full-size genome cDNA [20,21]. These "reverse genetics" techniques are powerful tools not only for basic research into viral properties, such as the characteristics of viral proteins, and mechanisms of replication, transcription and pathogenesis, but also for practical purposes, such as the development of new vaccines and viral vectors. As vector-based recombinant vaccines, human parainfluenza virus type III (HPIV III) vector-based, or Sendai virus vector-based vaccines have been evaluated [22,23].

Current measles vaccines used throughout the world were attenuated from the Edmonston strain, classified as genotype A [24]. The AIK-C strain of the measles vaccine was developed in 1976 in Japan from the Edmonston strain, by plaque cloning through passages in sheep kidney cells and chicken embryonic cells at 33 °C [25]. It shows optimal growth at 33 °C and little or no growth at 39 °C [21]. The safety and immunogenicity of the AIK-C measles vaccine were established through clinical trials [26–29]. Reverse genetics of the AIK-C live attenuated vaccine was performed and in this study, recombinant AIK-C MV vaccine strains encoding the RSV G or F protein were constructed, and immunogenicity and protective effects against RSV were investigated in cotton rats immunized with recombinant measles vaccines, expressing RSV G or F protein.

2. Materials and methods

2.1. Viral strains and cell cultures

The AIK-C seed strain for vaccine production was used. Wild-type strains of RSV subgroups A and B were isolated in HEp-2 cells from patients. Long and wild-type strains were used for the neutralization test (NT) against RSV subgroups A and B. 293T and HEp-2 cells were maintained in Eagle's MEM (Sigma–Aldrich, Dorset, UK) supplemented with 10% fetal bovine serum (FBS). Vero cells were maintained in Eagle's MEM supplemented with 5% FBS. B95a cells are marmoset B cell line, and maintained in RPMI-1640 medium (Sigma–Aldrich, Dorset, UK) supplemented with 10% FBS [30]. These media were supplemented with 4 mM L-glutamine, 10,000 IU/ml penicillin, and 10,000 μg/ml streptomycin.

2.2. Cloning of the RSV G and F genes

Genomic RNA was extracted from a clinical isolate of subgroup A and B, and the RSV genome was amplified by RT-PCR. The viral RNA was first converted to cDNA using a cDNA primer: 5′-ACACGATTTGCAATCAAACC-3′. The RSV G gene was amplified with 5′-GTTTCCATGGCCAAAACCAAGGACCAA-3′ and 5′-CCAAGCGGCCGCTAGTTTGTTGTGTTGGATGGAGA-3′, which amplified 894 bp. The RSV F gene was amplified with 5′-GTTGCCATGGAGTTGCCAATCCTCAA-3′ and 5′-TGTGGCGGCCGCTAACTAAATGCAATATTATTT-3′, which amplified 1722 bp. The F and G genes were cloned into pMV/20-77 using two restriction enzymes, Nco I and Not I (underlined sequences).

2.3. Construction of recombinant AIK-C

A schematic diagram of the strategy used for the construction of the recombinant cDNA plasmid is shown in Fig. 1. The full length plasmid was divided from two parts as previously reported. The first half contained the N, P, M and F genes from the leader sequence to the Pac I site at nucleotide position 7238 of the AIK-C genome. The second half contained the H and L regions from the Pac I site from position 7238 of the AIK-C genome to the trailer sequence. The full-length cDNA, pMVAIK, was constructed using these two plasmids [31].

The cloning vector for the RSV genome, pMVAIK/20-77, was constructed from positions 2040 (Sac II) to 7761 (EcoT22 I). The RSV G or F PCR product was digested with Nco I and Not I and ligated into pMVAIK/20-77, resulting in pMVAIK/20-77/RSV/G and pMVAIK/20-77/RSV/F, respectively. The pMVAIK/20-77/RSV/G or pMVAIK/20-77/RSV/F was digested with Sac II and Pac I and ligated into pMVAIK. Then, full-length infectious cDNA clones, pMVAIK/RSV/G and pMVAIK/RSV/F, were constructed.

2.4. Rescue of the infectious recombinant virus from cloned cDNA

Monolayers of 293T cells in 6-well plates were infected with the vaccinia virus MVAT7 pol, expressing T7 RNA polymerase. MVAT7 pol was derived from a highly attenuated and host range-restricted vaccinia virus, the Ankara strain [32]. Open reading frames of the N, P, and L genes were cloned downstream of the T7 promoter of pBluescript SK, and the expression plasmids pCIAN01, pCIAP01, and pCIAL01 were constructed [19,21]. After 1 h of adsorption, the cells were washed with Opti-MEM (GIBCO, Grand Island, NY, US) and transfected with 0.5 μg of pCIAN01, 0.25 μg of pCIAP01, 0.1 μg of pCIAL01, and 1.5 μg of pMVAIK/RSV with TransIT-LT1 Reagent (Mirus Bio Corporation, US). After incubation at 33 °C for 3 h, the medium containing the transfection reagent/plasmid complex was replaced with fresh MEM supplied with 5% FBS. The transfected cells were incubated at 33 °C in 5% CO_2 for 3 days. After 3 days, 293T cells were detached and co-cultured with B95a cells. When a demonstrable cytopathic effect (CPE) was observed, the supernatant and cell lysate were harvested and stocked.

2.5. Virus growth

To examine viral growth, B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F (m.o.i. = 0.02) and the plates were placed at temperatures of 33, 35, 37 and 39 °C. The culture fluids were obtained on days 1, 3, 5, and 7 of culture and infective titers were examined and expressed as $TCID_{50}$/ml in B95a cells.

2.6. Indirect immuno-staining and Western blotting

B95a cells were infected with MVAIK, MVAIK/RSV/G or MVAIK/RSV/F at m.o.i. of 0.01 in 24-well plates and cultured for two days at 33 °C. B95a cells were collected and subjected to indirect immuno-staining without fixation to detect the surface expression. Polyclonal antibodies against RSV raised in goat (Abcam, Cambridge, UK) were used and the cells incubated for 1 h at 37 °C. The cells were washed extensively with phosphate-buffered saline with 0.05% Tween 20 (PBST), and stained with second antibodies against goat IgG conjugated with FITC, raised in rabbit (Vector Laboratories, Burlingame, CA, US), and thereafter, mouse monoclonal antibody against MV HA protein (kindly supplied by Dr. Sato, National Institute of Infectious Diseases, Japan) was used and followed by second antibodies against mouse IgG conjugated with rhodamine raised in goat (Rockland Immunochemicals, Gilbertsville, PA, US).

Vero cells were infected with MVAIK, MVAIK/RSVF, and MVAIK/RSV/G and HEp-2 were infected with RSV subgroup A, Long

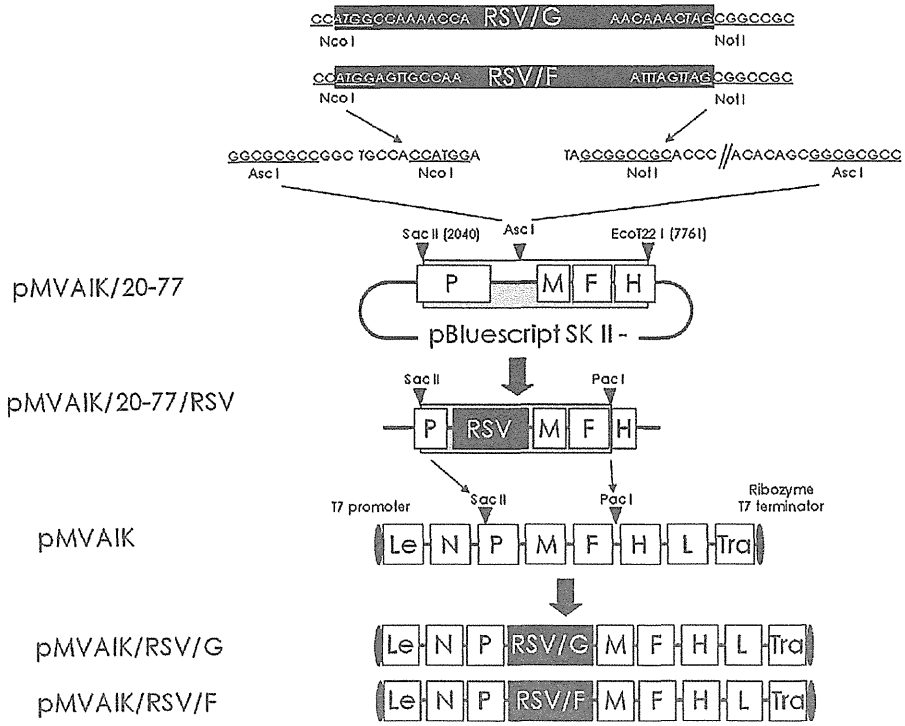

Fig. 1. Strategy for the construction of the recombinant AIK-C genome cDNAs having RSV protein genes. The recombinant AIK-C viral cDNAs expressing RSV G or F protein were constructed based on AIK-C cDNA (pMVAIK). pMVAIK/20-77 was constructed for the cloning of foreign genes. The Asc I site was introduced by adding GGCGCG after position 3432 of AIK-C and R1 and R2 sequences were added. The Nco I–Not I fragment of RSV G or F was cloned into pMVAIK/20-77, designed as pMVAIK/20-77/RSV. pMVAIK/20-77/RSV had unique restriction enzyme sites, Sac II and Pac I sites, located in the P gene and between the F and H gene. The DNA fragments between the Sac II and Pac I sites of pMVAIK/20-77/RSV/G and pMVAIK/20-77/RSV/F were inserted into pMVAIK using Sac II and Pac I sites. The recombinant plasmid constructs were designated pMVAIK/RSV/G and pMVAIK/RSV/F, respectively.

strain in a 24-well plate. Culture supernatants were collected and cells were freeze-thawed and total protein of 4 μg of supernatants and cell lysate was applied. Samples were subjected to Western blotting. Briefly, after SDS-PAGE, proteins were transferred to membrane (Immobilon; Millipore, Danvers, MA, US). Membranes were washed with PBST, incubated with an RSV polyclonal antibody raised in goats, washed again, and incubated with a donkey anti-goat IgG (H+L) conjugated with horse radish peroxidase (HRP). The final reaction was performed with a DAB SUBSTRATE KIT FOR PEROXIDASE (Vector Laboratories, Burlingame, CA, US) used as recommended by the manufacturer.

Culture medium of Vero cells infected with MVAIK/RSV/G or F was collected and fractionated through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient, 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was electrophoresed and analyzed by Western blotting, using RSV polyclonal antibodies and monoclonal antibodies against MV N protein.

2.7. Immunogenicity in experimental animals

Six-week-old cotton rats were purchased from Harlan (Indianapolis, IN, US) and Charles River (USA). Five cotton rats for each group were immunized intramuscularly with 1×10^6 $TCID_{50}$ of MVAIK, MVAIK/RSV/G or MVAIK/RSV/F. Serum samples were obtained immediately before and 1, 3, 5, 8, 12 and 16 weeks after immunization. Cotton rats immunized with MVAIK/RSV/G or F were boosted with the same dose after 16 weeks, and serum samples were collected one week after re-immunization (17 weeks).

2.8. Serology

Neutralization tests (NTs) against RSV were performed with the 50% plaque reduction assay, using Long strain and wild-type isolate of subgroup B. Briefly, serum samples were serially diluted by1:4, starting from a 1:10 dilution, and mixed with an equal volume of RSV (100 PFU) in MEM for 1 h at room temperature. The mixtures were inoculated on monolayers of HEp-2 cells in 24-well plates. Plates were incubated for 1 h at 37 °C in 5% CO_2 and then overlaid with MEM supplemented with glutamine, antibiotics, 5% fetal bovine serum and 0.5% agar. After incubation for six days at 37 °C in 5% CO_2, cells were fixed with 1% formalin. Agar was removed and cells were stained with neutral red. Plaque numbers were counted and NT antibody titers were calculated as the reciprocal of the serum dilutions that showed a 50% reduction of the plaque number.

For the particles agglutination (PA) test, gelatin particles were coated with purified measles virus antigen (Serodia®-Measles, Fuji Rebio, Tokyo, Japan). Sera were serially diluted two-fold, starting from a 1:10 dilution, and each serum dilution was mixed with an equal volume of gelatin particles to detect agglutination, according to the recommendations of the manufacturer. The PA antibody titers were expressed as the reciprocal of the serum dilution which induced particle agglutination.

2.9. Detection of the MV genome

Cotton rats were sacrificed 10 days after immunization with MVAIK/RSV/G and F, and samples of liver, kidney, spleen, lung, thymus, and nasal turbinate were obtained to detect the MV genome. The tissues were homogenized, and total RNA was

Table 1
Primer and probe sequences for the detection of the MVAIK N gene and RSV N gene by TaqMan real-time PCR.

Primers	Sequences (5′–3′)	Genomic position
RSV-Long-N-(+)	aatgctaaaagaaatgggagagg	
Probe	gctccaga	411–470
RSV-Long-N-(−)	ccacaatcaggagaatcatgc	
MV-AIK-C-N-(+)	caagatcagtagagcggttgg	
Probe	agcccaag	1212–1274
MV-AIK-C-N-(−)	ctttgatcaccgtgtagaaatga	

extracted using an RNeasy® Plus Mini Kit (QIAGEN, MD, US), as recommended. TaqMan PCR was performed in the MV N gene region. Reverse-transcribed real-time PCR was performed using a FastStart TaqMan® Probe Master (Roche Meylan, France), and LightCycler®480 System II (Roche Meylan, France) using 1 μg of extracted mRNA. cDNA was synthesized using an One Step PrimeScript® RT-PCR Kit (TaKaRa Bio, Otsu, Japan). The parameters used were 1 cycle of 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s, and 1 cycle of 40 °C for 30 s. Reactions were performed in triplicate and genome copy numbers were determined by referring to the results of serial dilution of the corresponding plasmid, pCIAN01. The primers used in TaqMan PCR are shown in Table 1.

2.10. Protection against RSV

Seven week-old cotton rats were immunized intramuscularly with MVAIK/RSV/F or MVAIK/RSV/G and, five weeks later, challenged with 10^6 PFU/0.5 ml of RSV subgroups A and B. They were sacrificed four days after the challenge and nasal wash, BAL, nasal turbinate, and lung tissues were obtained. Lung samples were divided into two portions, one for pathological examination, and another for recovering the infective particles and RSV genome.

Tissues were homogenized and 0.1 ml volumes of serial 10-fold dilutions of homogenized samples were placed on HEp-2 cells and overlaid with MEM 5% FBS and 0.5% agar. Plaque numbers were counted after incubation for six days at 37 °C and infectivity was expressed as the number of plaques. RNA was extracted from nasal wash, BAL, nasal turbinate and lung homogenate. cDNA was synthesized and reverse-transcribed real-time PCR was done at position 1212–1274 of the RSV N genome, using the primers and TaqMan probe listed in Table 1. The RSV genome copy number was calculated by referring to a linear regression assay of serial dilutions of the corresponding plasmid.

Lungs were inflated to their normal volumes with 4% formalin and submerged in formalin for overnight fixation. The fixed tissue was embedded in paraffin, sectioned, and stained with hematoxylin–eosin, and immuno-staining was performed using four clone blend monoclonal antibodies against RSV P, F, and N proteins (AdB Serotec, UK), and anti-mouse IgG conjugated with HRP.

3. Results

3.1. Characteristics of recombinant viruses

MVAIK/RSV/G and MVAIK/RSV/F were recovered from full-length recombinant cDNA and MVAIK from vector cDNA. B95a cells were infected with MVAIK, MVAIK/RSV/G and MVAIK/RSV/F at a m.o.i. of 0.02. The culture medium was harvested on days 1, 3, 5, and 7 at 33 °C and the results are shown in Fig. 2. Infectivity showed a peak titer of 10^5 $TCID_{50}$/ml 5 days after infection. MVAIK/RSV/G and MVAIK/RSV/F grew as well as MVAIK in B95a cells. AIK-C has temperature-sensitivity (*ts*), showing efficient virus

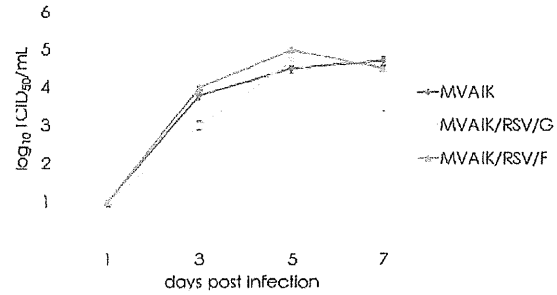

Fig. 2. Growth of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at m.o.i. of 0.02. Culture fluid was obtained on days 1, 3, 5, and 7 of culture at 33 °C. Infectivity is shown as mean titers of $TCID_{50}$/ml assayed in B95a cells. Error bars show 1.0 S.D.

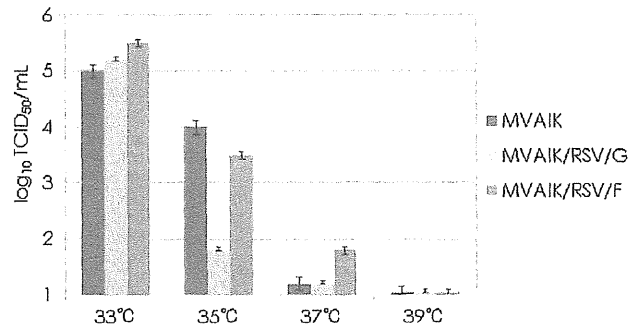

Fig. 3. Temperature sensitivity of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at a m.o.i. of 0.02. Culture fluid was obtained on day 5 and the infectivity at 33 °C, 35 °C, 37 °C, and 39 °C is shown as mean infectious titer ($TCID_{50}$/ml). Error bars show 1.0 S.D.

growth at 33 °C, but extremely poor at 39 °C, less than 10^{-4} in comparison with the result at 33 °C. MVAIK/RSV/G and MVAIK/RSV/F were examined for virus growth at 33, 35, 37 and 39 °C. The culture supernatants were harvested on day 7 of the culture and infectivity was examined. Both MVAIK/RSV/G and MVAIK/RSV/F showed 10^5 $TCID_{50}$/ml at 33 °C, and MVAIK/RSV/F grew little at 37 °C. But, however, no infectious virus was detected at 39 °C, and the *ts* phenotype was maintained (Fig. 3).

3.2. Detection of RSV G or F protein

B95a cells were infected with MVAIK/RSV/F, MVAIK/RSV/G, and MVAIK at a m.o.i. of 0.01. Live cells were stained with monoclonal antibodies against measles HA and polyclonal antibodies against RSV and visualized with second antibodies conjugated with rhodamine or FITC, as shown in Fig. 4. RSV F and MV HA proteins were observed diffusely on the surface of B95a cells infected with MVAIK/RSV/F. RSV G protein was detected in speckled pattern together with MV HA protein on the surface of B95a cells infected with MVAIK/RSV/G.

Culture medium and cell lysate were examined for the expression of RSV G and F by Western blotting and the results are shown in Fig. 5. Live vector virus MVAIK and RSV were used for the negative and positive controls. RSV G and F proteins were detected in both supernatant and cell lysate infected with MVAIK/RSVF, and MVAIK/RSV/G, similar to those infected with RSV (Fig. 5, Panel A).

Culture fluid was collected and fractionated through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient, 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was electrophoresed and analyzed by Western blotting, using RSV polyclonal antibodies and a monoclonal antibody

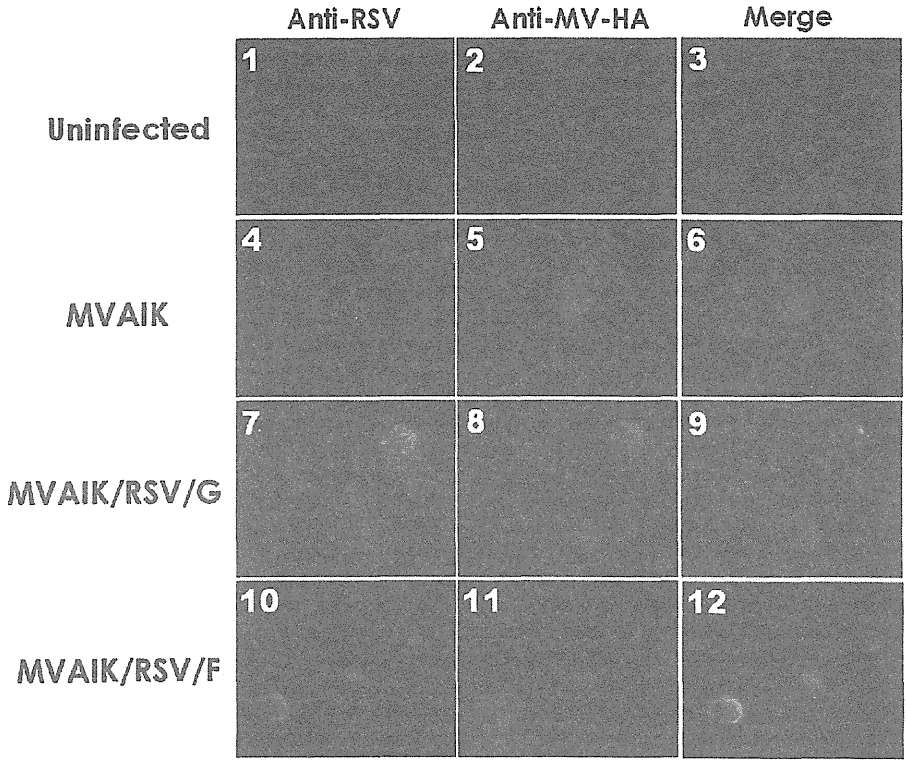

Fig. 4. Expression of MV HA and RSV G or F protein. B95a cells were infected with MVAIK (panels 4 and 5), MVAIK/RSV/G (panels 7 and 8) or MVAIK/RSV/F (panels 10 and 11) at a m.o.i. of 0.01 in 24-well plate and cultured for two days at 33 °C. Uninfected B95a cells are shown in panels 1 and 2. B95a cells were collected and subjected to live cell staining without fixation to detect the surface expression. The expression of RSV (panels 1, 4, 7, and 10) and MV HA protein (panels 2, 5, 8, and 11) are shown. Panels 3, 6, 9 and 12 are merged images.

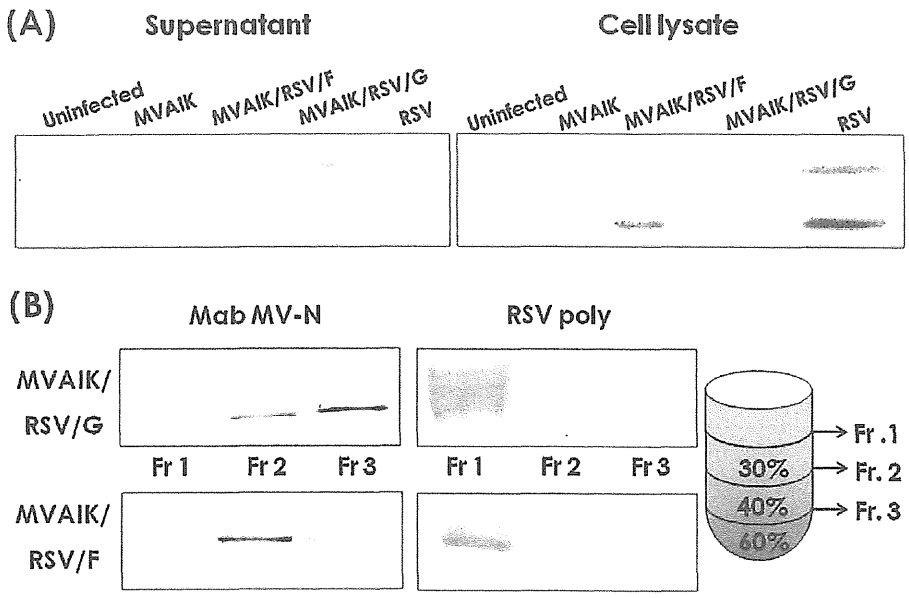

Fig. 5. Results of Western blotting of culture supernatant, cell lysate, and purified recombinant measles viral particles. (A) Vero cells were infected with MVAIK, MVAIK/RSVF, and MVAIK/RSV/G and HEp-2 cells were infected with RSV subgroup A, Long strain, and were cultured in 1 ml in a 24-well plate. Just before the appearance of CPE, culture media was replaced with serum free medium (VP-SFM). 1 ml of culture medium was harvested and 100 μl of PBS was added in plate. Cells were freeze-thawed and cell lysate was clarified. As for the Western blotting, 1/30 of initial supernatants and 1/100 of cell lysate were subjected for experiments. They were stained with polyclonal antibodies against RSV. (B) Infectious particles were obtained through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient of 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was analyzed by Western blotting, using RSV polyclonal antibodies and monoclonal antibodies against MV N protein.

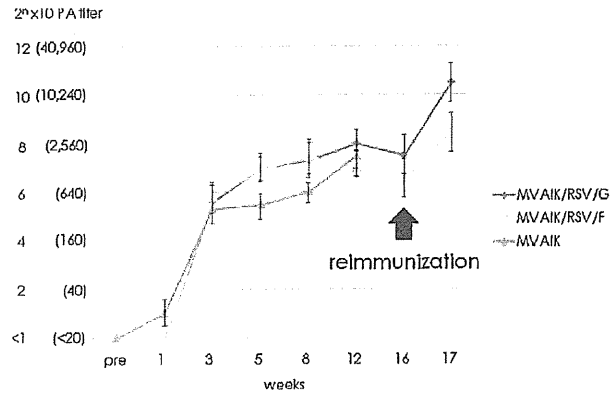

Fig. 6. Serological response of PA antibodies against MV. PA antibody titers were examined, using Serodia®-Measles. PA titers are expressed as $2^n \times 10$. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. Five cotton rats were immunized and followed for 12 weeks. Mean PA titers ±1.0 S.D. are shown. Two rats for each were reimmunized at the 16th week.

against the MV N protein. RSV G or F was detected in Fraction 1, and, whereas the MV N protein was detected in Fractions 2 and 3 (Fig. 5, Panel B). Accordingly, RSV G or F protein translated from the inserted gene was considered not to be incorporated into MV particles.

3.3. Immunogenicity of recombinant measles viruses

The recombinant viruses, MVAIK/RSV/G and MVAIK/RSV/F, were inoculated into cotton rats to confirm the immunogenicity intramuscularly of the inserted RSV G or F protein. Five cotton rats were immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F for each study group and serum samples were obtained before and 1, 3, 5, 8, 12, and 16 weeks after immunization. The results are shown in Fig. 6. PA antibodies against MV were detected three weeks after the immunization in all animals. High levels of PA antibody, $2^{6-8} \times 10$ (1:640–1:2560), were maintained until 16th week in those immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. Two rats were reimmunized 16 weeks after the first immunization and sera were obtained one week after the reimmunization for each group. PA antibodies increased from $2^{7.5\pm1.5}$ to $2^{10.5\pm1.5} \times 10$ in the MVAIK/RSV/G group, and from $2^{6.5\pm1.5}$ to $2^{8.5\pm1.5} \times 10$ in the MVAIK/RSV/F group. PA antibodies against MV increased after the reimmunization by four to eight-fold.

The results for NT antibodies against RSV are shown in Fig. 7. In the cotton rats immunized with MVAIK/RSV/G, NT antibodies against RSV subgroup A were detected one week after immunization but the mean titer began to decrease 5 week after immunization. The mean NT titers against RSV subgroup A decreased to undetectable levels 12 weeks after the immunization. In the MVAIK/RSV/F group, NT antibodies against RSV subgroup A were detected one week after the immunization in all animals with a mean titer of $10^{2.0\pm0.7}$. High titers were observed at the 5th week with a mean of $10^{2.6\pm1.0}$. Levels of these antibodies were maintained until 16th week.

In this experiment, RSV source of the recombinant MVAIK/RSV/F or MVAIK/RSV/G was derived from the RSV subgroup A wild type. Cross immunity against RSV subgroup B was further investigated. In cotton rats immunized with MVAIK/RSV/F, NT antibodies against RSV subgroup B were detected at the 3rd week with a mean titer of 150 ($10^{2.1}$) and maintained for 16 weeks. However, cross-reactive antibodies against RSV subgroup B were not detected in the cotton rats immunized with MVAIK/RSV/G.

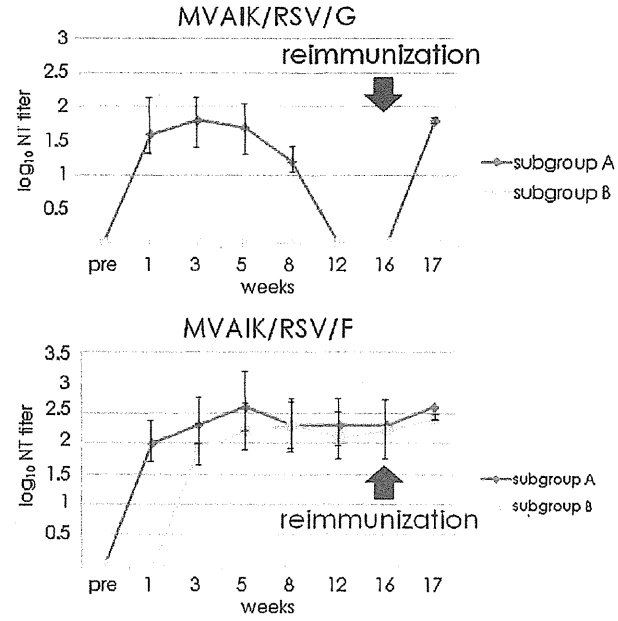

Fig. 7. Development of NT antibodies against RSV. NT antibodies were examined using the RSV Long strain (Subgroup A) and wild-type RSV subgroup B strain. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization. 50% plaque reduction NT titers are expressed 10^n and are shown as mean NT titers with 1.0 S.D. The upper panel shows the immune response after immunization with MVAIK/RSV/G. The lower panel shows the results after immunization with MVAIK/RSV/F.

In the cotton rats immunized with MVAIK/RSV/F, NT antibodies against RSV subgroups A and B increased after reimmunization by two fold, but not significantly. As for the rats immunized with MVAIK/RSV/G, NT antibodies against RSV subgroup A were boosted from an undetectable level before the reimmunization to $10^{1.8\pm0.1}$, but those against RSV subgroup B were not detected.

3.4. Protection against RSV challenge

The peak response against RSV was observed five weeks after immunization. Three cotton rats were immunized with MVAIK/RSV/F and MVAIK/RSV/G and challenged with the homologous RSV subgroup A (Long strain) and heterologous subgroup B (wild-type). No infectious virus was recovered from nasal wash and BAL but RSV genome was detected. RSN genome copy number was slightly lower in immunized groups but not significant (data not shown). The recovery of infectious virus and genome copy numbers from lung tissues are shown in Fig. 8. $10^{5.4}$ and $10^{4.5}$ PFU of infectious virus were recovered from 20 mg of lung tissue in two cotton rats of the control group challenged with RSV subgroup A, but no infectious virus was recovered in three cotton rats immunized with MVAIK/RSV/F. Meanwhile, $10^{4.5}$, $10^{2.8}$ and $10^{3.3}$ PFU of infectious virus were recovered in cotton rats immunized with MVAIK/RSV/G.

As for challenge with RSV subgroup B, $10^{5.0-5.8}$ PFU of RSV was recovered from lung infected with RSV subgroup B in non-immunized rats. In cotton rats immunized with MVAIK/RSV/F, virus titers were slightly lower, $10^{4.4-4.5}$ PFU but $10^{5.0-5.3}$ PFU from their lung tissues in the cotton rats immunized with MVAIK/RSV/G. There was no significant reduction in RSV N gene copy number.

For histopathological examinations, lung tissues were obtained four days after the challenge with RSV subgroups A and B and the results of HE staining and immuno-staining against RSV antigens are shown in Fig. 9. The non-immunized rat challenged with RSV subgroup A showed prominent interstitial pneumonia

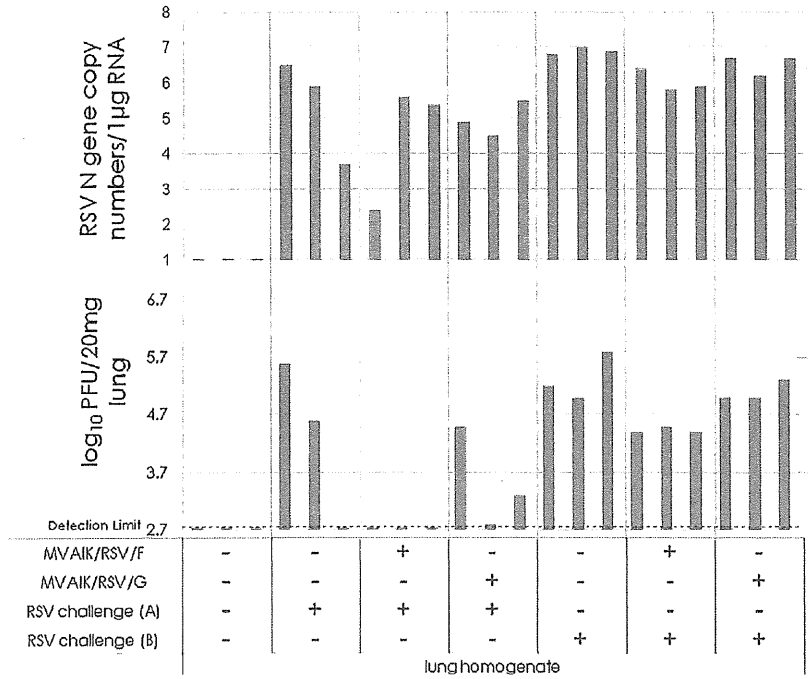

Fig. 8. Recovery of RSV infectious virus and genome copy numbers after challenge with RSV subgroups A and B. Three cotton rats were investigated in the normal control group, non-immunized group, and group immunized with MVAIK/RSV/F or MVAIK/RSV/G. Animals non-immunized group, and group immunized with MVAIK/RSV/F or MVAIK/RSV/G were challenged with 1.0×10^6 PFU of the homologous RSV Long strain and wild-type subgroup B five weeks later. Virus infectivity was monitored in lung homogenate, and RSV infectivity is shown as PFU in 20 mg of lung tissue. And 1 μg of total RNA of lung tissue was used for real-time PCR, and each column represents individual result.

(panel 2; thickening of alveolar wall, and infiltration of inflammatory mononuclear cells) with RSV antigens in bronchial epithelial cells (panel 6). In cotton rat immunized with MVAIK/RSV/F showed very mild inflammation (panel 3), though most sections were normal, without RSV antigen in bronchial tissue after RSV challenge with subgroup A (panel 7). In cotton rat immunized with MVAIK/RSV/G, moderate interstitial pneumonia was observed with a small amount of RSV antigen (panels 4 and 8).

As for the challenge with RSV subgroup B, histological findings in non-immunized rat challenged with subgroup B were similar to the results challenged with RSV subgroup A. The results of immunostaining are shown. Large amounts of RSV antigen were detected in non-immunized rat (panel 9). Small amounts of RSV antigens were

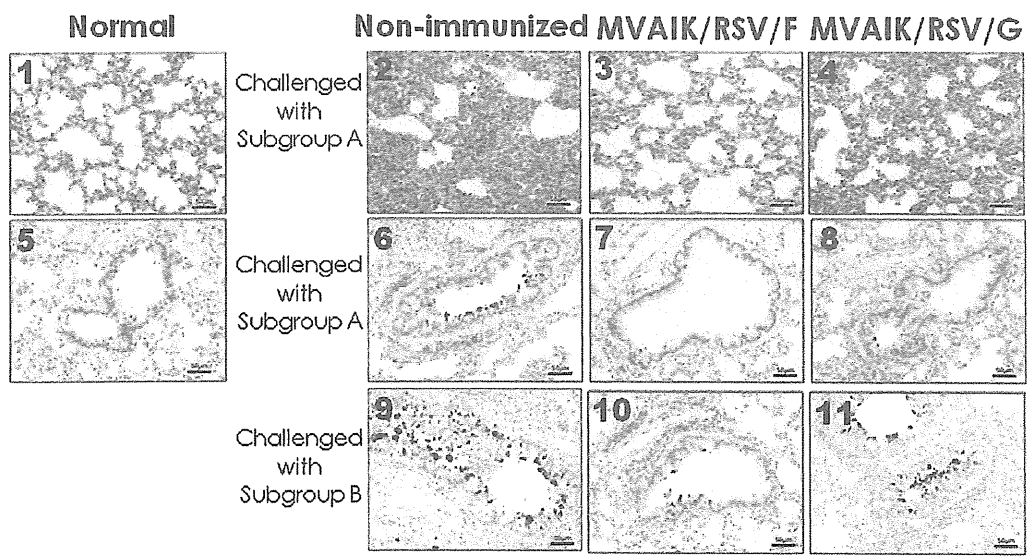

Fig. 9. Pulmonary histopathology in cotton rats challenged with RSV subgroups A and B. Cotton rats were immunized intramuscularly with MVAIK/RSV/F (panels 3, 7, and 10) or MVAIK/RSV/G (panels 4, 8, and 11) and then challenged five weeks later, with RSV subgroup A (panels 2, 3, 4, 6, 7, and 8) and subgroups B (panels 9, 10 and 11). They were sacrificed four days after the challenge. Histological examination was performed by HE staining of lung tissues (panels 1, 2, 3, and 4) and the results of immuno-staining of bronchiolar regions are shown in panels 5, 6, 7, 8, 9, 10, and 11. Immuno-staining was performed using four clone blend monoclonal antibodies against RSV P, F, and N proteins and anti-mouse IgG conjugated with HRP. HE staining and immuno-staining of normal control are shown in panels 1 and 5.

detected in MVAIK/RSV/F group (panel 10) in comparison with MVAIK/RSV/G group (panel 11). Finding of RSV antigens were well correlated with the results of the recovery of infectious virus from lung tissues. Inoculated virus would be cleared and demonstrated a mild pathological finding in rats immunized with MVAIK/RSV/F.

4. Discussion

RSV is a clinically important cause of respiratory tract infections, especially among high-risk infants, immunocompromised hosts, and the elderly. Despite a serious disease burden, there is no licensed vaccine for RSV. Initial efforts to develop a vaccine involved FI-RSV which unexpectedly enhanced the disease in clinical trials in RSV-naive children [33]. FI-RSV failed to induce a secretory IgA response after pareteral administration without inducing a CTL response, which was a serious drawback of the inactivated vaccine. The defeated F protein would not induce Th1 response and the aluminium-precipitated vaccine induced only Th2 response. The allergic reaction to this vaccine would be caused by the Th2-prone reaction [34]. Several subunit vaccines were investigated, but failed to generate effective antibodies. A live attenuated vaccine has the advantage of inducing humoral and cellular immune responses similar to a natural infection. Temperature-sensitive (*ts*) and cold-adapted (*ca*) RSV vaccine strains have been developed by conventional attenuation methods. Over the last 40 years, cautious and deliberate progress has been made toward developing a RSV vaccine using various experimental approaches, including live attenuated strains and vector-based and viral protein subunit vaccine candidates. But the balance between the safety and immunogenicity is a key issue to the development of a live attenuated vaccine, and the (*ts*) RSV vaccine candidate resulted in insufficient attenuation, causing similar respiratory illness [35]. Based on a vaccine candidate having the *ts* phenotype, several recombinant vaccine candidates were developed by deletion of the SH gene or NS1 gene or mutation by reverse genetics. These recombinant RSV vaccines induced sufficient immune response in chimpanzees [36]. Another approach involved the application of reverse genetics to express RSV protein in a recombinant vector-based vaccine. The first vector-based candidate was evaluated using vaccinia virus. Recombinants expressing RSV F or G was highly immunogenic, induced protection in mouse but provided inconsistent protection in chimpanzees [37]. MVA strain of vaccinia-based recombinants expressing RSV G and F protein were immunogenic in rodent but not in rhesus monkey model [38]. Several vector-based live vaccine platforms were established using HPIV-III and Sendai virus [23,39]. Through preceding experiments, the F protein is known to be more effective than G. But there were no experiences for clinical usage and the HPIV-III-based recombinant vaccine was poorly immunogenic in human clinical trials.

In this report, reverse genetics using the AIK-C live attenuated measles vaccines were developed. A recombinant measles virus vector-based vaccine was established using the Schwartz strain, expressing the West Nile virus [40]. As well as the Schwartz strain, the AIK-C measles vaccine is a further attenuated vaccine strain having the *ts* phenotype, and its safety and immunogenicity has been confirmed [26,29]. Thus, in this report, AIK-C was used for a live virus vaccine-vector. Expression of the RSV G or F protein was confirmed by indirect immuno-staining of B95a cells infected with MVAIK/RSV/G or F with polyclonal and monoclonal antibodies against the F protein. By Western blotting, the G or F protein was detected in culture medium and cell lysate of B95a cells infected with MVAIK/RSV/G or F. The RSV G and F proteins were considered not to be incorporated into MV particles because theoretically they had no binding site for the MV M protein. MV envelop proteins bound the M protein [41]. The genetic stability of the vaccine candidate was examined and inserted genes for RSV G and F were stable even after 15 passages.

The recombinant measles virus (MVAIK) triggered an immune response three weeks after vaccination in cotton rats. Levels of these antibodies were maintained for 16 weeks. The same was observed after immunization with MVAIK/RSV/G or F. To investigate the viral growth, samples of nasal turbinate, lung, thymus, spleen, liver, kidney, and bone marrow were obtained 10 days after immunization, but no infectious virus was recovered. Total RNA was extracted and RT-real time PCR was performed to detect the measles N gene by real-time PCR. The MV genome was detected only in thymus in cotton rats immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F (data not shown). Infectious virus was recovered from inguinal superficial lymph nodes three days after infection in the previous study [42]. NT antibody titers against RSV were investigated, using RSV Long (subgroup A) and wild-type subgroup B. MVAIK/RSV/G or MVAIK/RSV/F induced the production of NT antibodies against RSV subgroup A from one week after vaccination in cotton rats. Antibody titers were higher after immunization with MVAIK/RSV/F than with MVAIK/RSV/G. RSV has distinctly different subgroups, A and B. The G or F gene of subgroup A was used in this study. Therefore, the cross reaction of NT against subgroup B was investigated. MVAIK/RSV/G did not generate NT antibodies against RSV subgroup B, but MVAIK/RSV/F induced production of cross-reactive NT antibodies against RSV subgroups A and B. The predicted amino acid sequence of the RSV F protein used in this study exhibited 98.6% homology among F proteins of subgroup A strains and 90.8% in comparison with those of subgroup B strains. The predicted amino acid sequence of RSV G protein has 86.9% homology among subgroup A strains but 49.7% in comparison with subgroup B. Thus, F protein was relatively conserved between subgroups A and B but the G protein of RSV was variable and thought not suitable as a vaccine antigen. Recently, a humanized monoclonal antibody against the RSV F protein was used for prevention of serious RSV infections in young infants having cardiac and pulmonary disorders, with a low birth weight, or born prematurely. In this study, recombinant MVAIK/RSV/G or F was administered intramuscularly and induced sufficient NT antibodies. Secretory IgA antibodies and CTL response were not examined but it protected against the challenge with homologous RSV subgroup A. In non-immunized cotton rats, $10^{5.4}$ and $10^{4.5}$ PFU of infectious virus were recovered from 20 mg of lung tissue four days after the RSV challenge. But those immunized with MVAIK/RSV/F were protected, without recovery of infectious virus from the lung tissues. And they did not demonstrate interstitial pneumonia. Cross reactive NT antibodies were demonstrated after immunization with MVAIK/RSV/F but its protective effect is not sufficient against subgroup B, demonstrating slightly lower levels (approximately 1/10 of non-immunized control) of the recovery of infectious virus. Protective effects of MVAIK/RSV/G were poor in comparison with MVAIK/RSV/F similar to the serological responses.

As for the experimental animal models, transgenic mice expressing human CD46 with the knock out of type I interferon (IFN) receptor gene were used to evaluate the immunogenicity of a recombinant MV vaccine candidate produced using the West Nile virus [40], SARS corona virus [43], hepatitis B virus [44] and HIV [45]. Efficient immune responses were reported, but the IFN system is the most important signal for innate immunity. In the case of the RSV vaccine candidate, innate immunity modified the adaptive immunity, and, therefore, cotton rats without gene manipulation were used in these experiments [46].

Recombinant MV vaccine-based vectors have practical limitation for timing of immunization. In young infants, maternal conferred immunity would interfere with vaccine effects. In field trials, AIK-C gave efficient sero-conversion and induction of cell-mediated immunity even when the vaccine was given at the age

of six months [26,27]. They demonstrated more than 80% seroconversion rate to overcome maternal conferred immunity and the safety was similarly confirmed, suggesting no evidence of immunesuppression. RSV infection was observed even after six months of age, and, therefore, MVAIK/RSV/F would be applicable for six months to provide protective immunity both against RSV and measles especially for developing countries.

As for the effective protection against RSV infection, intranasal administration is desired. But we have no experience of intranasal administration of AIK-C vaccine, and, in our previous experiments, the recombinant MVAIK did not induce serum NT against MV through intranasal administration because of the strict *ts* phenotype in cotton rat model, having high body temperature [21,42]. Therefore, the comparative studies are planning to investigate the immunogenicity and challenge tests in monkeys immunized with MVAIK/RSV/F.

In conclusion, a new MV vaccine-strain-based RSV vaccine candidate was demonstrated to confer protection against RSV in cotton rats. The xenogeneic recombinant might induce simultaneously protective immunity against backbone-MV and inserted-RSV infections. Recombinant MVAIK expressing RSV F protein is a promising candidate and protective effects should be confirmed in monkey model, considering the immunization routes.

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Elucidation of the full genetic information of Japanese rubella vaccines and the genetic changes associated with *in vitro* and *in vivo* vaccine virus phenotypes

Noriyuki Otsuki*, Hitoshi Abo, Toru Kubota, Yoshio Mori, Yukiko Umino, Kiyoko Okamoto, Makoto Takeda, Katsuhiro Komase

Department of Virology 3, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan

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ABSTRACT

Rubella is a mild disease characterized by low-grade fever, and a morbilliform rash, but causes congenital defects in neonates born from mothers who suffered from rubella during the pregnancy. After many passages of wild-type rubella virus strains in various types of cultured cells, five live attenuated rubella vaccines were developed in Japan. An inability to elicit anti-rubella virus antibodies in experimentally infected animals was used as an *in vivo* marker phenotype of Japanese rubella vaccines. All Japanese rubella vaccine viruses exhibit a temperature-sensitive (ts) phenotype, and replicate very poorly at a high temperature. We determined the entire genome sequences of three Japanese rubella vaccines (Matsuba, TCRB19, and Matsuura), thereby completing the sequencing of all five Japanese rubella vaccines. In addition, the entire genome sequences of three vaccine progenitors were determined. Comparative nucleotide sequence analyses revealed mutations that were introduced into the genomes of the TO-336 and Matsuura vaccines during their production by laboratory passaging. Analyses involving cellular expression of viral P150 nonstructural protein-derived peptides revealed that the N1159S mutation conferred the ts phenotype on the TO-336 vaccine, and that reduced thermal stability of the P150 protease domain was a cause of the ts phenotype of some rubella vaccine viruses. The ts phenotype of vaccine viruses was not necessarily correlated with their inability to elicit humoral immune responses in animals. Therefore, the molecular mechanisms underlying the inability of these vaccines to elicit humoral responses in animals are more complicated than the previously considered mechanism involving the ts phenotype as the major cause.

1. Introduction

Rubella is a communicable and ordinarily mild disease that is characterized by low-grade fever, a short-lived morbilliform rash, and lymphadenopathy [1]. A German physician originally designated this disease 'German measles', since it resembles measles but is much less severe [1]. Arthralgia and arthritis are common complications of rubella, particularly in adolescent and adult females. Rubella has received much attention following a report that congenital cataracts were associated with rubella infection of mothers during pregnancy [2]. Subsequent studies indicated that sensorineural hearing loss and cardiovascular defects are also common in neonates born from mothers who suffered from rubella, especially during the early phase of pregnancy [1].

Rubella virus (RuV) was first isolated in 1962 [3,4], and belongs to the genus *Rubivirus* in the family *Togaviridae*. After many passages of wild-type (wt) RuV strains in various types of cultured cells, live attenuated rubella vaccines were developed [1,5,6]. The first rubella vaccine was licensed in the United States in 1969 [7]. A total of nine vaccines (HPV-77, RA27/3, Cendehill, BRD-2, Matsuba, TCRB19, KRT, Matsuura, and TO-336) have been developed to date [1,7]. Among these, five vaccines were developed in Japan [6].

An increased growth rate in certain cultured cells has been used as an *in vitro* marker phenotype of Japanese rubella vaccines [6]. In addition, an inability to elicit anti-RuV antibodies in experimentally infected guinea pigs and rabbits has been used as an *in vivo* marker phenotype of Japanese rubella vaccines [6]. The Minimum Requirements for Biological Products (MRBP) announced by the Ministry of Health, Labour and Welfare, Japan, defines the *in vivo* phenotype as a marker phenotype of rubella vaccines [8,9]. All lots of commercially used Japanese rubella vaccines must receive a national test by the National Institute of Infectious Diseases, Japan, to verify this phenotype as a marker test [8,9]. Later, it was recognized that all Japanese rubella vaccines exhibit a temperature-sensitive (ts) phenotype, meaning that they replicate very poorly at a high temperature (39 °C), whereas wt strains can replicate well at that temperature [5]. Although understanding of the molecular bases for acquisition of these *in vitro* and *in vivo* vaccine virus pheno-

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 562 8941.
E-mail address: otsuki@nih.go.jp (N. Otsuki).

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types is crucial for quality control of vaccines, they have been poorly elucidated. In the present study, we performed various analyses to elucidate the genetic changes introduced into the genomes of rubella vaccines during passages under laboratory conditions and to show importance of these changes in determining the *in vitro* and *in vivo* vaccine virus phenotypes. Our data in the present study provide basic and solid information for the genetic changes of rubella vaccines as well as a novel insight into the understanding of molecular bases for the vaccine phenotypes.

2. Materials and methods

2.1. Cells and viruses

RK13 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 8% bovine serum. After infection with RuV, the cells were cultured in MEM containing 2% bovine serum. When transfected with plasmids, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mM non-essential amino acids.

Five licensed vaccines (KRT, Matsuba, TCRB19, TO-336, and Matsuura) were passaged once or twice in RK13 cells to obtain sufficient amounts of stocks. These vaccines were termed as KRT, Matsuba.vac, TCRB19, TO-336.vac, and Matsuura.vac, respectively, in the present study. Three wt strains, TO-336.GMK5, Matsuba.GMK3, and Matsuura.B3, isolated in Japan in the late 1960s were also passaged in RK13 cells once or twice to obtain sufficient amounts of virus stocks. The wt RVi/Hiroshima.JPN/01.03 strain isolated in Japan in 2003 was passaged four times in RK13 cells. A TO-336.vac-derived mutant clone that replicated well at a high temperature was generated as follows. RK13 cells were infected with TO-336.vac and incubated at 39 °C. A clone that replicated well at this temperature was plaque-purified and propagated in RK13 cells at 39 °C. The obtained clone was designated TO-336.rev.

2.2. Sequencing

Viral RNAs were extracted from each virus stock using a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Several cDNA fragments covering the entire virus genome were amplified using a One-step RT-PCR Kit (Qiagen KK, Tokyo, Japan). The primers used for amplification will be provided upon request. To determine the 5′ terminus of each viral genome, a 5′ RACE system (Invitrogen, Carlsbad, CA) was used. To amplify the 3′ terminus of the viral genome, an RNA PCR Kit (AMV) ver. 3.0 (Takara Bio, Shiga, Japan) was used with an oligo (dT) adaptor primer. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen KK) and a QIAquick PCR Purification Kit (Qiagen KK). The nucleotide sequences of the purified PCR products were determined using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a capillary sequencer. Some PCR products were cloned into the pCR2.1 vector (Invitrogen), and the nucleotide sequences of more than three clones were analyzed to determine a consensus sequence.

2.3. Sequence data analysis

The entire genome sequences of 13 RuV strains were used for comparisons. Data for seven RuV strains, Therien [10] (GenBank M15240), RA27/3 [11] (GenBank L78917), RVi/Matsue.JPN/68 [12] (GenBank AB222609), TO-336wt [13] (GenBank AB047330), TO-336.vac [13] (GenBank AB047329), M33 [11,14] (GenBank X72393 and X05259), and KRT [12] (GenBank AB222608) were reported previously. Corrected nucleotide sequence data were used for the Therien and M33 strains [11], rather than the original data (GenBank M15240, X72393, and X05259). The entire genome nucleotide sequences of six RuV strains, TCRB19, Matsuba.GMK3, Matsuura.B3, Matsuura.vac, TO-336.GMK5, and Matsuba.vac, were determined in the present study, and registered in GenBank under accession numbers AB588188, AB588189, AB588190, AB588191, AB588192, and AB588193, respectively. Analyses were performed using the GENETYX-MAC ver. 13.0.14 software (Genetyx, Tokyo, Japan).

2.4. Phylogenic analysis

The phylogenic relationships of the RuV strains were analyzed by drawing a neighbor-joining tree with a kimura-2-parameter model with 739 nucleotides (nt) of the E1 regions of 44 RuV strains using a CLUSTALW ver. 1.83 application (http://www.ddbj.nig.ac.jp/Welcome-j.html). These 739 nt correspond to the minimum acceptable window defined by the World Health Organization [15]. The reliability of the tree was estimated with 1000 iterations of bootstrapping. Another phylogenic tree was similarly constructed using the entire genome sequences of six RuV strains and the neighbor-joining method with a kimura-2-parameter model.

2.5. Growth kinetics analysis

Monolayers of RK-13 cells in 12-well plates were incubated with RuV strains at a MOI of 0.01 for 1 h. After the incubation, the cells were washed three times with phosphate-buffered saline and cultured in 1 ml of MEM containing 2% bovine serum at 35 °C or 39 °C. The culture medium was collected at 0, 1, 2, 3, and 5 days post-infection (p.i.). The virus titers were determined by plaque assays.

2.6. Plaque assay

Monolayers of RK13 cells in 6-well plates were incubated with 0.1 ml of samples that had been serially diluted by 10-fold for 1 h at room temperature. After the incubation, the cells were cultured in 3 ml of MEM containing 2% bovine serum, 0.5% agarose, and 40 μg/ml DEAE-dextran (0.5% agarose-MEM) at 35 °C for 7 days. After this culture period, 2 ml of 0.5% agarose-MEM containing 0.01% neutral red was overlaid on each well. The number of plaques were counted at 2 or 3 days after this procedure.

2.7. Plasmid construction

Plasmids encoding nonstructural protein (NSP)-derived peptides corresponding to amino acid positions 994–1548 ($NSP_{994–1548}$) [16] were constructed as follows. First-strand cDNAs were synthesized from purified viral RNA extracts from the TO-336.vac and KRT strains using SuperScript III reverse transcriptase (Invitrogen) and the 1548 reverse primer (5′-TATGAATTCGCCTACATGGATGCAGGC-3′) [16]. DNA fragments spanning nucleotide positions 994–1548 of the RuV genome were amplified by PCR using the 1548 reverse primer and the 994 forward primer (5′-AATGGATCCATGGACCACCGCCCGGCTGC-3′) [16]. The amplified DNA fragments were inserted into the mammalian cell expression plasmid pCMV-3tag-1a (Stratagene, Carlsbad, CA), in the downstream of a CMV promoter, using restriction enzyme recognition sites for *BamH*I and *EcoR*I, such that $NSP_{994–1548}$ was expressed as a peptide fused with three FLAG tags. Single point mutations were introduced into pCMV-3tag-1a encoding $NSP_{994–1548}$ using a KOD-plus- Mutagenesis Kit (Toyobo, Osaka, Japan).

2.8. Protein expression using plasmids and detection by immunoblotting

RK13 cells were transfected with pCMV-3tag-1a encoding $NSP_{994-1548}$ using the Lipofectamine LTX Plus reagent (Invitrogen) and cultured at 35 °C or 39 °C for 1 day or 3 days. In another experiment, transfected cells were cultured at 35 °C for 1 day, and subsequently, *de novo* protein synthesis was blocked by culturing the cells in medium containing 0.1 mg/ml cycloheximide at 35 °C or 39 °C for 2 days. After cell lysis, polypeptides were separated by electrophoresis in a polyacrylamide gel (10–20% gradient) in the presence of 0.1% sodium dodecyl sulfate and transferred onto nitrocellulose membranes. The membranes were incubated with anti-FLAG or anti-tubulin (clone B-5-1-2) antibodies (Sigma) followed by incubation with a peroxidase-conjugated secondary antibody. Peptides were then detected using an ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). The signal intensities were quantified using an LAS 1000plus Image Analyzer and the Image Gauge software (Fuji Film, Tokyo, Japan).

2.9. Detection of antibody titers in animals infected with RuV as a marker test

Specific pathogen-free female Hartley guinea pigs (weighing 300–400 g; 4, 8, or 12 animals per group) were infected subcutaneously with 5000 PFU of RuV. At 5 weeks p.i., the animals were euthanized and blood samples were obtained. The serum hemagglutination inhibition (HI) antibody titers were determined using goose erythrocytes and the hemagglutination antigen of RuV (Denka Seiken, Tokyo, Japan). Prior to the analyses, the serum specimens were treated with kaolin to remove nonspecific inhibitors and absorbed with goose erythrocytes to remove nonspecific hemagglutinins. After these treatments, the initial dilution of the samples was 1:8.

3. Results

3.1. TO-336.GMK5 and Matsuura.B3 strains are progenitors of or closely related to the progenitors of the currently used rubella vaccine strains

The growth kinetics of TO-336.GMK5, Matsuba.GMK3, and Matsuura.B3 were analyzed at 35 °C and 39 °C, and compared with those of the vaccine strains. The RVi/Hiroshima.JPN/01.03 wt strain was also evaluated as a control. After infection at 35 °C, all the tested viruses grew well and reached infectious titers that ranged from 10^4 to 10^6 PFU/ml at 5 days p.i. (Fig. 1A). At 39 °C, TO-336.GMK5 and Matsuura.B3 grew productively and were similar to the RVi/Hiroshima.JPN/01.03 wt strain (Fig. 1B). In contrast, all of the vaccine strains showed abortive infections (Fig. 1B). Although Matsuba.GMK3 was able to replicate at 39 °C, its growth was severely restricted (Fig. 1B). These data show that TO-336.GMK5 and Matsuura.B3 have retained wt phenotypes with the capacity to grow at a high temperature, while Matsuba.GMK3 has a ts phenotype, although it is less severe than those of the vaccine strains.

Phylogenic analyses using 739 nt of the E1 regions (nucleotide positions 8731–9469) of 44 RuV strains revealed the relationships of the Matsuba.GMK3, TO-336.GMK5, and Matsuura.B3 strains with other RuV wt and vaccine strains (Fig. 2). The data suggested that TO-336.GMK5 was a direct progenitor strain of the currently used TO-336.vac strain. Similarly, the data indicated that Matsuura.B3 was closely related to a progenitor of the currently used Matsuura.vac strain (Fig. 2). On the other hand, Matsuba.GMK3 was apparently unrelated to the Matsuba.vac strain (Fig. 2).

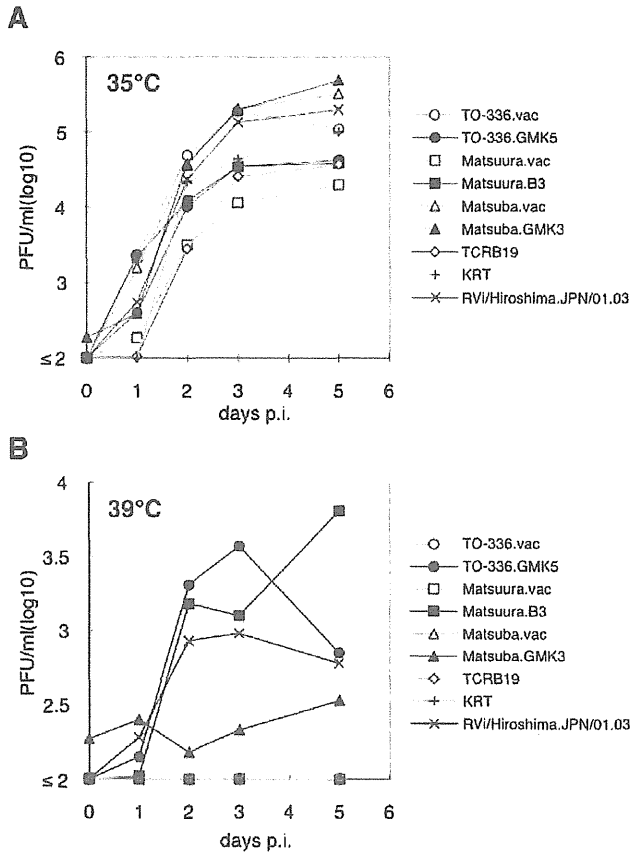

Fig. 1. Growth kinetics of RuV wt strains and vaccines at 35 °C and 39 °C. RK-13 cells were infected with RuV strains at a MOI of 0.01 and incubated at 35 °C (A) or 39 °C (B). The infectious titers of the culture supernatants were determined by plaque assays. Open circles, open squares, open triangles, open diamonds, and crosses indicate TO-336.vac, Matsuura.vac, Matsuba.vac, TCRB19, and KRT, respectively. Filled circles, filled squares, filled triangles, and saltires indicate TO-336.GMK5, Matsuura.B3, Matsuba.GMK3, and RVi/Hiroshima.JPN/01.03, respectively.

3.2. Japanese rubella vaccine strains have deletions in untranslated regions

The entire genome sequences of the TO-336.GMK5, Matsuba.GMK3, and Matsuura.B3 strains and three vaccine strains (Matsuba.vac, TCRB19, and Matsuura.vac) were determined. With the clarification of these sequences, the entire genome sequences became available for 13 RuV strains. These sequences included all five Japanese rubella vaccine (KRT [12], TO-336.vac [13], Matsuura.vac, TCRB19, Matsuba.vac), a US vaccine (RA27/3 [11]), and seven wt strains (Therien [10], M33 [11,14] (Gilliam S., GenBank X72393), TO-336wt [13], RVi/Matsue.JPN/68 [12], TO-336.GMK5, Matsuba.GMK3, and Matsuura.B3). Previous studies have indicated that the genome length of RuV is 9762 nt excluding the 5′ cap and 3′ poly(A) tract, and that the genome consists of a 40-nt 5′ untranslated region (UTR), a 6348-nt open reading frame (ORF) encoding two NSPs (P150 and P90), a 123-nt UTR, a 3189-nt ORF encoding three structural proteins (SPs) (C, E2, and E1), and a 62-nt 3′ UTR [11–13]. The genomes of Matsuba.GMK3, Matsuura.B3, and Matsuura.vac were also 9762 nt in length, and showed the same organization as the previously reported RuV strains [11–13]. On the other hand, the genome lengths of two Japanese vaccine

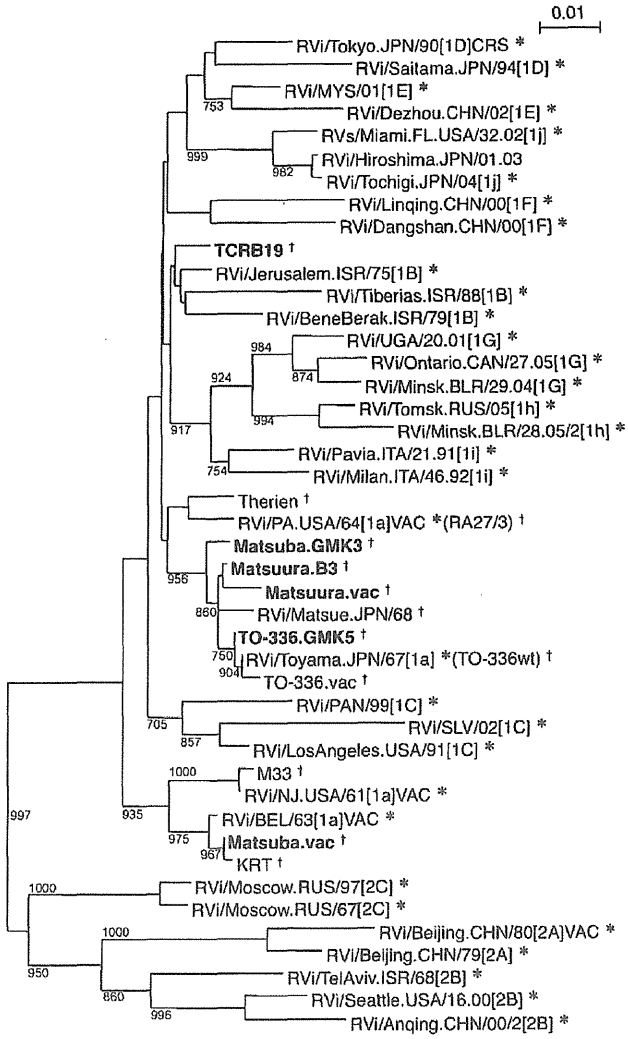

Fig. 2. Phylogenic tree of the 44 RuV strains. A phylogenic tree was drawn on the basis of 739 nt (positions 8731–9469) in the E1 regions of 44 RuV strains using a neighbor-joining method with a kimura-2-parameter model. Boldface characters indicate the strains whose entire genome sequences were determined in the present study. Asterisks indicate World Health Organization reference strains. Daggers indicate strains whose entire genome sequences are currently available. The genotypes based on the World Health Organization nomenclature are shown in square brackets. Bootstrap values above 700 (1000 replications) are shown on the phylogenic tree.

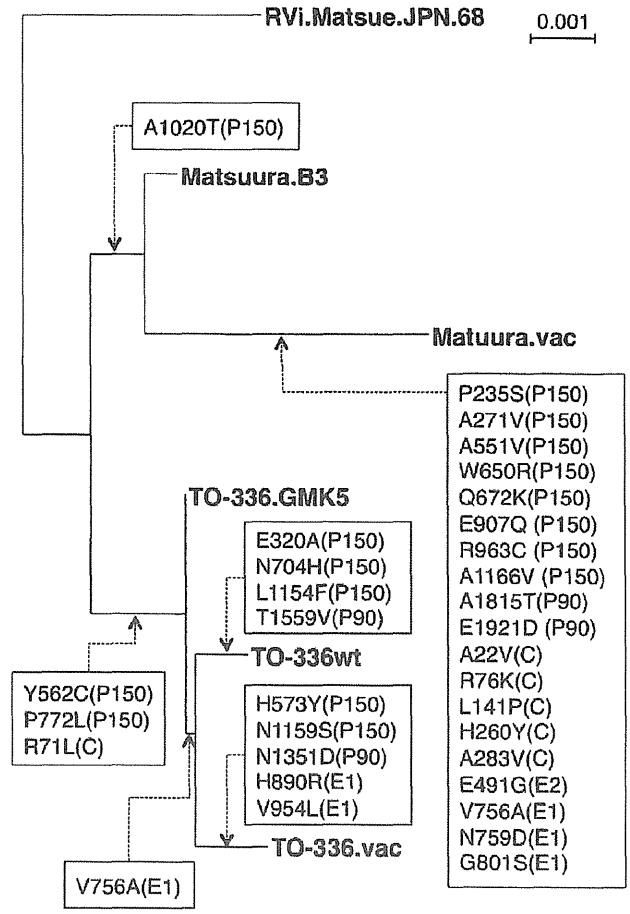

Fig. 3. Phylogenic relationships and amino acid substitutions in the Matsuura and TO-336 RuV strains. A phylogenic tree was drawn on the basis of the entire genome sequences of the Matsuura.B3, Matsuura.vac, TO-336.GMK3, TO-336 wt, TO-336.vac, and RVi/Matsue.JPN68 strains using a neighbor-joining method with a kimura-2-parameter model. The tree was rooted by the RVi/Matsue.JPN68 strain. Amino acid substitutions are indicated in boxes at the predicted points where they were introduced into the Matsuura and TO-336 strains.

strains, Matsuba.vac and TCRB19, were shorter by one nucleotide because of a single deletion in the UTR between the ORFs for the NSPs and SPs (junction UTR). The junction UTR is predicted to form secondary structures with a series of stem-loops [17] and to regulate subgenomic RNA synthesis [18]. Matsuba.vac and TCRB19 had a deletion (Δ) at nucleotide positions 6415 and 6479, respectively. Although neither position appeared to be directly involved in the stem-loop formation, this does not rule out the possibility that these deletions affect the functions of the junction UTR.

3.3. TO-336.vac has acquired six amino acid substitutions under the attenuation process

Kakizawa et al. [13] performed a sequence comparison analysis between TO-336.vac and a TO-336 wt strain (referred to as TO-336wt). They identified 10 amino acid differences between TO-336wt and TO-336.vac [13], while only six amino acid changes were found between TO-336.vac and TO-336.GMK5 (Figs. 3 and 4). A phylogenic tree drawn on the basis of the entire genome sequences revealed that TO-336.GMK5 was a progenitor of both TO-336.vac and TO-336wt (Fig. 4). The data suggested that TO-336wt was a descendant of TO-336.GMK5 with a different passage history from TO-336.vac (Fig. 3). These data are consistent with a previous report regarding the passage history of TO-336wt [19]. A comparison of the amino acid sequences and the phylogenic tree data suggested that TO-336.vac had acquired six substitutions, H573Y and N1159S in P150, N1351D in P90, and V756A, H890R, and V954L in E1, during the passage history of the virus (Fig. 3). N1159S and N1351D were located in the protease domain of P150 and the helicase domain of P90, respectively [20–23] (Fig. 4). Zhou et al. [24] recently reported that a 32-mer peptide region (amino acid positions 1152–1183) within the protease domain acts as a calmodulin-binding domain (CaMBD) that mainly adopts a helical structure and plays crucial roles in the protease activity and virus infectivity. N1159S was located in this helical structure. The V756A substitution in E1 was also found in TO-336wt (Figs. 3 and 4B), and was therefore not reported by Kakizawa et al. [13]. TO-336wt possessed four additional substitutions (E320A, N704H, and L1154F in P150 and T1559V in P90) (Fig. 3). These mutations were unique to TO-336wt

(Fig. 4). Kakizawa et al. [13] reported that TO-336.vac also possesses a unique residue (arginine) at position 501 of P150. However, this residue was not found in our analyses. The TO-336.vac strain used in the present study possessed the same residue as TO-336.GMK5 and TO-336wt at that position.

3.4. Matsuura.vac has acquired 19 amino acid substitutions under the attenuation process

From the nucleotide sequence data, 19 amino acid differences were predicted between Matsuura.B3 and Matsuura.vac

A

		MT																					
		3	42	235	271	289	295	320	323	362	407	427	433	466	473	483	491	503	514	529	541	551	555	562
Wt	M33	K	T	P	A	V	T	E	W	S	S	D	F	L	K	T	A	V	P	R	A	A	L	Y
	Matsuba.GMK5	K	T	P	A	L	T	E	R	S	S	D	F	L	K	T	A	V	P	R	A	A	L	Y
	Matsue.JPN68	K	T	P	A	V	T	E	R	S	G	D	F	F	K	T	A	A	P	R	A	A	L	Y
	TO-336wt	K	T	P	A	V	T	A	R	S	S	D	F	L	K	T	A	A	P	R	A	A	L	C
	TO-336.GMK5	K	T	P	A	V	T	E	R	S	S	D	F	L	K	T	A	A	P	R	A	A	L	C
	Matsuura.B3	K	T	P	A	V	T	E	R	S	S	D	F	L	K	T	A	A	P	R	A	A	L	Y
	Therien	K	T	P	A	V	T	E	R	C	G	D	L	L	K	T	E	V	Q	R	A	A	P	Y
Vac	Matsuba.vac	K	T	P	A	V	A	E	R	S	S	D	F	L	K	T	A	A	P	R	A	A	L	Y
	KRT	K	T	P	A	V	A	E	R	S	S	D	F	L	K	A	A	A	P	R	A	A	L	Y
	TO-336.vac	K	T	P	A	V	T	E	R	S	S	D	F	L	K	T	A	A	P	R	A	A	L	C
	Matsuura.vac	K	T	S	V	V	T	E	R	S	S	D	F	L	K	T	A	A	P	R	A	V	L	Y
	TCRB19	K	T	P	A	V	T	E	R	S	S	D	F	L	Q	T	A	V	P	R	A	A	L	Y
	RA27/3	R	S	P	A	V	T	E	R	S	S	G	F	L	K	T	A	V	P	H	V	A	L	Y

		573	584	604	606	650	672	674	697	699	702	704	715	717	720	722	732	739	740	751	756	758	767	772
Wt	M33	H	G	R	Y	W	K	I	R	T	E	N	G	L	T	P	R	H	S	V	R	Q	V	P
	Matsuba.GMK5	H	E	R	F	W	Q	I	G	A	D	N	G	S	T	S	R	H	L	A	P	A	A	P
	Matsue.JPN68	H	E	R	F	W	Q	I	G	A	D	N	G	S	T	P	R	H	L	A	P	A	A	P
	TO-336wt	H	E	R	F	W	Q	I	G	A	D	H	G	S	T	P	R	H	L	A	P	A	A	L
	TO-336.GMK5	H	E	R	F	W	Q	I	G	A	D	N	G	S	T	P	R	H	L	A	P	A	A	L
	Matsuura.B3	H	E	R	F	W	Q	I	G	A	D	N	G	S	T	P	R	H	L	A	P	A	A	P
	Therien	H	E	R	F	W	Q	I	G	A	D	N	R	S	A	P	R	H	S	A	P	A	A	P
Vac	Matsuba.vac	H	E	R	F	W	Q	V	G	A	D	N	G	L	T	P	R	P	S	V	P	A	A	P
	KRT	H	E	R	F	W	Q	V	G	A	D	N	G	L	T	P	R	P	S	V	P	A	A	P
	TO-336.vac	Y	E	R	F	W	Q	I	G	A	D	N	G	S	T	P	R	H	L	A	P	A	A	L
	Matsuura.vac	H	E	R	F	R	K	I	G	A	D	N	G	S	T	P	R	H	L	A	P	A	A	P
	TCRB19	H	E	C	F	R	Q	I	G	A	D	N	G	P	T	P	R	H	S	A	P	A	A	P
	RA27/3	H	E	R	F	W	Q	I	G	A	D	N	G	S	T	S	C	H	S	A	P	A	A	P

											X								Pro					
		774	775	777	784	790	791	795	799	801	865	874	900	907	930	958	961	963	1002	1007	1020	1042	1115	1117
Wt	M33	T	T	E	H	V	Y	G	S	K	D	I	R	E	R	A	A	R	S	D	A	Y	Q	M
	Matsuba.GMK5	T	S	G	H	A	Y	D	P	K	D	T	Q	E	C	A	A	R	T	D	A	Y	Q	M
	Matsue.JPN68	T	S	G	H	A	Y	D	S	K	D	T	R	E	C	A	A	R	S	D	A	Y	Q	M
	TO-336wt	T	S	G	H	A	Y	D	P	K	D	T	R	E	C	A	A	R	S	D	A	Y	Q	M
	TO-336.GMK5	T	S	G	H	A	Y	D	P	K	D	T	R	E	C	A	A	R	S	D	A	Y	Q	M
	Matsuura.B3	T	S	G	H	A	Y	D	P	K	D	T	R	E	C	A	A	R	S	D	T	Y	Q	M
	Therien	I	P	G	D	A	C	G	S	R	N	T	R	E	C	T	V	R	S	D	A	Y	H	M
Vac	Matsuba.vac	T	S	E	H	V	Y	G	S	K	D	T	R	E	C	A	V	R	S	G	A	H	Q	M
	KRT	T	S	E	H	V	Y	G	S	K	D	T	R	E	C	A	V	R	S	G	A	H	Q	M
	TO-336.vac	T	S	G	H	A	Y	D	P	K	D	T	R	E	C	A	A	R	S	D	A	Y	Q	M
	Matsuura.vac	T	S	G	H	A	Y	D	P	K	D	T	R	Q	C	A	A	C	S	D	T	Y	Q	M
	TCRB19	T	S	G	D	A	Y	G	S	K	D	T	R	E	C	T	A	R	S	D	A	C	Q	M
	RA27/3	T	S	G	D	A	Y	G	S	K	D	T	R	E	R	T	A	R	S	D	A	Y	Q	V

		Pro									Hel								RdRp				
		1140	1154	1159	1166	1177	1190	1191	1199	1209	1337	1351	1393	1403	1466	1497	1559	1583	1639	1767	1815	1921	1979
Wt	M33	V	L	N	A	K	H	E	W	P	I	N	D	A	G	T	T	L	R	T	A	E	N
	Matsuba.GMK5	V	L	N	A	K	H	E	R	P	V	N	D	A	E	T	T	S	R	A	A	E	S
	Matsue.JPN68	V	L	N	A	K	H	E	R	P	V	N	D	A	E	T	T	S	R	A	A	E	S
	TO-336wt	V	F	N	A	K	H	E	R	P	V	N	D	A	E	T	V	S	R	A	A	E	S
	TO-336.GMK5	V	L	N	A	K	H	E	R	P	V	N	D	A	E	T	T	S	R	A	A	E	S
	Matsuura.B3	V	L	N	A	K	H	E	R	P	V	N	D	A	E	T	T	S	R	A	A	E	S
	Therien	A	L	N	A	K	H	E	R	P	V	N	D	R	E	T	T	S	R	A	A	E	S
Vac	Matsuba.vac	V	L	N	A	K	H	E	R	P	V	N	D	A	E	I	T	S	C	A	A	E	S
	KRT	V	L	N	A	K	H	E	R	P	V	N	D	A	E	I	T	S	R	A	A	E	S
	TO-336.vac	V	L	S	A	K	H	E	R	P	V	D	D	A	E	T	T	S	R	A	A	E	S
	Matsuura.vac	V	L	N	V	K	H	E	R	P	V	N	D	A	E	T	T	S	R	A	T	D	S
	TCRB19	V	L	N	A	K	R	E	R	P	V	N	D	A	E	T	T	S	R	A	A	E	S
	RA27/3	V	L	N	A	R	H	K	R	S	V	N	E	A	E	T	T	S	R	A	A	E	S

Fig. 4. Comparison of the amino acid residues among seven RuV wt strains and six vaccines. The amino acid sequences were compared among 13 RuV strains (seven wt strains and six vaccines), and residues with variations were noted. (A) NSPs. (B) SPs. Shaded symbols indicate amino acid residues in minority groups among the 13 strains. Numbers indicate the amino acid positions. Known or predicted functional domains [20,22,44,45] are indicated in the top rows. MT: methyltransferase domain; X: X domain; Pro: protease domain; Hel: helicase domain; RdRp: RNA-dependent RNA polymerase domain; C_{SP}: signal peptide of the capside protein; $E2_{TM}$: transmembrane domain of E2; $E1_{TM}$: transmembrane domain of E1.

B

		C																			
		11	18	22	26	34	48	60	64	67	69	71	72	76	87	95	141	175	226	254	260	283
Wt	M33	D	A	A	G	S	T	P	R	A	A	R	K	R	S	K	L	I	T	T	H	A
	Matsuba.GMK5	D	A	A	E	S	S	R	G	G	G	R	R	R	S	K	L	T	T	S	H	V
	Matsue.JPN68	A	A	A	E	S	S	R	G	G	G	R	R	R	S	K	L	T	T	S	H	A
	TO-336wt	D	A	A	E	S	S	R	G	G	G	L	R	R	S	K	L	T	T	S	H	A
	TO336.GMK5	D	A	A	E	S	S	R	G	G	G	L	R	R	S	K	L	T	T	S	H	A
	Matsuura.B3	D	A	A	E	S	S	R	G	G	G	R	R	R	S	K	L	T	T	S	H	A
	Therien	D	A	A	E	S	S	R	G	G	G	R	R	R	T	K	L	T	T	S	H	A
Vac	Matsuba.vac	G	A	A	E	P	S	R	G	G	G	R	K	R	S	K	L	T	T	S	H	A
	KRT	G	A	A	E	P	S	R	G	G	G	R	K	R	S	K	L	T	S	S	H	A
	TO-336.vac	D	A	A	E	S	S	R	G	G	G	L	R	R	S	K	L	T	T	S	H	A
	Matsuura.vac	D	A	V	E	S	S	R	G	G	G	R	R	K	S	K	P	T	T	S	Y	V
	TCRB19	D	A	A	E	S	S	R	G	G	G	R	R	R	S	E	L	T	T	S	H	A
	RA27/3	D	T	A	E	S	T	R	G	G	G	R	R	R	T	K	L	T	T	S	H	A

		C_p	E2															$E2_{ns}$		E2	$E2_{ns}$
		292	306	307	313	314	319	361	404	405	411	412	413	422	446	486	491	505	534	535	539	558
Wt	M33	A	A	D	M	P	R	H	S	L	Y	I	A	P	Y	I	E	T	S	L	F	A
	Matsuba.GMK5	A	A	D	T	L	R	H	P	L	S	T	T	A	H	V	E	T	S	L	V	A
	Matsue.JPN68	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	E	T	S	L	L	A
	TO-336wt	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	E	T	S	L	L	A
	TO336.GMK5	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	E	T	S	L	L	A
	Matsuura.B3	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	E	T	S	L	L	A
	Therien	T	A	D	T	L	G	Y	P	L	S	T	T	A	H	V	E	A	S	L	L	A
Vac	Matsuba.vac	A	V	H	T	P	R	H	S	L	S	T	T	P	H	V	E	T	P	P	L	A
	KRT	A	V	H	T	P	R	H	S	L	S	T	T	P	H	V	E	T	P	P	L	A
	TO-336.vac	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	E	T	S	L	L	A
	Matsuura.vac	A	A	D	T	L	R	H	P	F	S	T	T	A	H	V	G	T	S	L	L	A
	TCRB19	A	A	D	T	L	R	H	P	L	S	T	T	A	H	V	E	T	S	L	L	A
	RA27/3	A	A	D	T	L	R	H	P	L	S	T	T	A	H	V	E	T	S	L	L	T

		E1																		$E1_{hy}$
		587	599	609	650	756	759	785	792	801	873	890	915	919	953	954	959	962	980	991	1041
Wt	M33	T	T	G	T	V	N	L	Y	G	I	H	A	A	T	V	V	F	Q	T	P
	Matsuba.GMK5	T	T	R	T	V	N	L	Y	G	I	H	A	A	T	V	V	F	Q	S	L
	Matsue.JPN68	T	T	R	T	V	N	L	Y	G	I	H	A	A	T	V	V	F	Q	S	L
	TO-336wt	T	T	R	T	A	N	L	Y	G	I	H	A	A	T	V	V	F	Q	S	L
	TO336.GMK5	T	T	R	T	V	N	L	Y	G	I	H	A	A	T	V	V	F	Q	S	L
	Matsuura.B3	T	T	R	T	V	N	L	Y	G	I	H	A	A	T	V	V	F	Q	S	L
	Therien	T	A	R	A	V	N	L	Y	G	I	H	A	T	T	V	L	V	Q	T	P
Vac	Matsuba.vac	A	T	R	T	V	D	M	Y	G	I	H	T	A	T	V	V	F	Q	S	L
	KRT	A	T	R	T	V	D	M	Y	G	I	H	T	A	T	V	V	F	R	S	L
	TO-336.vac	T	T	R	T	A	N	L	Y	G	I	R	A	A	T	L	V	F	Q	S	L
	Matsuura.vac	T	T	R	T	A	D	L	Y	S	I	H	A	A	T	V	V	F	Q	S	L
	TCRB19	T	A	R	T	V	N	L	Y	G	M	H	A	A	A	V	V	F	Q	S	L
	RA27/3	T	A	R	T	V	N	L	H	G	I	H	A	T	T	L	I	F	Q	S	L

Fig. 4. (Continued).

(Figs. 3 and 4). Except for the A1020T substitution in P150, Matsuura.B3 possessed the identical amino acid sequence to the consensus sequence among the other three wt strains, TO-336.GMK5, RVi/Matsue.JPN/68, and Matsuba.GMK3, in the same cluster (Figs. 2 and 4). These data suggest that Matsuura.vac acquired the 19 amino acid substitutions under the attenuation process (Fig. 3). Eight of these substitutions were in P150 (Figs. 3 and 4). Among these substitutions, P235S was in the methyltransferase (MT) domain [20,22], E907Q and R963C were in the X domain with unknown functions [20,21], and A1166V was in the protease domain [20–23] (Fig. 4A). A1166V was also located in the helical structure of the CaMBD (amino acid positions 1152–1183) in the protease domain [24]. Two substitutions (A1815T and E1921D) were found in P90, and both were located in the RNA-dependent RNA polymerase domain [20,21] (Fig. 4A). The C, E2, and E1 SPs had five, one, and three substitutions, respectively (Fig. 4B).

3.5. No common substitutions are found in vaccine strains, but some substitutions are shared by specific vaccine strains

Matsuba.vac and KRT shared many amino acid substitutions (Fig. 4). TCRB19 and RA27/3 also shared some amino acid substitutions (Fig. 4). However, despite these common features of the Japanese rubella vaccine strains, no consensus amino acid changes were found among them (Fig. 4). Regarding the molecular determinant for the ts phenotype, Sakata et al. [12] suggested that a Y1042H substitution in P150 is responsible for the ts phenotype of KRT. Matsuba.vac possessed the same substitution (Fig. 4A). Interestingly, TCRB19 also had a tyrosine-to-cysteine substitution at the same position (Fig. 4A). Therefore, KRT, Matuba.vac, and TCRB19 may exhibit the ts phenotype via the same molecular mechanism.

3.6. TO-336.vac has acquired wt phenotypes by second-site mutations in the protease domain of P150

A TO-336.vac-derived mutant clone, designated TO-336.rev, was generated by passages of TO-336.vac in RK13 cells at 39 °C. Unlike TO-336.vac, TO-336.rev was able to replicate in RK13 cells at 39 °C, and its virus titer at 5 days p.i. was as high as that of TO-336.GMK5 (Fig. 5). The entire genome sequence of TO-336.rev was determined, and compared with that of TO-336.vac. A total of nine nucleotide substitutions were found in the genome of TO-336.rev (Table 1). Two of these substitutions were non-synonymous, and both were located in the protease domain of P150 (Table 1). These mutations caused asparagine-to-threonine and alanine-to-valine substitutions at amino acid positions 1126 and 1277 (N1126T

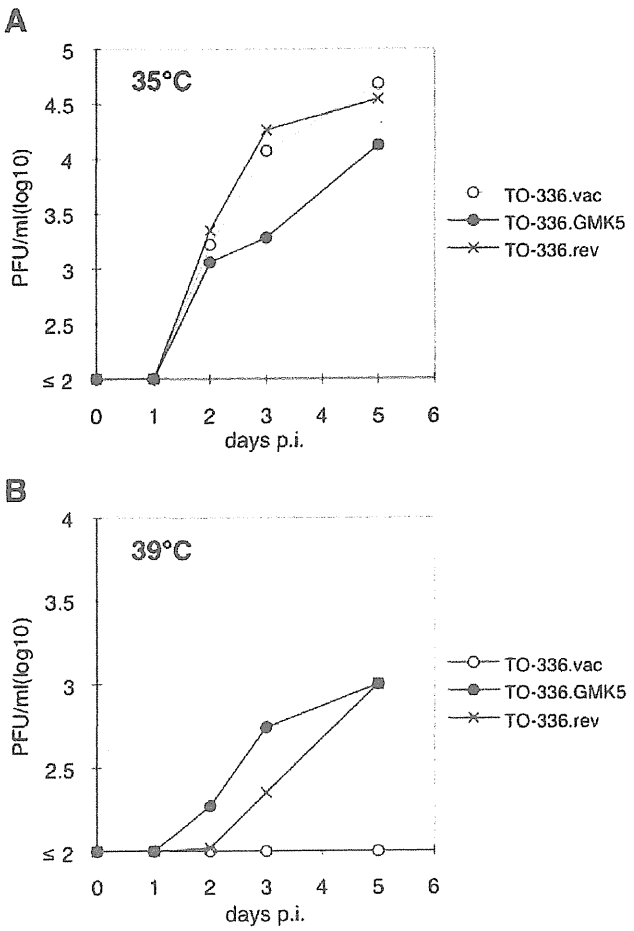

Fig. 5. Growth kinetics of TO-336.rev at 35 °C and 39 °C. RK-13 cells were infected with RuV strains at a MOI of 0.01 and incubated at 35 °C (A) or 39 °C (B). The infectious titers of the culture supernatants were determined by plaque assays. Closed and open circles indicate TO-336.GMK5 and TO-336.vac, respectively. Saltires indicate TO-336.rev.

and A1277V), respectively (Table 1). TO-336.vac possessed three amino acid substitutions in the NSPs compared with TO-336.GMK5 (Figs. 3 and 4A). Therefore, N1126T and A1277V were not direct reversions to the residues of TO-336.GMK5, but instead were second-site mutations that rendered TO-336.vac able to grow at 39 °C. These data brought the protease domain of the P150 protein to our attention.

3.7. Reduced stability of an NSP-derived peptide at 39 °C is correlated with reduced virus growth at that temperature (ts phenotype)

Chen et al. [16] analyzed the protease activities of RuV NSPs using NSP-derived peptides of varying lengths. We used one of these NSP-derived peptides corresponding to amino acid positions 994–1548 possessing FLAG tags at the amino-terminus ($NSP_{994–1548}$). Since the peptide retains the ability to cleave itself behind the amino acid position 1301 [16], the anti-FLAG antibody was expected to detect both uncleaved and cleaved forms of the peptide. The $NSP_{994–1548}$ peptide of TO-336.vac (TO-336.vac-$NSP_{994–1548}$) and that containing the residues of TO-336.GMK5 at positions 1159 and 1351 (TO-336.vac-$NSP_{994–1548}$(S1159N/D1351N)) were expressed in cells at 35 °C or 39 °C (Fig. 6A, TO-336.vac and S1159N/D1351N). A TO-336.vac-$NSP_{994–1548}$ mutant (C1152S), which lacks the protease activity, was expressed as a control (Fig. 6A, C1152S). Both the TO-336.vac-$NSP_{994–1548}$ and TO-336.vac-$NSP_{994–1548}$(S1159N/D1351N) were cleaved efficiently, and much stronger signals were detected for cleaved forms, when compared to the signals for uncleaved forms, at both temperatures (Fig. 6A). These data showed that the protease activity of TO-336.vac is not significantly affected by the mutations at positions 1159 and 1351. However, it was noted that the expression level of TO-336.vac-$NSP_{994–1548}$ became lower than those of TO-336.vac-$NSP_{994–1548}$(S1159N/D1351N) at 39 °C (Fig. 6A). It was more evident, when expression levels were analyzed at 3 days posttransfection (Fig. 6B) than at 1 day posttransfection (Fig. 6A). TO-336.vac-$NSP_{994–1548}$ peptides with the residues of TO-336.rev at positions 1126 and/or 1277 also showed similar protease activities (Fig. 6C). These peptides with various mutations were expressed in cells at 35 °C, and subsequently cultured

Table 1
Nucleotide and amino acid differences between TO-336.vac and the related viruses.

Region	Nucleotide				Amino acid			
	Position	TO-336.GMK5	TO-336.vac	TO-336.rev	Position	TO-336.GMK5	TO-336.vac	TO-336.rev
5′ UTR	36	U	C	C	N/A			
P	448	U	C	C	136	–	–	–
150	1327	U	C	C	429	–	–	–
	1708	U	C	C	556	–	–	–
	1757	C	U	U	573	His	Tyr	Tyr
	3417	A	A	C	1126	Asn	Asn	Thr
	3516	A	G	G	1159	Asn	Ser	Ser
	3781	C	C	U	1247	–	–	–
	3793	C	C	A	1251	–	–	–
	3870	C	C	U	1277	Ala	Ala	Val
	3946	U	U	C	1302	–	–	–
P90	4091	A	G	G	1351	Asn	Asp	Asp
J-UTR	6463	C	C	A	N/A			
C	6583	C	U	U	24	–	–	–
	6649	C	U	U	46	–	–	–
	6958	C	U	U	149	–	–	–
E1	8778	U	C	C	756	Val	Ala	Ala
	9013	U	U	C	834	–	–	–
	9180	A	G	G	890	His	Arg	Arg
	9371	G	C	C	954	Val	Leu	Leu
3′	9712	U	U	C	N/A			
UTR	9742	C	C	U	N/A			

N/A: not applicable, –: silent mutation.

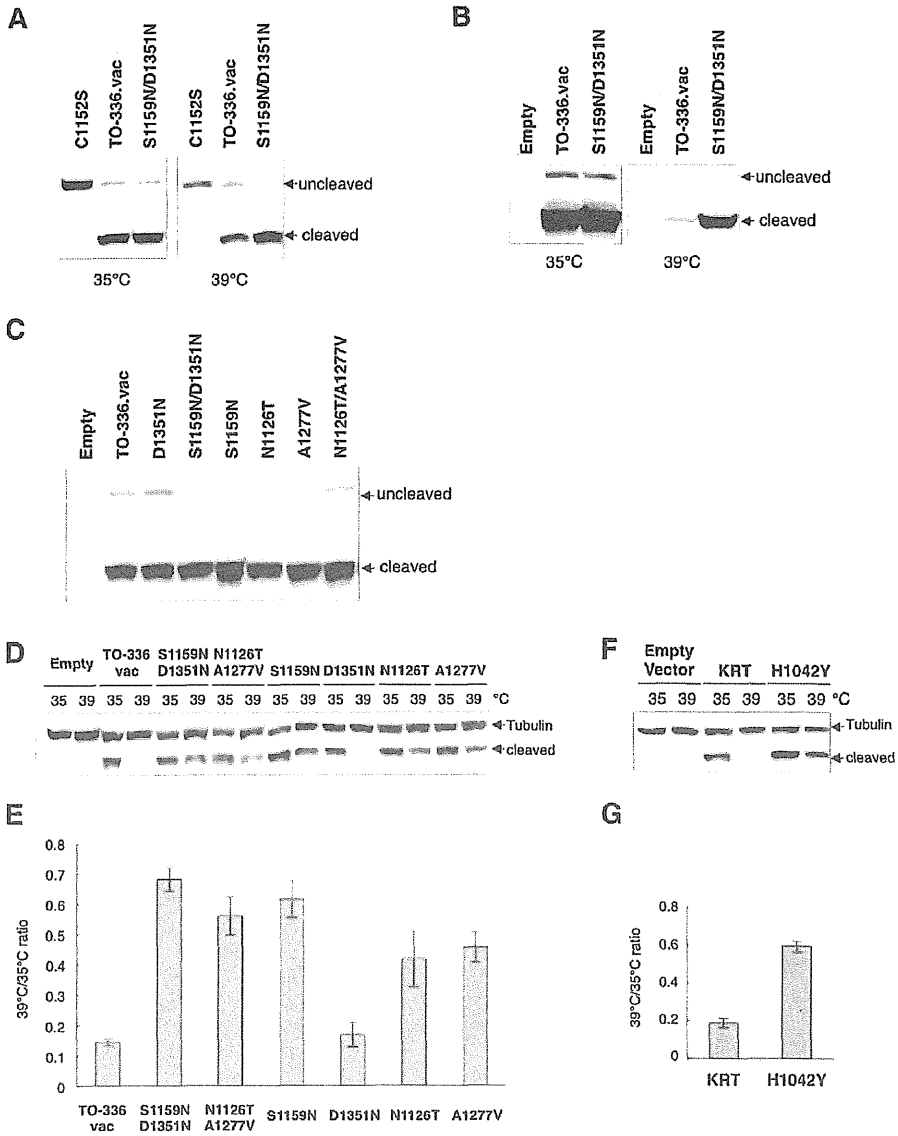

Fig. 6. Analysis of the thermal stabilities of NSP-derived peptides. (A and B) Using expression plasmids, an $NSP_{994-1548}$ peptide of TO-336.vac (TO-336.vac-$NSP_{994-1548}$) and that possessing the amino acid substitutions S1159N and D1351N (TO-336.vac-$NSP_{994-1548}$(S1159N/D1351N)) were expressed in cells at 35 °C or 39 °C. TO-336.vac-$NSP_{994-1548}$ possessing a C1152S mutation was also expressed as a control to show an uncleaved form of the peptide. Expression levels were analyzed at 1 day (A) and 3 days (B) after transfection of the plasmids. Empty; an expression plasmid lacking an $NSP_{994-1548}$ insert. (C) TO-336.vac-$NSP_{994-1548}$ peptides possessing the amino acid substitutions S1159N, D1351N, D1126T, and A1277 V individually or in combination were expressed in cells at 35 °C, and expression levels were analyzed at 1 day after transfection of the plasmids. Empty; an expression plasmid lacking an $NSP_{994-1548}$ insert. (D) An $NSP_{994-1548}$ peptide of TO-336.vac (TO-336.vac-$NSP_{994-1548}$) and those possessing the amino acid substitutions S1159N, D1351N, D1126T, and A1277 V individually or in combination was expressed in cells at 35 °C using expression plasmids. After 1 day of culture with the expression plasmids at 35 °C, the cells were cultured with cycloheximide to inhibit *de novo* protein synthesis for 2 days at 35 °C or 39 °C. Tubulin was detected as an internal control. Empty; an expression plasmid lacking an $NSP_{994-1548}$ insert. (E) Quantification of the data shown in (D). The ratios of the expression levels of the NSP peptides(cleaved form) at 39 °C and 35 °C are shown. The data represent the means ± standard errors of triplicate experiments. (F) $NSP_{994-1548}$ of KRT (KRT-$NSP_{994-1548}$) and that possessing an H1042Y substitution (KRT-$NSP_{994-1548}$(H1024Y)) were subjected to the same experiments described for (D). (G) Quantification of the data shown in (F). The data represent the means ± standard errors of triplicate experiments.

with cycloheximide to inhibit *de novo* synthesis of the peptides for 2 days at 35 °C or 39 °C (Fig. 6D). Fig. 6E shows the ratios of expression levels of the peptide ($NSP_{994-1301}$) at 39 °C and 35 °C (39 °C/35 °C ratios). Only cleaved forms, $NSP_{994-11301}$, were shown, since uncleaved forms were barely detectable. These data showed that TO-336.vac-$NSP_{994-1301}$ was unstable at 39 °C (Figs. 6D and E, TO-336.vac), whereas TO-336.vac-$NSP_{994-1301}$(S1159N/D1351N) was stable (Figs. 6D and 7E, S1159N/D1351N). A peptide containing the residues of TO-336.rev at positions 1126 and 1277 (TO-336.vac-$NSP_{994-1301}$(N1126T/A1277V)) also exhibited high stability at 39 °C (Figs. 6D and E, N1126T/A1277V). The effects of individual mutations at positions 1159, 1351, 1126, and 1277 were analyzed. The data revealed that the peptide possessing S1159N was as stable as TO-336.vac-$NSP_{994-1301}$(S1159N/D1351N) (Figs. 6D and E, S1159N). The D1351N substitution had no effect on the stability of $NSP_{994-1301}$ at 39 °C (Figs. 6D and E, D1351N). These data suggest that the N1159S mutation causes the ts phenotype of TO-336.vac, and that N1351D only exerts a neutral effect. On the other hand, both types of peptides with N1126T or A1277V showed moderate stability at 39 °C (Figs. 6D and E, N1126T and A1277V). These data

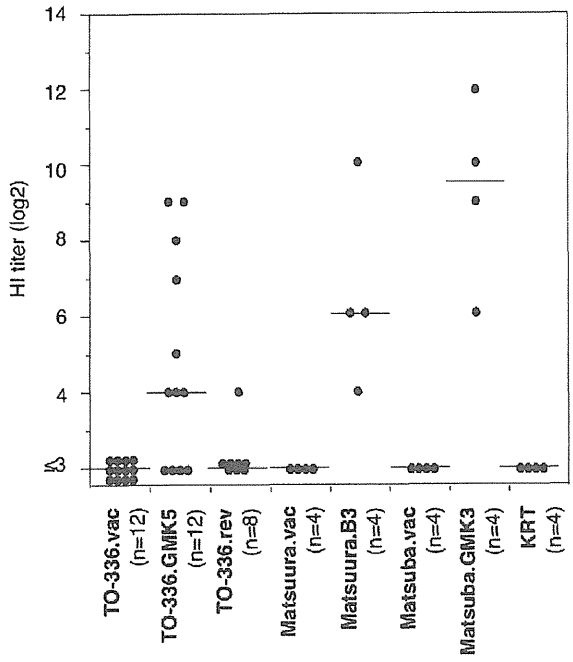

Fig. 7. Production of HI antibodies in guinea pigs inoculated with RuV strains. Guinea pigs (4, 8, or 12 animals per RuV strain) were subcutaneously inoculated with 5000 PFU of RuV. The serum HI antibody titers were measured at 5 weeks p.i. The horizontal bars indicate the median values of the HI antibody titers of the animals inoculated with each RuV strain.

suggest that both of the mutations contribute to the phenotypic reversion of TO-336.rev.

The Y1042H substitution in P150 is known to be responsible for the ts phenotype of KRT [12]. The $NSP_{994-1548}$ peptide of KRT (KRT-$NSP_{994-1548}$) was also subjected to the expression analysis. A strong signal for KRT-$NSP_{994-1301}$ was detected in cells incubated at 35 °C, while only a faint signal was detected in cells incubated at 39 °C (Figs. 6F and G, KRT). A histidine residue at amino acid position 1042 of NSP was replaced with tyrosine, the wt residue. This mutation (H1042Y) made the KRT-$NSP_{994-1301}$ stable at 39 °C (Figs. 7F and G, H1042Y). Therefore, the amino acid residues at 1042, 1126, 1159, and 1277, which are located in the protease domain, seem to be individually involved in the thermal stability of $NSP_{994-1301}$.

Collectively, the data suggest that substitutions in the protease domain may be predisposed to cause thermal lability of the NSPs, conferring the ts phenotype on some rubella vaccine strains.

3.8. A high growth capacity of RuV in cultured cells at a high temperature is neither essential nor sufficient to elicit antibody responses in guinea pigs

Japanese rubella vaccines lack the ability to elicit anti-RuV antibodies in experimentally infected guinea pigs and rabbits [6]. This is used as an *in vivo* marker phenotype of Japanese rubella vaccine strains [6]. According to the protocol for a marker test of rubella vaccines documented in the MRBP [8,9], 5000 PFU of each wt or vaccine strain was inoculated subcutaneously into guinea pigs, and their HI antibody titers were analyzed at 5 weeks after the inoculation. HI antibody titers were undetectable in the sera of all animals inoculated with vaccine strains (TO-336.vac, Matsuura.vac, Matsuba.vac, and KRT) (Fig. 7). The TO-336.GMK5 and Matsuura.B3 strains induced anti-RuV antibody responses in 66.7% and 100% of the inoculated animals, respectively (Fig. 7). It was noteworthy that all of the animals inoculated with Matsuba.GMK3, which showed a moderate ts phenotype (Fig. 1B), produced high HI titers (Fig. 7). The median value of the HI titers induced by Matsuba.GMK3 was even higher than those induced by TO-336.GMK5 and Matsuura.B3 (Fig. 7). The reversion mutant, TO-336.rev, which was able to replicate at a high temperature (Fig. 5B), hardly induced any antibody responses in the animals (Fig. 7). Most of the animals were seronegative, and only one of eight animals showed a low HI titer (Fig. 7). These findings demonstrate that the ability of RuV to grow at a high temperature was not necessarily correlated with the potency to elicit humoral immune responses in guinea pigs.

4. Discussion

Many vaccine strains for live attenuated vaccines have been successfully generated by adaptation of clinical isolates through numerous passages in various cultured cells [6,25–28]. During this process, the viruses have often been propagated at low temperatures (29–35 °C), and have acquired the ts phenotype [6,26,28]. Although these adaptations often reduce viral virulence, the molecular mechanisms of the attenuation have been poorly elucidated. Comparisons of nucleotide and amino acid sequences between vaccine strains and their progenitors provide basic and solid information toward understanding of the molecular bases that underlie the attenuation and the acquisition of other unique phenotypes of vaccine strains. In the present study, we determined the entire nucleotide sequences of the progenitors of currently used rubella vaccine strains. Unfortunately, the detailed records of the old isolates were unavailable. The passage histories of two viruses, however, could be predicted from their strain names, since the names of vaccine progenitors are usually designated on the basis of their passage history [6]. TO-336.GMK5 and Matsuba.GMK3 seemed to have been isolated in GMK cells and passaged in these cells five and three times, respectively. However, the history of Matsuura.B3 was unclear. In addition to these viruses, the entire genome nucleotide sequences of three Japanese rubella vaccines (Matsuba.vac, TCRB19, and Matsuura.vac) were determined. Phylogenic analyses confirmed that TO-336.GMK5 and Matsuura.B3 were progenitors or closely related progenitors of the currently used TO-336.vac and Matsuura.vac strains, respectively, whereas Matsuba.GMK3 was apparently unrelated to the currently used Matsuba.vac strain. However, it could be the progenitor of a vaccine candidate that has not been licensed. Comparative analyses of these strains and other RuV strains provided full lists of the mutations introduced into the genomes of TO-336.vac and Matsuura.vac during their passages under laboratory conditions. Matsuura.vac had acquired greater number of amino acid substitutions than TO-336.vac. This may be caused by differences in the host cell types used to produce these vaccine strains and/or the numbers of passages in these cells. TO-336.vac was generated after seven passages in GMK cells, followed by 20 passages in guinea pig kidney cells and three passages in rabbit kidney cells at 29–32 °C [6,19]. Matsuura.vac was generated after 14 passages in GMK cells, 65 passages in chick embryo amniotic cavities, and 11 passages in Japanese quail embryo fibroblasts at 32–35 °C [6,19].

A single amino acid substitution, Y1042H, has been demonstrated to be responsible for the ts phenotype of the KRT vaccine strain [12]. This mutation is located in the protease domain of P150 [20]. TO-336.vac became able to grow at a high temperature by acquiring second-site mutations in the protease domain. Therefore, we focused on the mutations in the protease domain for determining the ts phenotype. The protease domain possesses a cysteine-rich Ca^{2+} and Zn^{2+}-binding domain, which is essential for the protease activity and virus replication [29,30]. This domain contains a CaMBD with an alpha-helical structure, which also plays important roles in the protease activity and virus