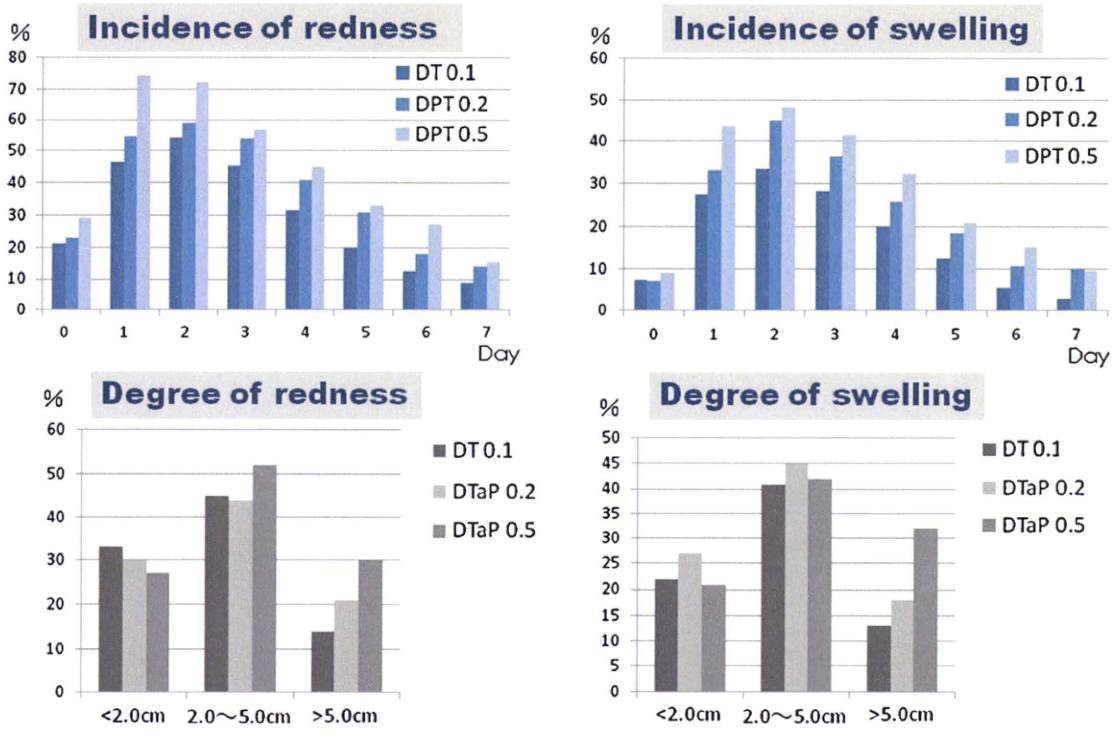

Fig. 4. Onset of local redness and swelling, and the severity of adverse events.

three groups, but 0.5 ml of DTaP had a tendency to induce a serious local reaction (redness and swelling) >5.0 cm.

3.4. Immunogenicity

Study group 1, in whom paired serum samples were examined, consisted of 266 subjects with serological examination, 29 with 0.1 ml of DT, 119 with 0.2 ml of DTaP, and 118 with 0.5 ml of DTaP. The sero-positivity of antibodies for diphtheria toxoid >0.1 was 60.9% (162/266), 90.6% (241/266) for tetanus toxoid >0.01, 54.13% (144/266) for PT >10, and 82.33% (219/266) for FHA > 10 EIA units. Antibodies against PT were markedly reduced at the age of 11–12 years.

The results of sero-positivity and GMT are shown in Table 4. The sero-positivity of PT and FHA and their GMT were the same before and after immunization in the DT 0.1 ml group. After immunization, the sero-positivity against PT increased from 52.1 to 95% in the DTaP 0.2 ml group and from 55.1 to 95.8% in the DTaP 0.5 ml group. The GMT of PT antibodies after immunization with 0.2 ml of DTaP was 89.05 (95% CI: 70.54–112.41), and there was no significant difference after immunization with 0.5 ml of DTaP, being 102.74 (95% CI: 82.91–127.32). Sero-positivity against FHA increased from 85.7 to 100% in the DTaP 0.2 ml group and from 78.8 to 98.3% in the DTaP 0.5 ml group. The GMT of antibodies against FHA was 252.82 (95% CI: 214.29–298.27) after immunization with 0.2 ml of DTaP and 302.06 (95% CI: 254.2–358.93) after immunization with 0.5 ml of DTaP, without a significant difference. Sero-positivity against diphtheria toxoid was 55.9–66.4% before immunization and increased to 100% in all three groups. The GMT of antibodies against diphtheria toxoid was 40.14 (95% CI: 28.28–56.96), 45.17 (95% CI: 35.59–57.32), and 46.78 (95% CI: 35.73–61.24) in the DT 0.1 ml, DTaP 0.2 ml, and DTaP 0.5 ml groups, respectively. As for the antibodies against tetanus toxoid, 86.2–94.1% sero-positivity before immunization increased to 100%. The GMT of antibodies against tetanus toxoid after vaccination with 0.2 ml of DTaP was 18.02 (95% CI: 14.90–21.80), similar to the 20.96 (95% CI: 13.37–32.84) after immunization with 0.1 ml of DT. However, the GMT of antibodies against tetanus toxoid was 27.12 (95% CI: 22.79–32.27) after immunization with 0.5 ml of DTaP, higher than those in DT 0.1 ml and DTaP 0.2 ml groups.

3.5. Difference in immunogenicity of different brands

There was no significant difference in immunogenicity against PT and FHA after immunization with 0.2 or 0.5 ml of DTaP. Risk ratios of a local reaction to 0.5 ml of DTaP compared to 0.1 ml of DT were higher than that to 0.2 ml of DTaP. GMTs after immunization with different brands of DTaP are shown in Fig. 5. A volume of 0.2 ml of DTaP contained 1.2–9.4 μg of PT, 9.4–20.6 μg of FHA, 6–6.6 Lf of diphtheria toxoid, and 1.0 Lf of tetanus toxoid. A volume of 0.1 ml of DT contains similar amounts of tetanus and diphtheria toxoid antigens in different brands and compared with 0.2 ml of each DTaP brand. 29 were immunized with 0.1 ml DT, 26 with 0.2 ml of Takeda DTaP, 26 with Biken, 19 with Kaketsu, 19 with Kitasato, and 29 with Denka. There was no significant difference in GMTs of antibodies against diphtheria toxoid after immunization with the five different brands in comparison with that induced after immunization with 0.1 ml of DT. The GMT against tetanus toxoid after immunization with Kitasato was higher than that after 0.1 ml of DT. As for the pertussis antigens, the GMT of PT antibodies after immunization with Takeda or Denka vaccine was lower than those induced after the other brands. These two brands contained lower amounts of PT antigen. The GMT against FHA after immunization with Denka was slightly lower than the others, not reflecting the concentration of vaccine material.

4. Discussion

Pertussis is an infectious disease affecting young infants and children, leading to severe illness in very young infants,

Table 4
Immunogenicity of DT and DTaP.

	DT 0.1 ml		DTaP 0.2 ml		DTaP 0.5 ml	
	Sero+ rate GMT pre (95% CI)	Sero+ rate GMT post (95% CI)	Sero+ rate GMT pre (95% CI)	Sero+ rate GMT post (95% CI)	Sero+ rate GMT pre (95% CI)	Sero+ rate GMT post (95% CI)
Anti-PT	58.6% 10.8 (6.38–18.29)	58.6% 13.93 (8.98–21.61)	52.1% 12.11 (9.21–15.94)	95% 89.05 (70.54–112.41)	55.1% 10.88 (8.27–14.32)	95.8% 102.74 (82.91–127.32)
Anti-FHA	82.8% 24.92 (16.34–38.00	86.2% 31.2 (22.43–43.42)	85.7% 33.73 (27.32–41.64	100% 252.82 (214.29–298.27)	78.8% 25.83 (20.67–32.28)	98.3% 302.06 (254.2–358.93)
Anti-D	58.6% 0.23 (0.11–0.471)	100% 40.14 (28.28–56.96)	66.4% 0.22 (017–0.30)	100% 45.17 (35.59–57.32)	55.9% 0.16 (0.12–0.24)	100% 46.78 (35.73–61.24)
Anti-T	86.2% 0.47 (0.28–0.81)	100% 20.96 (13.37–32.84)	94.1% 0.87 (0.70–1.09)	100% 18.02 (14.90–21.80)	88.1% 0.59 (0.44–0.79)	100% 27.12 (22.79–32.27)

causing whoop, staccato, apnea, and choking with sputa. To prevent the disease, acellular pertussis vaccines have been used in many developed countries. However, the acellular vaccine did not confer a long-lasting antibody response after vaccination and so in the late 1990s several pertussis outbreaks occurred in young adults [10–16]. The diagnosis of pertussis in adults was difficult because they only demonstrated mild atypical symptoms, showing a prolonged cough without whooping [24–26]. The adult patients showing a prolonged cough were not suspected to have pertussis because general physicians believed that pertussis was a disease only affecting children. They were, therefore, undiagnosed, and the number of patients with pertussis was underreported. In addition, they were not treated and transmitted pertussis to young infants before DTaP immunization [27]. The adult pertussis vaccine trial was conducted in 2781 subjects consisting of 1391 received the acellular pertussis vaccine and 1390 received the control vaccine. Ten patients of pertussis were diagnosed by culture, PCR, or serological responses and nine were in the control group and one in acellular pertussis vaccine group. An incidence of 370–450 cases per 100,000 person-years was noted in the control group aged 15–65 years and the acellular pertussis vaccine was protective in the same age group [28]. These adult patients with pertussis were considered to be an infectious source for transmission to young infants in household contact. Through such household contacts, even vaccinated children who had been completely immunized showed typical pertussis, and the most likely source of infant

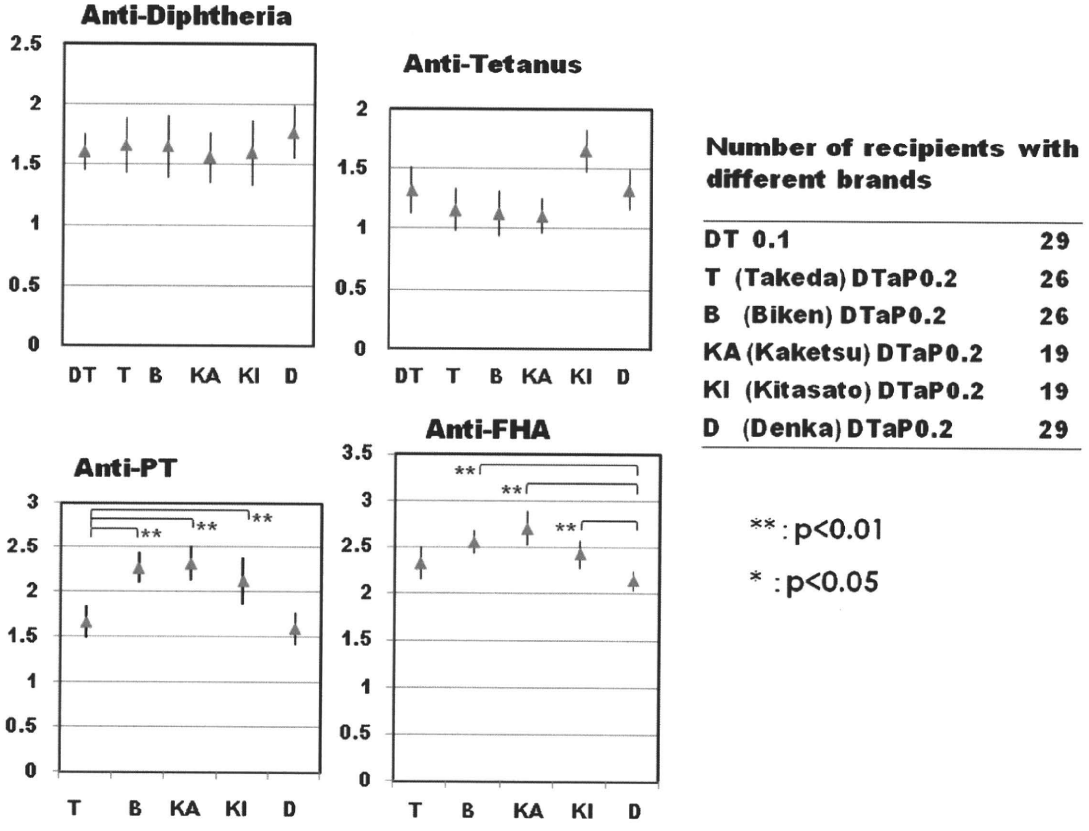

Fig. 5. GMTs of anti-D, T PT, and FHA antibodies after immunization with different brands of DTaP at 0.2 ml.

infection was reported, being a sibling (41%), mother (38%), and father (17%). To control pertussis, Tdap was developed and recommended as the booster in teenagers and young adults [15]. It is necessary to maintain a high level of immunity in all generations [29,30]. Thus, Tdap was newly recommended for all generations from 19 to 64 years as well as teenagers [17,18].

DTaP was first developed in Japan and has been used since 1981 [4]. Some pertussis patients were reported sporadically in Japan, and a survey of 89 households showed that the source of infection was an adult in approximately 11% and the secondary attack rate was 10%, confirmed by serological responses with asymptomatic infection [31]. The estimated efficacy of DTaP was 84% (95% CI: 71–91%) in children aged 2–8 years. Since vaccine-induced immunity waned 6–10 years after immunization, immunization with vaccines including pertussis components was proposed for both children and adults [32]. Adult patients with pertussis have gone undiagnosed and, therefore, the disease burden of pertussis has been neglected. In 2007–08, there were several outbreaks in universities, schools, and other facilities, and the number of reported cases of pertussis increased. Most of the patients were over 15 years of age and, the number of patients aged less than 1 year increased.

To control pertussis, an active immunization strategy should be implemented. Some ideas were considered to import Tdap, as well as change the immunization schedule. The immunization schedule of DTaP in Japan is 4 doses in young children only, being one or two times fewer doses in comparison with the schedule of DTaP in the EU and US. The components of Tdap (Adacel and Boostrix) were 2.5–8 μg of PT, 5–8 μg of FHA, 2.5–3 μg of pertactin, 2–2.5 Lf of diphtheria toxoid, and 5 Lf of tetanus toxoid. The five brands of DTaP in Japan have different formulations of components, as shown in Table 1. The B-type DTaP has only two components (Biken and Kaketsu) and T-type vaccines contain several other components besides PT and FHA (Takeda, Denka, and Kitasato). A dose of 0.1 ml of DT was scheduled at the age of 11–12 years. The concentration of tetanus toxoid in 0.2 ml of DTaP was similar to that in 0.1 ml of DT, but that of diphtheria toxoid was higher than that in 0.1 ml of DT. In comparison with Tdap used abroad, 0.2 ml of DTaP contained higher amounts of diphtheria toxoid and there was no significant difference in the incidence of adverse local reactions and serological response. Also, 0.2 ml of DTaP contains lower contents of tetanus toxoid and they induced efficient antibodies against tetanus toxoid. As for the antigen content of pertussis components, the PT antigen content varies from 1.2 to 9.4 μg, and the FHA content from 9.4 to 20.6 μg in 0.2 ml of different brands of DTaP. The GMT of antibodies against PT and FHA showed no significant difference after immunization with 0.2 or 0.5 ml of DTaP, but when comparing the GMT after immunization among different brands with different antigen concentrations, DTaP with higher antigen content did not always induce higher antibody titers. A lower-level serological response was observed in those immunized with a lower antigen content, but sero-positivity (protection levels > 10) was almost 100% after immunization with different bands of DTaP. DTaP with higher antigen content induced more marked serological responses at 4 years of age on booster immunization, but the difference was ten-times for PT antigen and five-times for FHA [33].

In the late 1990s, the resurgence of pertussis might have been associated with multi-factorial events: waning immunity, increased awareness, inappropriate vaccination schedule, improved diagnostic methods, and variant strains evading immunity acquired by immunization [8,34–36]. There have been several reports on mutation of the PT gene and it is still controversial which antigens are related to promoting immunity or reducing the severity of symptom [37,38]. Antibodies against PT reduced susceptibility to pertussis and those against pertactin or Fim2/3 were protective antibodies [39]. Protective immunity was considered to be induced by multiple components [40].

In many developed countries, the control of pertussis is complicated because of the difficulty in case identification, limited persistence of vaccine-acquired immunity, and transmission from unrecognized very mild patients or asymptomatic cases. In Japan, the number of pertussis patients has been increasing and resurgence in very young infant due to household contact was reported [41]. In this report, safe and effective immunization was achieved by 0.2 ml of DTaP instead of 0.1 ml of DT. The booster immunization with pertussis components should be implemented to achieve more effectively control the epidemiology of pertussis in Japan.

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

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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

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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

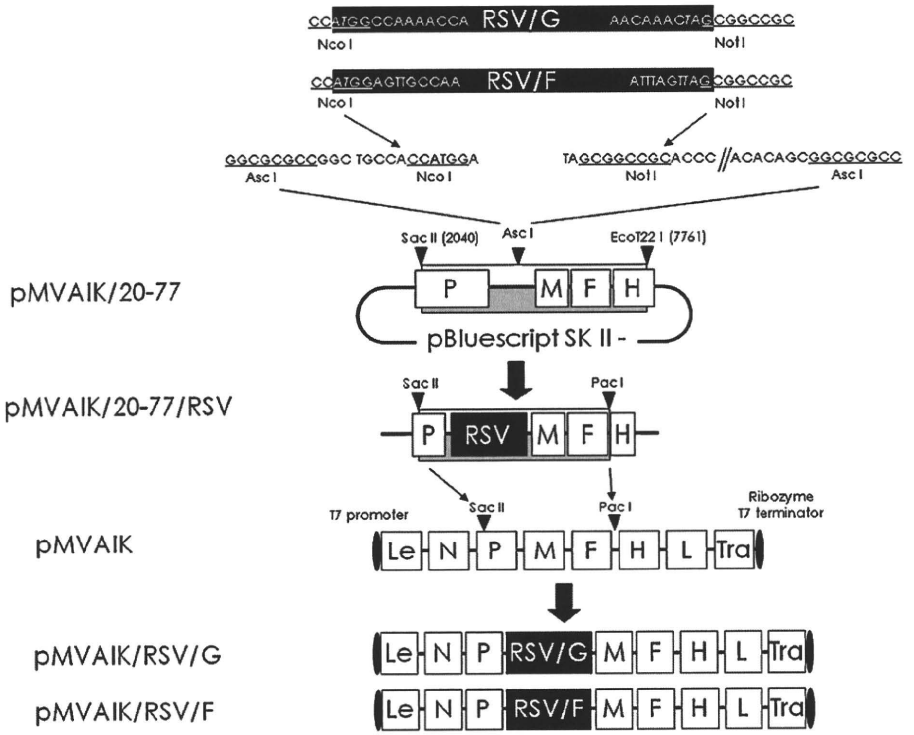

Fig. 1. Strategy for the construction of the recombinant AIK-C genome cDNAs having RSV protein genes. The recombinant AIK-C viral cDNAs expressing RSV G or F protein were constructed based on AIK-C cDNA (pMVAIK). pMVAIK/20-77 was constructed for the cloning of foreign genes. The Asc I site was introduced 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

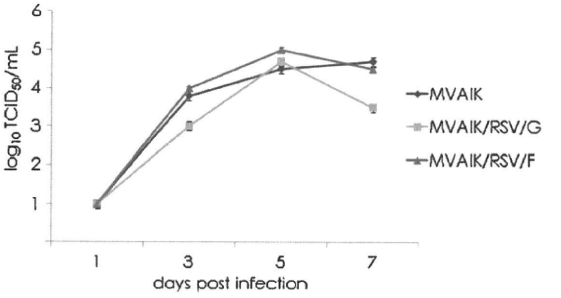

Fig. 2. Growth of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at m.o.i. of 0.02. Culture fluid was obtained on days 1, 3, 5, and 7 of culture at 33 °C. Infectivity is shown as mean titers of $TCID_{50}$/ml assayed in B95a cells. Error bars show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

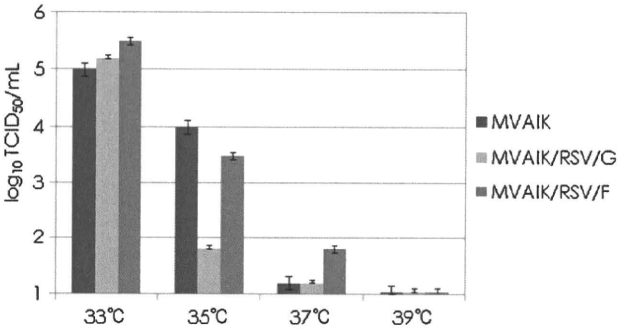

Fig. 3. Temperature sensitivity of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at a m.o.i. of 0.02. Culture fluid was obtained on day 5 and the infectivity at 33 °C, 35 °C, 37 °C, and 39 °C is shown as mean infectious titer ($TCID_{50}$/ml).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

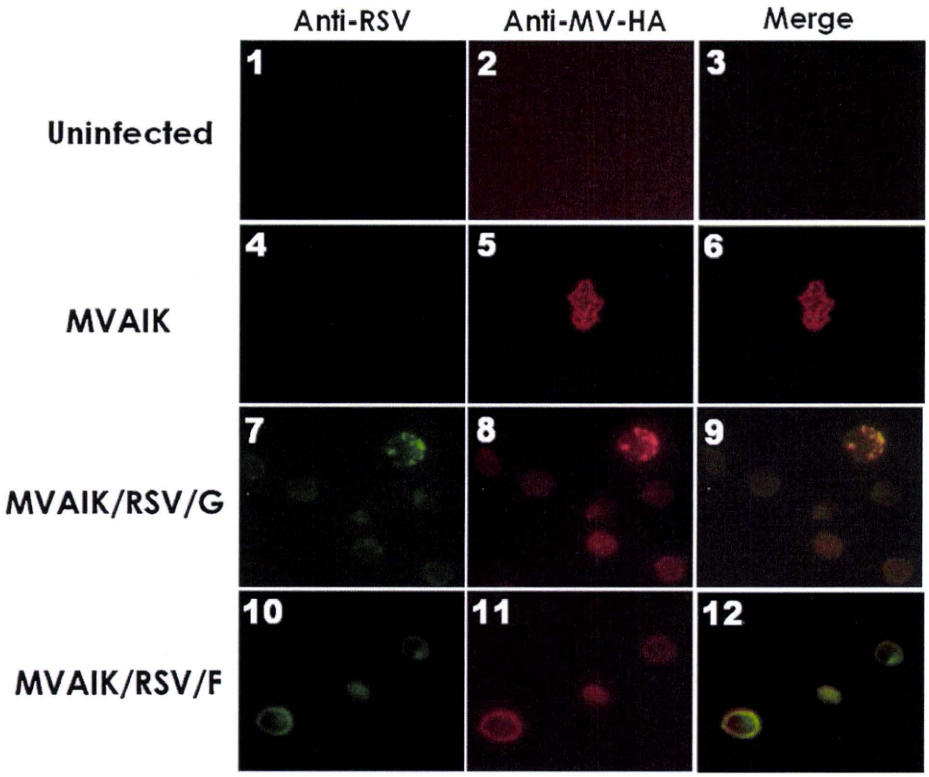

Fig. 4. Expression of MV HA and RSV G or F protein. B95a cells were infected with MVAIK (panels 4 and 5), MVAIK/RSV/G (panels 7 and 8) or MVAIK/RSV/F (panels 10 and 11) at a m.o.i. of 0.01 in 24-well plate and cultured for two days at 33 °C. Uninfected B95a cells are shown in panels 1 and 2. B95a cells 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.

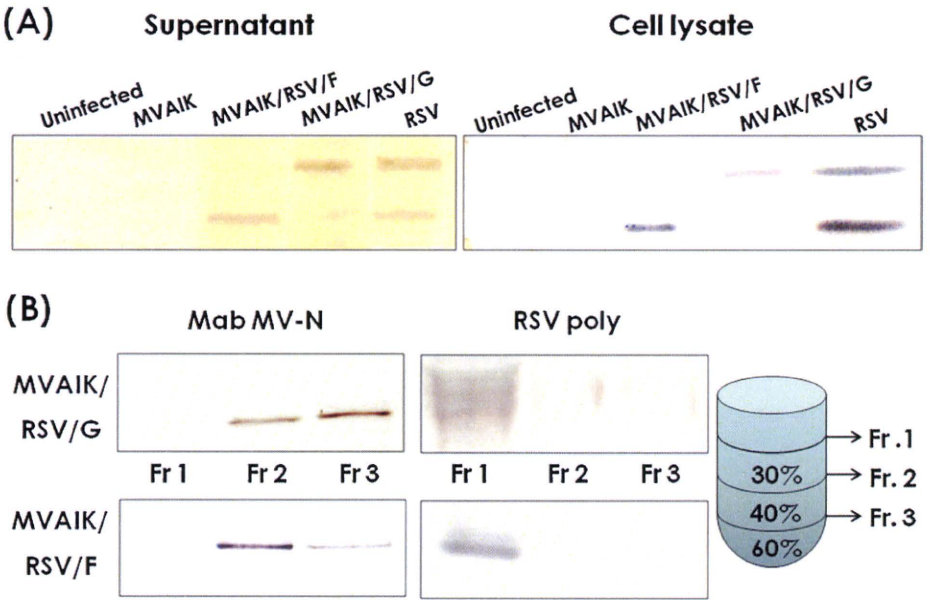

Fig. 5. Results of Western blotting of culture supernatant, cell lysate, and purified recombinant measles viral particles. (A) Vero cells were infected with MVAIK, MVAIK/RSVF, and MVAIK/RSV/G and HEp-2 cells were infected with RSV subgroup A, Long strain, and were cultured in 1 ml in a 24-well plate. 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.

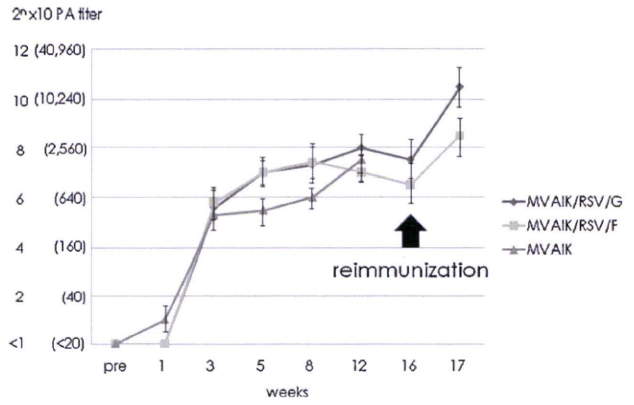

Fig. 6. Serological response of PA antibodies against MV. PA antibody titers were examined, using Serodia®-Measles. PA titers are expressed as $2^n \times 10$. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. Five 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 $2^{7.5 \pm 1.5}$ to $2^{10.5 \pm 1.5} \times 10$ in the MVAIK/RSV/G group, and from $2^{6.5 \pm 1.5}$ to $2^{8.5 \pm 1.5} \times 10$ 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 $10^{2.0 \pm 0.7}$. High titers were observed at the 5th week with a mean of $10^{2.6 \pm 1.0}$. 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.

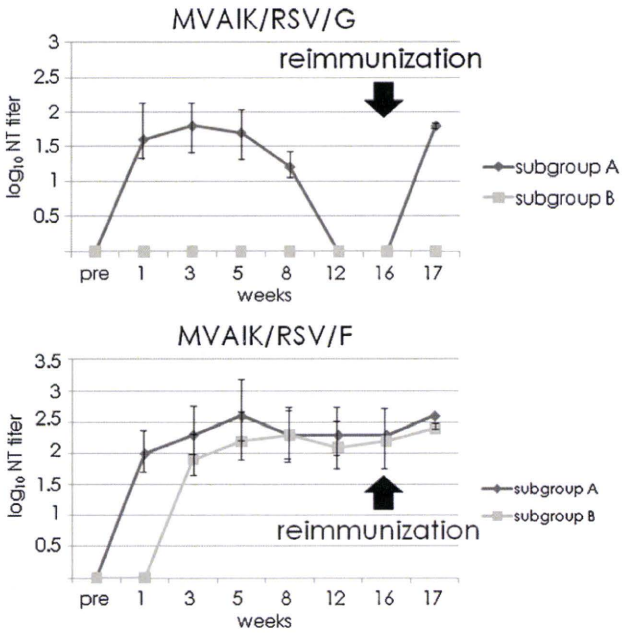

Fig. 7. Development of NT antibodies against RSV. NT antibodies were examined using the RSV Long strain (Subgroup A) and wild-type RSV subgroup B strain. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization. 50% plaque reduction NT titers are expressed 10^n 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 $10^{1.8 \pm 0.1}$, 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

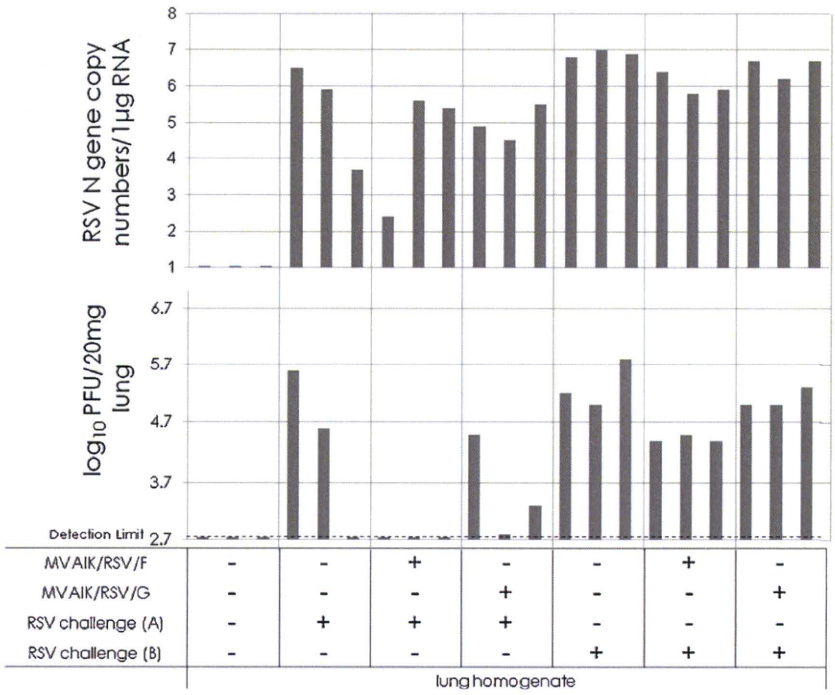

Fig. 8. Recovery of RSV infectious virus and genome copy numbers after challenge with RSV subgroups A and B. Three cotton rats were investigated in the normal control group, non-immunized group, and group immunized with MVAIK/RSV/F or MVAIK/RSV/G. Animals non-immunized group, and group immunized with 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

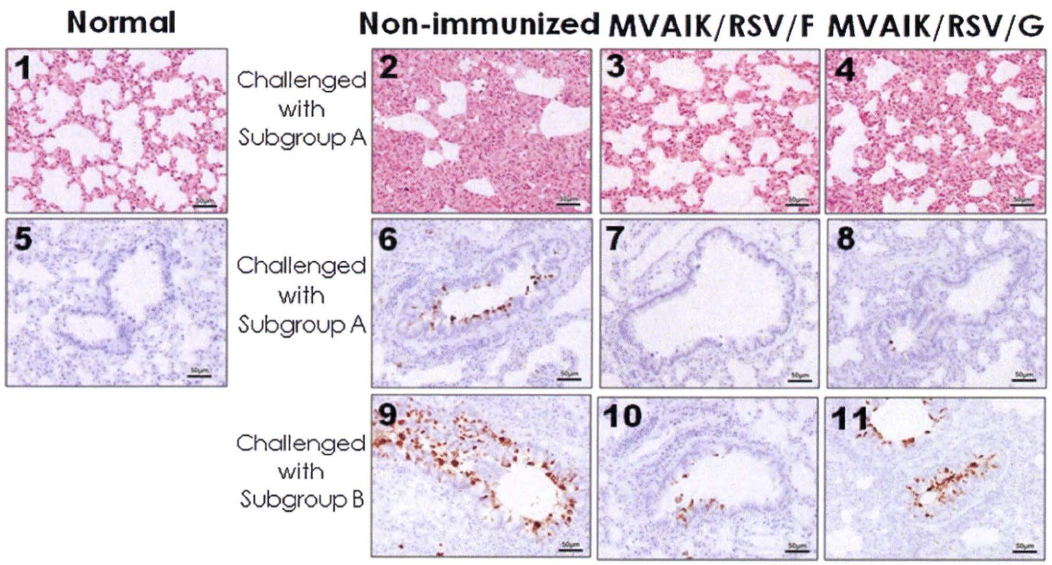

Fig. 9. Pulmonary histopathology in cotton rats challenged with RSV subgroups A and B. Cotton rats were immunized intramuscularly with MVAIK/RSV/F (panels 3, 7, and 10) or MVAIK/RSV/G (panels 4, 8, and 11) and then challenged five weeks later, with RSV subgroup A (panels 2, 3, 4, 6, 7, and 8) and subgroups 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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LETTERS
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Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines

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Nanotechnology is an innovative method of freely controlling nanometre-sized materials[1]. Recent outbreaks of mucosal infectious diseases have increased the demands for development of mucosal vaccines because they induce both systemic and mucosal antigen-specific immune responses[2]. Here we developed an intranasal vaccine-delivery system with a nanometre-sized hydrogel ('nanogel') consisting of a cationic type of cholesteryl-group-bearing pullulan (cCHP). A non-toxic subunit fragment of *Clostridium botulinum* type-A neurotoxin BoHc/A administered intranasally with cCHP nanogel (cCHP-BoHc/A) continuously adhered to the nasal epithelium and was effectively taken up by mucosal dendritic cells after its release from the cCHP nanogel. Vigorous botulinum-neurotoxin-A-neutralizing serum IgG and secretory IgA antibody responses were induced without co-administration of mucosal adjuvant. Importantly, intranasally administered cCHP-BoHc/A did not accumulate in the olfactory bulbs or brain. Moreover, intranasally immunized tetanus toxoid with cCHP nanogel induced strong tetanus-toxoid-specific systemic and mucosal immune responses. These results indicate that cCHP nanogel can be used as a universal protein-based antigen-delivery vehicle for adjuvant-free intranasal vaccination.

Beginning in 2003, an enormous research initiative—Grand Challenges in Global Health—has been organized worldwide with the support of the Bill and Melinda Gates Foundation and the US National Institutes of Health. Its aim is to overcome the global infectious disease problems affecting human health today[3]. The development of a new-generation needle-free mucosal vaccine has been proposed as one of the initiative's most important goals, because it can elicit antigen-specific systemic humoral and cellular immune responses and simultaneously induce mucosal immunity, especially in the aero-digestive and reproductive tracts[2–4]. FluMist, which is composed of cold-adapted trivalent live influenza viruses, is a well-known example as the first advanced intranasal vaccine to be used in US public health, in 2003 (ref. 5). Since then, tremendous efforts have been made to further develop intranasal vaccine technology. Subunit intranasal vaccination is expected to be the safest strategy, because it should have a low risk of causing unfavourable and undesired biological reactions[6]. However, intranasal administration of a subunit antigen alone is generally insufficient for induction of antigen-specific immune responses. As a result, an adjuvant such as a bacterial toxin generally needs to be added, but these toxins are poorly tolerated by humans[7].

Cholera toxin and heat-labile enterotoxin have been extensively used as potent mucosal adjuvants in experimental animal studies because of their multiple immune-potentiating functions: they activate immunocompetent cells, including dendritic cells and B cells, and thus induce antigen-specific mucosal immunity[7–9]. However, a human clinical trial carried out in Switzerland from 2000 to 2001 to develop an intranasal influenza vaccine with inactivated influenza virus combined with a small amount of heat-labile enterotoxin was withdrawn because the co-administered heat-labile enterotoxin was suspected of causing Bell's palsy, a rare condition, in vaccinated subjects[10]. In addition, a separate study in mice demonstrated that the toxin-based adjuvant migrated into, and accumulated in, the olfactory tissues[11]. As a result of these safety issues, the development of intranasal vaccines employing the co-administration of toxin-based adjuvants has rapidly declined. Further scientific and technological innovations that will help the development of safe but effective adjuvant-free intranasal vaccines are, therefore, of high priority in global health.

Application of biomaterials, such as polymer nanoparticles and liposomes, has a great potential in vaccine development and immunotherapy[12–14]. In particular, nanometre-sized (<100 nm) polymer hydrogels (nanogels) have attracted growing interest as nanocarriers, especially in drug-delivery systems[15,16]. We have developed a new method of creating a series of functional nanogels through self-assembly of associating polymers[17]. One of these polymers, the cholesteryl-group-bearing pullulan (CHP) forms physically crosslinked nanogels by self-assembly in water[18–22] (Fig. 1a and Supplementary Fig. S1). The CHP nanogels trap various proteins by mainly hydrophobic interactions[23] and acquire chaperon-like activity because the proteins are trapped inside a hydrated nanogel polymer network (nanomatrix) without aggregating and are gradually released in the native form[20,24]. These properties make the CHP nanogel a superior nanocarrier for protein delivery, especially in the area of cancer vaccine development[25,26]. In fact, recent successful clinical studies have clearly shown that subcutaneous injection of CHP nanogel carrying the cancer antigen HER2 (CHP–HER2) or NY-ESO-1 (CHP–NY-ESO-1) effectively

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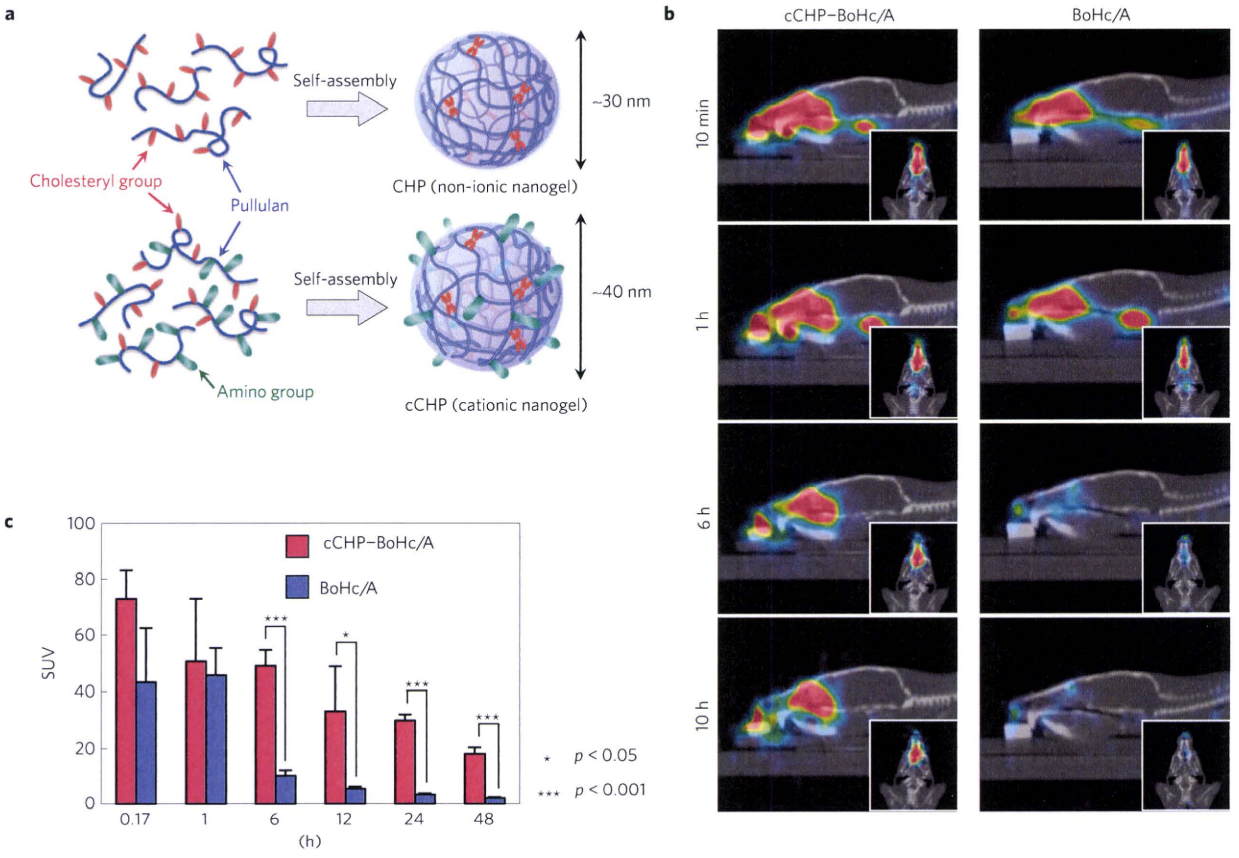


Figure 1 | Use of cCHP nanogel as a new antigen-delivery vehicle for intranasal vaccination. a, cCHP nanogel was generated from a cationic type of cholesteryl-group-bearing pullulan. **b**, Superimposition of sagittal and transverse (photo insets) PET images on the corresponding computed tomography images showed that intranasally administered cCHP nanogels carrying [^{18}F]-labelled BoHc/A were effectively delivered to the nasal mucosa. **c**, Direct quantitative study with [^{111}In]-labelled BoHc/A further demonstrated that BoHc/A was retained in the nasal tissues for more than two days after intranasal immunization with cCHP nanogel. In contrast, most naked BoHc/A disappeared from the nasal cavity within 6 h after administration.

induces antigen-specific CD8$^+$ cytotoxic T lymphocyte responses and antibody production[21,22]. Therefore, the technological successes have been extended to the use of a CHP nanogel strategy to develop adjuvant-free intranasal vaccines that can induce antigen-specific protective immunity against infectious diseases.

To demonstrate the effectiveness of CHP nanogel as a new vehicle for adjuvant-free intranasal vaccines, we prepared and used an *Escherichia-coli*-derived recombinant non-toxic receptor-binding fragment (heavy-chain C terminus) of *C. botulinum* type-A neurotoxin subunit antigen Hc (BoHc/A) as a prototype vaccine antigen because the immunogenicity of BoHc/A has already been demonstrated elsewhere[27,28]. In the initial study for evaluation of BoHc/A quality, because the antigen was highly purified, only a negligible amount of endotoxin with no *in vivo* biological effects on immunocompetent cells was detected (Supplementary Table S1; ref. 29). *C. botulinum* has been defined as a category A bioterrorism agent by the US Centers for Disease Control and Prevention because of the strong neural toxicity of *C. botulinum*-producing neurotoxin (BoNT), which could enable the bacterium to be disseminated as a biological weapon. Thus, the development of an effective vaccine—especially a mucosal vaccine—against BoNT is important for global deterrence of bioterrorism[30].

We intranasally immunized mice with CHP nanogel carrying BoHc/A (CHP–BoHc/A). It should be noted that the levels of endotoxin carried by the CHP nanogel were undetectable (Supplementary Table S1). Subsequent quality analyses of CHP–BoHc/A to confirm the nanometre-scale size uniformity and complex formation by dynamic light scattering (DLS) and fluorescence response energy transfer (FRET) analyses showed that the CHP nanogel continuously formed the nanoparticles after the incorporation of BoHc/A (Supplementary Fig. S2). However, intranasally administered CHP–BoHc/A was no better than naked BoHc/A for inducing BoNT/A-specific antibody responses (Supplementary Fig. S3a,b). These results suggest that CHP–BoHc/A is delivered minimally to the upper respiratory immune system because the mucosal tissues are tightly covered by an epithelial layer. In support of this hypothesis, the use of CHP nanogel did not enhance the BoHc/A uptake by nasal dendritic cells when compared to intranasal administration of naked BoHc/A (Supplementary Fig. S3c). Therefore, we next developed an endotoxin-free cationic type of CHP (cCHP) nanogel containing 15 amino groups per 100 glucose units (Fig. 1a and Supplementary Fig. S1 and Table S1) to improve the antigen-delivery efficacy of CHP nanogel to the anionic epithelial cell layer. DLS and FRET analyses showed that the cCHP nanogel possessed similar structural characteristics to the CHP nanogel because it maintained nanoscale size uniformity even after the incorporation of BoHc/A (Supplementary Fig. S2). In addition, consistent with its positive zeta-potential (Supplementary Table S2), it strongly interacted with the membranes of HeLa cells (Supplementary Fig. S4a) and was subsequently taken up into the cells by endocytosis (Supplementary Fig. S4b). These results are consistent with our previous finding that cCHP nanogel effectively delivered several proteins into cells *in vitro*[31]. Furthermore, an *in vivo*

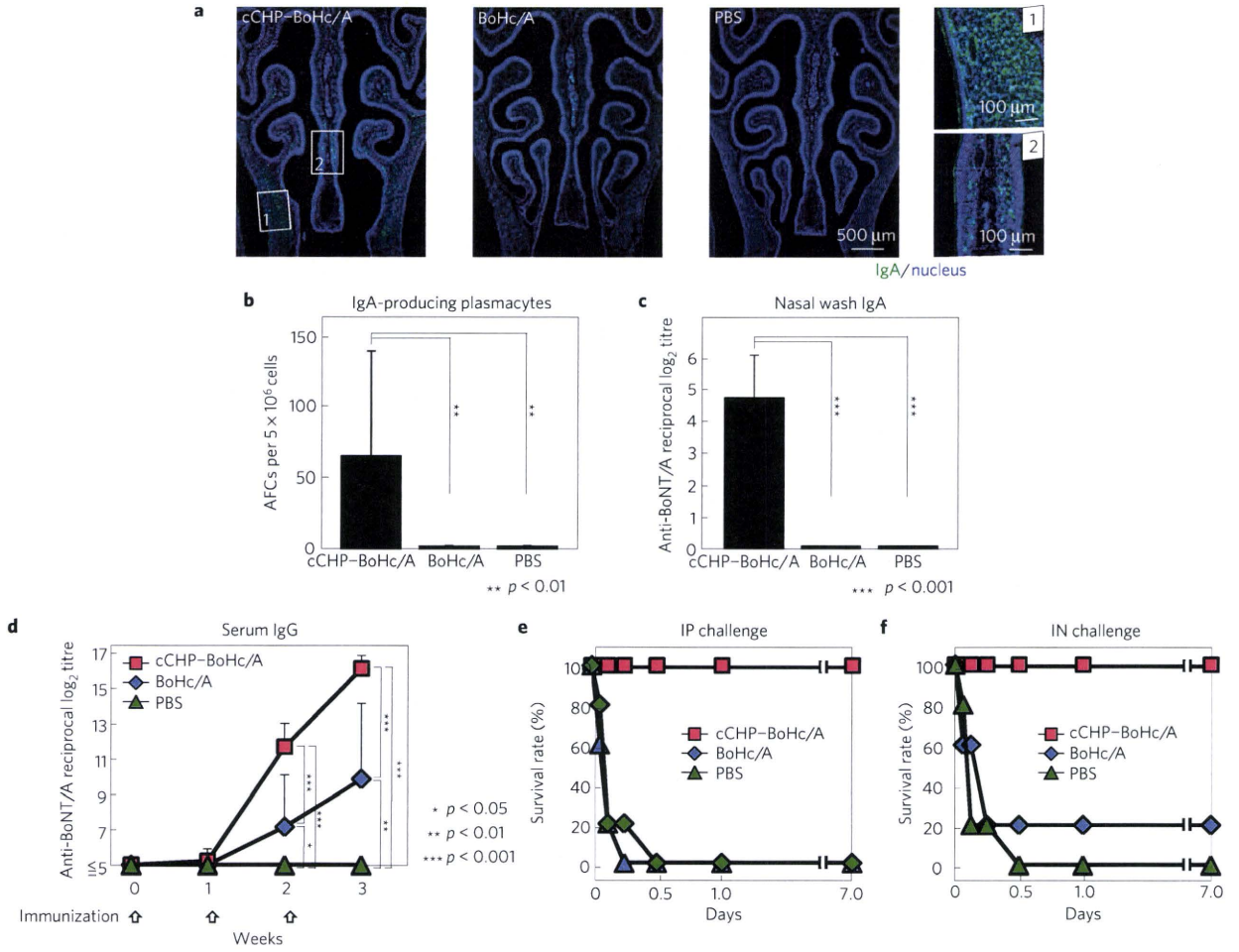


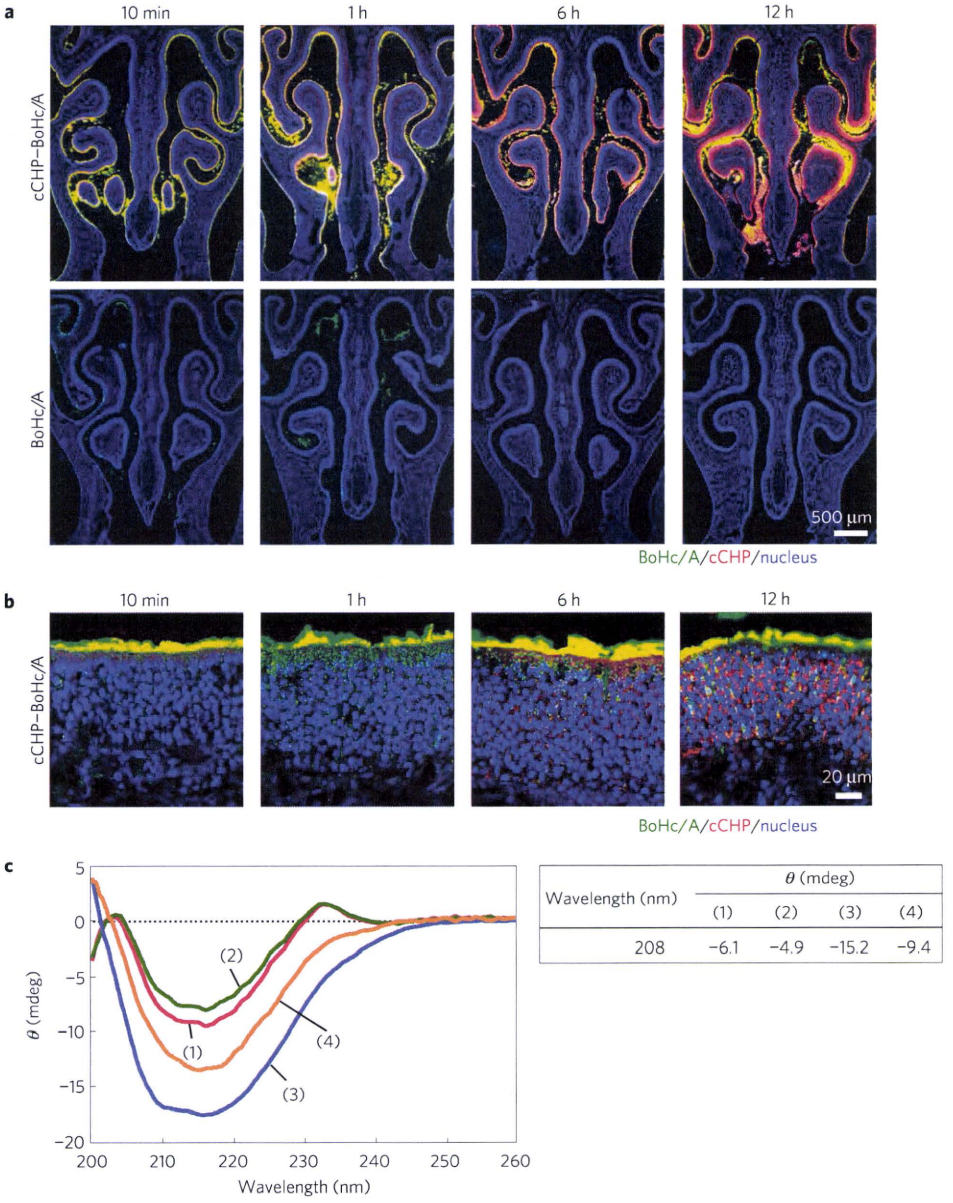
Figure 2 | Efficiency of BoHc/A with cCHP nanogel. a,**b**, BoNT/A-specific IgA-producing cells (or antibody-forming cells: AFCs) were effectively induced and recruited in the lamina propria and paranasal sinuses of the nasal mucosa 1 week after final immunization with cCHP-BoHc/A. **c**, Vigorous BoNT/A-specific IgA antibody responses were observed in nasal washes collected from mice intranasally immunized with cCHP-BoHc/A, but not from those given naked BoHc/A or control PBS. **d**, Strong BoNT/A-specific serum IgG antibody responses were induced by intranasal immunization with cCHP-BoHc/A. **e**,**f**, Mice intranasally vaccinated with cCHP-BoHc/A were completely protected from both intraperitoneal challenge with BoNT/A and intranasal exposure to the progenitor toxin.

imaging study using small-animal positron emission tomography (PET) and X-ray computed tomography showed clearly that intranasally administered cCHP nanogel carrying [^{18}F]-labelled BoHc/A was effectively delivered to, and continuously retained by, the nasal mucosa. In contrast, most of the [^{18}F]-labelled BoHc/A administered intranasally without cCHP nanogel disappeared from the nasal cavity within 6 h (Fig. 1b and Supplementary Fig. S5). A direct counting assay using a different radioisotope [^{111}In] with a long half-life (2.805 days) further demonstrated that BoHc/A was retained in the nasal cavity for more than two days when administered intranasally with cCHP nanogel (Fig. 1c).

To explore the efficacy of cCHP nanogel as a new adjuvant-free delivery vehicle for intranasal vaccination, we next tested whether intranasal immunization with cCHP–BoHc/A would effectively induce BoNT/A-specific mucosal IgA antibody responses. A histochemical study showed that the numbers of IgA-committed B cells markedly increased in the lamina propria and paranasal sinuses of the nasal passages on intranasal immunization with cCHP–BoHc/A, but not with naked BoHc/A or control PBS (Fig. 2a). A subsequent enzyme-linked immunosorbent spot study analysing mononuclear cells isolated from the nasal cavities of cCHP–BoHc/A-immunized mice directly confirmed induction of BoNT/A-specific IgA-producing cells (Fig. 2b). Furthermore, high titres of BoNT/A-specific IgA antibodies were detected in only those nasal washes collected from mice immunized with cCHP–BoHc/A, not with naked BoHc/A or control PBS (Fig. 2c).

As mucosal vaccination induces two-layered immunity (that is, in both the systemic and the mucosal compartments)[2,4], our next experiments were designed to determine whether BoNT/A-specific serum antibody responses were induced by intranasal immunization with cCHP–BoHc/A. Vigorous BoNT/A-specific serum IgG antibody responses were induced in cCHP–BoHc/A-vaccinated mice but not in mice immunized with naked BoHc/A or control PBS (Fig. 2d). To confirm the broad utility of this strategy with cCHP nanogel, we next evaluated the efficacy of intranasal administration of cCHP nanogel carrying a second prototype vaccine antigen, tetanus toxoid (cCHP–TT). As we expected, high titres of tetanus-toxoid-specific serum IgG as well as mucosal IgA antibodies were induced by intranasal administration of cCHP–TT (Supplementary Fig. S6). These findings indicate that the cCHP nanogel can be used universally as a new protein antigen delivery vehicle for intranasal vaccines.

We next carried out toxin-challenge experiments to confirm the ability of intranasal immunization with cCHP–BoHc/A to



Wavelength (nm)	θ (mdeg)			
	(1)	(2)	(3)	(4)
208	−6.1	−4.9	−15.2	−9.4

Figure 3 | Chaperone-like activity of cCHP nanogel facilitates effective delivery of vaccine antigen into the nasal mucosa. a, Intranasally administered cCHP-BoHc/A but not naked BoHc/A was effectively attached to the apical membrane of nasal epithelium. **b**, BoHc/A was subsequently released from the cCHP nanogel and transported into the epithelial layer. **c**, Circular dichroism analysis showed that the ellipticity (θ) value of BoHc/A, which was decreased to −15.2 mdeg after the BoHc/A was incorporated into cCHP nanogel, recovered to −9.4 mdeg after the release of BoHc/A from the cCHP nanogel by treatment with methyl-β-cyclodextrin. (1) Native BoHc/A, (2) BoHc/A heated for 5 h at 45 °C, (3) BoHc/A incubated with cCHP nanogel for 5 h at 45 °C, (4) cCHP-BoHc/A treated with methyl-β-cyclodextrin for 1 h at 25 °C.

neutralize BoNT/A and its progenitor *in vivo*. BoNT produced by *C. botulinum* usually forms a large complex called progenitor toxin with non-toxic accessory components, such as haemagglutinin, which are involved in binding to the mucosal epithelium[32]. It has been suggested that, on infection, the progenitor toxin binds to the mucosal epithelium; BoNT/A is then released into the blood circulation after detaching from these accessory components and finally interacts with nerve cells, causing botulism[33]. After intraperitoneal (i.p.) challenge with BoNT/A (500 ng, 5.5×10^4 i.p. LD_{50}, where LD_{50} represents the dose lethal to 50% of animals tested), mice intranasally immunized with cCHP–BoHc/A survived without any clinical signs, whereas those that had received naked BoHc/A or control PBS almost immediately developed neurological signs and died within half a day (Fig. 2e). Furthermore, mice intranasally immunized with cCHP–BoHc/A were completely protected from the effects of intranasal exposure to the progenitor toxin (10 μg, 2×10^5 i.p. LD_{50}) (Fig. 2f). Thus, the intranasal vaccine formulation of cCHP–BoHc/A effectively induces both systemic and mucosal protective immunity against lethal exposure to both BoNT/A and its progenitor without the co-administration of mucosal adjuvant.

To directly address how cCHP nanogel initiates and accelerates the immune responses against incorporated vaccine antigen without the use of a mucosal adjuvant, we next carried out a series of histochemical studies with tetramethylrhodamine isothiocyanate (TRITC)-conjugated cCHP nanogel carrying Alexa-Fluor-647-conjugated BoHc/A. As we expected, within 1 h of intranasal

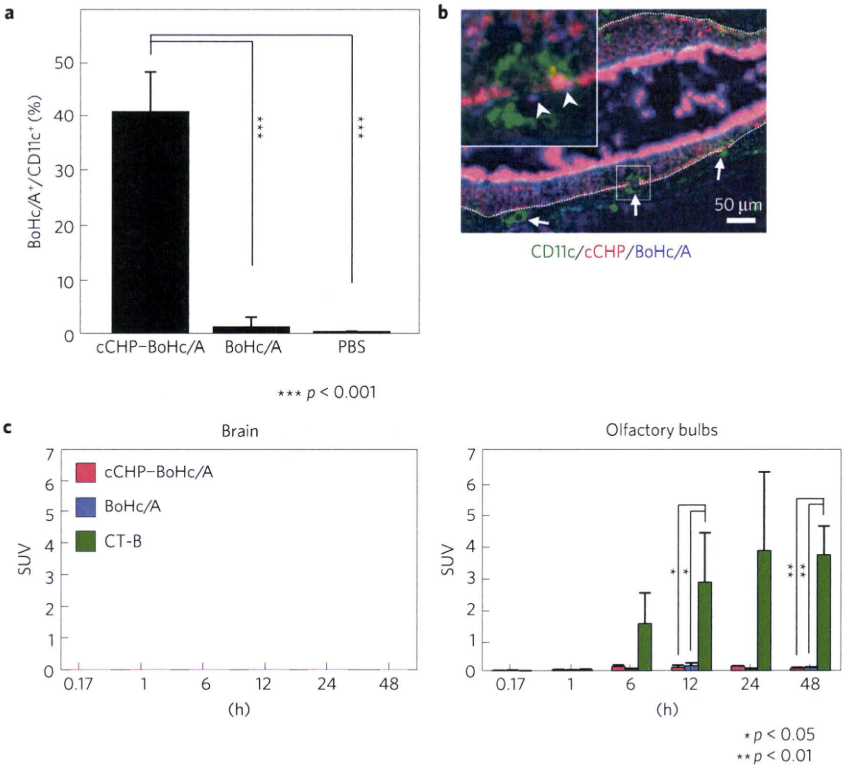


Figure 4 | Antigen delivered to dendritic cells by cCHP nanogel stimulates the nasal immune system but does not accumulate in the CNS. a,b, Flow cytometric (**a**) and immunohistochemical analyses (**b**) showed that BoHc/A released from cCHP nanogel was effectively taken up by $CD11c^+$ dendritic cells located in the epithelial layer and lamina propria of the nasal cavity, as shown by arrowheads. $CD11c^+$ dendritic cells and the basal layer of nasal epithelium in **b** are shown by arrows and dotted lines, respectively. **c**, The radioisotope counting assay showed that intranasally administered cCHP nanogel carrying [^{111}In]-labelled BoHc/A did not accumulate in the olfactory bulbs or brain. In contrast, [^{111}In]-labelled cholera toxin B subunit (CT-B), used as a positive control, accumulated in the olfactory bulbs from 6 h after administration.

administration, antigen-coupled fluorescence signals were observed in antigen-sampling M cells recognized by our previously established monoclonal antibody NKM 16-2-4 (ref. 34), in the nasopharynx-associated lymphoid tissues, which are inductive tissues for the airway mucosal immune system[4] (Supplementary Fig. S7a). However, because the nasal epithelium is anatomically widespread, cCHP–BoHc/A was universally distributed in the apical membrane of the nasal epithelium, and its density was much greater than that detected in the follicle-associated epithelium of nasopharynx-associated lymphoid tissues (Fig. 3a and Supplementary Fig. S7b). Examination of high-magnification images revealed that the cCHP–BoHc/A was internalized into the nasal epithelium immediately after the intranasal administration; BoHc/A was then detached gradually from the cCHP nanogel in a controlled manner in the nasal epithelial cells (Fig. 3b). In this regard, we previously showed that the proteins encapsulated by nanogels were released by protein exchange in the presence of excess amounts of other proteins, such as cellular components or enzymes[31]. In fact, the *in vitro* circular dichroism analysis showed that the secondary structure of BoHc/A was changed after the molecule was incorporated into the CHP nanogel but recovered after it was released (Fig. 3c). These results suggest that the cCHP nanogel acts as an artificial chaperone for intranasal vaccine antigen, leading to the induction of antigen-specific respiratory immune responses. In support of our hypothesis, the flow cytometric and immunohistochemical analyses showed that, within 6 h after administration of the BoHc/A with cCHP nanogel, the BoHc/A released from the nasal epithelium by exocytosis was effectively taken up by $CD11c^+$ dendritic cells located in both the epithelial layer and the lamina propria of the nasal cavity (Fig. 4a,b). It should be emphasized that the immunological role of cCHP nanogel is just to convey the vaccine antigen into the respiratory immune system effectively; it does not provide adjuvant-like activity to dendritic cells, because the bone-marrow-derived naive dendritic cells cultivated with cCHP nanogel did not enhance the expression of the co-stimulatory and antigen-presentation molecules (Supplementary Fig. S8). Moreover, nasal dendritic cells spontaneously expressed these molecules, probably because of chronic stimulation by inhaled environmental antigens, and their expression levels were not changed by intranasal administration with cCHP–BoHc/A (Supplementary Fig. S9). Therefore, the optimum antigen delivery offered by cCHP nanogel to activated nasal dendritic cells over a wide area of the nasal mucosa would be an effective strategy for inducing antigen-specific protective immune responses.

As the most important issue in intranasal vaccine development is to overcome safety concerns about the potential dissemination of intranasal vaccine antigens to the central nervous system (CNS), we carried out an *in vivo* tracer study with [^{111}In]-labelled BoHc/A. When cCHP nanogel carrying [^{111}In]-labelled BoHc/A was administered intranasally, no transition into the olfactory bulbs or brain was observed over a two-day period after administration (Fig. 4c). In contrast, when [^{111}In]-labelled cholera toxin B subunit, which can reach and accumulate in olfactory tissues[11], was administered intranasally with the same dose of radioisotope as used with the cCHP–BoHc/A, the radioisotope count in the olfactory bulbs was significantly higher than with cCHP nanogel holding [^{111}In]-labelled BoHc/A (Fig. 4c). These results support the hypothesis that cCHP nanogel administered intranasally possesses

no risk of redirecting the vaccine antigen into the CNS when administered intranasally and, therefore, can be used as a safe delivery vehicle for intranasal vaccines.

In essence, the nanogel antigen delivery system now opens up a new avenue for the creation of adjuvant-free intranasal vaccines. Taken in terms of its validity in leading to the induction of effective immune responses at both systemic and mucosal compartments without a concern for the deposition of vaccine antigen into the CNS, it would provide a unique and attractive vaccine strategy for the control of respiratory infectious diseases (for example, influenza).

Methods

Animals. Female BALB/c mice between 6 and 8 weeks old were maintained in the experimental animal facilities at the Institute of Medical Science of The University of Tokyo and at Hamamatsu Photonics K.K. All experiments were carried out according to the guidelines provided by the Animal Care and Use Committees of the University of Tokyo and Hamamatsu Photonics K.K.

Preparation of nanogel vaccine. CHP or cCHP nanogel synthesized as described previously[31,35] was mixed for 5 h at 45 °C at a 1:1 molecular ratio with vaccine antigen (BoHc/A expressed by *E. coli* or tetanus toxoid; kindly provided by the Research Foundation for Microbial Diseases of Osaka University). The FRET was determined by an FP-6500 fluorescence spectrometer (Jasco) with fluorescein isothiocyanate (FITC)-conjugated BoHc/A and TRITC-conjugated CHP or cCHP nanogel. The DLS of CHP or cCHP carrying, or not carrying BoHc/A, and the zeta-potential of BoHc/A with or without cCHP nanogel were determined with a Zetasizer Nano ZS instrument (Malvern Instruments). The circular dichroism spectra of BoHc/A before and after being incorporated into the cCHP nanogel, and after release from the cCHP nanogel by treatment with 15 mM of methyl-β-cyclodextrin, were obtained by using a J-720 spectropolarimeter (Jasco). To determine the cellular uptake *in vitro*, HeLa cells were treated with 10 nM of CHP or cCHP nanogel carrying FITC-conjugated BoHc/A, or of FITC-conjugated naked BoHc/A, for 4 h and analysed by flow cytometry with FACSCalibur (Becton Dickinson).

***In vivo* imaging study and radioisotope counting assay.** cCHP nanogel incorporating [^{18}F]-labelled BoHc/A was administered intranasally to mice and the distribution of radioisotope in the nasal cavity was determined by using a small-animal PET system (Clairvivo PET, Shimadzu Corporation)[36]. The radioisotope signals were measured for 10 h after administration and were superimposed on the image obtained by a small-animal X-ray computed tomography scanner (Clairvivo CT, Shimadzu Corporation). The images were analysed by using a PMOD software package (PMOD Technologies) and expressed as standardized uptake values (SUV) calculated from radioactivity in the volumes of interest. To trace the antigen for longer, [^{111}In]-labelled naked BoHc/A was administered intranasally with or without cCHP nanogel and the radioisotope counts in the nasal mucosa, olfactory bulbs and brain were directly measured by a γ-counter (1480 WIZARD, PerkinElmer) 10 min, 1, 6, 12, 24 and 48 h after administration. As a control, [^{111}In]-labelled cholera toxin B subunit[37] was administered intranasally. SUV was calculated as radioactivity (c.p.m.) per gram of tissue divided by the ratio of injection dose (1×10^6 c.p.m.) to body weight.

Immunization study. CHP or cCHP nanogel (each 88.9 μg for BoHc/A or 78.5 μg for tetanus toxoid) carrying BoHc/A (10 μg) or tetanus toxoid (30 μg), or the same amount of naked BoHc/A or tetanus toxoid dissolved in 15 μl of PBS, was administered intranasally to mice on three occasions at 1-week intervals. Sera were collected before, and 1 week after, each immunization, and nasal wash samples were taken 1 week after final immunization for antigen-specific enzyme-linked immunosorbent assay as described previously[34,38]. Mononuclear cells were isolated from the nasal passages 1 week after the final immunization and subjected to antigen-specific enzyme-linked immunosorbent spot analysis as shown in a previous study[38].

Neutralizing assay. To analyse the toxin-neutralizing activity of cCHP–BoHc/A-induced serum IgG and nasal IgA antibodies, the immunized mice were intraperitoneally challenged with 500 ng of BoNT/A (5.5×10^4 i.p. LD_{50}) diluted in 100 μl of 0.2% gelatin/PBS or intranasally exposed to 10 μg (in 10 μl PBS, 5 μl per nostril) of *C. botulinum*type-A progenitor toxin (2×10^5 i.p. LD_{50}, Wako). Clinical signs and survival rates were observed for 7 days, as described previously[34,38].

Histochemistry and flow cytometric analyses. Frozen sections of nasal tissues prepared from immunized mice were stained with FITC-conjugated anti-mouse IgA (BD Biosciences). To determine the distribution of cCHP–BoHc/A after intranasal administration, either TRITC-conjugated cCHP nanogel carrying Alexa-Fluor-647-conjugated BoHc/A, or Alexa-Fluor-647-conjugated naked BoHc/A, was administered intranasally and the sections of nasal tissues were stained with FITC-conjugated NKM 16-2-4 (ref. 34) or biotinylated anti-CD11c (BD Biosciences). For CD11c staining, the sections were then treated with streptavidin/horseradish peroxidase diluted 1:1000 (Pierce) followed by tyramide–FITC (PerkinElmer Life and Analytical Sciences). All sections were finally counterstained with 4,6-diamidino-2-phenylindole (Sigma) and analysed under a confocal laser-scanning microscope (TCS SP2, Leica) or a fluorescence microscope (BZ-9000, Keyence). To determine the antigen uptake by dendritic cells, cCHP nanogel carrying Alexa-Fluor-647-conjugated BoHc/A, Alexa-Fluor-647-conjugated naked BoHc/A or control PBS was administered intranasally. After 6 h, mononuclear cells were isolated from the nasal passages and stained with FITC-conjugated CD11c (BD Biosciences). The frequency of BoHc/A$^+$ CD11c$^+$ cells was analysed by flow cytometry.

Data analysis. Data are expressed as means ± standard deviation. All analyses for statistically significant differences were carried out by Tukey's *t*-test, with significance indicated by *p* values of <0.001 (***), <0.01 (**) and <0.05 (*).

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