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ORIGINAL ARTICLE

Antibodies against mumps virus component proteins

Keita Matsubara · Satoshi Iwata · Tetsuo Nakayama

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Abstract The neutralization (NT) test is regarded as the most reliable method for detection of protective antibodies, but is labor-intensive and time consuming. Enzyme-linked immunosorbent assay (EIA) is frequently used in sero-epidemiological studies because of its simplicity and ease of use. In this study, immunofluorescent (IF) antibodies against nucleocapsid (N), fusion (F), and hemagglutinin–neuraminidase (HN) proteins were investigated in comparison with NT and EIA antibodies. The antibody against N protein was dominant in serum samples obtained from patients with a previous history of mumps infection. Titers of antibodies against F and HN proteins were very low. Many serum samples were positive for EIA but negative for NT, and no significant correlation was noted between NT and EIA antibodies. Among the three component proteins, correlation of EIA and IF antibodies with N protein was relatively good. After vaccination with mumps vaccine, EIA positivity was closely related to the IF antibodies against N protein, and after vaccination NT-positive sera became positive for IF antibodies against F and HN proteins. IF antibodies against F and HN proteins were considered to have a strong association with NT antibodies, and those against N protein were considered to have a strong association with EIA antibodies.

Keywords Neutralization (NT) test · Enzyme-linked immunosorbent assay (EIA) · Fusion (F) protein · Hemagglutinin–neuraminidase (HN) protein · Nucleocapsid (N) protein

Introduction

Mumps virus is a member of the genus *Ruburavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, and is a major source of epidemic parotitis in childhood [1]. Symptoms of infection include swelling and tenderness in one or both salivary glands, and fever. Although the prognosis is benign, complications include aseptic meningitis, encephalitis, epididymo-orchitis, and oophoritis. Mumps virus is transmitted via droplet infection or direct contact with saliva. The incubation period is usually 16–18 days, and infectivity lasts from several days before the onset of illness until the parotid swelling disappears [2]. Individuals with asymptomatic infection also excrete infectious virus, and are sources of further transmission.

Among patients with typical parotid swelling, mumps infection is easily diagnosed, and virological examination is rarely needed. However, when aseptic meningitis occurs without other symptoms, it is necessary to distinguish the mumps virus from other pathogens, for example enteroviruses. Laboratory-based diagnosis of mumps infection depends on serological examination, isolation of the virus, and detection of the viral genome by polymerase chain reaction (PCR) assay. Isolation of the virus is regarded as the best method for diagnosis of mumps,

K. Matsubara (✉)
Department of Pediatrics, National Hospital Organization Tokyo Medical Center, Higashigaoka 2-5-1, Meguro-ku, Tokyo 152-8902, Japan
e-mail: kmatsuba@384.jp

S. Iwata
Center for Infectious Diseases and Infection Control, Keio University School of Medicine, Tokyo, Japan

T. Nakayama
Laboratory of Viral Infection, Kitasato Institutes for Life Sciences, Tokyo, Japan

and reverse transcription (RT)-PCR is highly sensitive and specific [3, 4], but few laboratories in Japan can perform both examinations. Therefore serological testing is commonly used, by use of the complement fixation (CF) test, the hemagglutination inhibition (HI) test, the virus neutralization (NT) test, or enzyme-linked immunosorbent assay (EIA). The CF and HI tests are less sensitive and specific than the NT test and EIA, and another disadvantage of the HI test is cross-reaction of the antibodies with other paramyxoviruses including parainfluenza type 3 virus [5, 6].

The NT test is regarded as the most reliable indicator of immunity against virus infection, but it is very laborious, difficult to perform, and time-consuming [7, 8]. EIA is simple, rapid, and appropriate for automation, and so ideally applied for large-scale serological surveillance [7]. Diagnosis involves detection of the immunoglobulin-M (IgM) antibody in the acute stage, or a more than fourfold increase in the immunoglobulin-G (IgG) antibody in paired serum samples. Many studies on EIA of mumps virus have been published, and some have reported that the assay is more sensitive than the NT test [9–13]. However, the NT test can detect functionally protective antibodies, whereas EIA can detect IgG or IgM-binding antibodies only [8]. EIA may not be suitable for protection against infection. It is important to evaluate NT antibodies and to investigate antibodies against the component proteins of mumps virus. The mumps virus genome encodes seven proteins; nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin–neuraminidase (HN), and large (L) proteins. F and HN proteins are enveloped proteins located in the outer membrane. Antibodies directed against these two proteins have been shown to neutralize the virus [14–19]. Therefore, it is necessary to evaluate the association of the NT test and EIA with antibodies against these component proteins.

Materials and methods

Serum samples

Serum samples were obtained every year from new students of the nursing school in Ashikaga city, Tochigi prefecture, for health assessment. The purpose of the study was explained and written informed consent was obtained from 299 participants. Thirty-one individuals had no history of immunization with mumps vaccine or clinically apparent mumps infection, so EIA antibody titers were negative. They received mumps vaccine after informed consent was obtained. Serum samples were obtained 4–6 weeks later and stocked at −20°C.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (EIA) was performed with the mumps IgG-EIA Kit (Denka Seiken, Tokyo, Japan). Briefly, purified viral particle antigens were coated on a 96-well plate and 100 μl serum diluted 1:200 was added to each well. After incubation with the serum sample for 60 min, anti-human IgG antibody labeled with peroxidase was added and stained, as recommended in the instruction manual. The kit contained reference standards relevant to NT titers, negative, $2^{1.0}$, $2^{2.0}$, $2^{3.0}$, $2^{5.0}$, $2^{6.0}$, and $2^{7.0}$. The EIA antibody titer was determined by referring to a linear regression curve obtained from the references. Sera with an EIA titer of $\geq 2^{2.0}$ were considered EIA-positive.

NT test

Serum samples were treated at 56°C for 30 min to inactivate the complement. Wild-type Mp/Tokyo.JPN/40.02 (genotype G) was used as the challenge virus. Then, 100 μl serial twofold dilutions in Eagle's medium containing 2% fetal calf serum (FCS) was mixed with an equal volume of virus (100 $TCID_{50}$/100 μl). After neutralization at 37°C for 90 min in a 5% CO_2 incubator, the mixtures were placed on a monolayer of Vero cells in a 96-well plate, in duplicate, and kept at 37°C in a 5% CO_2 incubator. The plate was observed for cytopathic effects (CPE) under a light microscope until day 7 of culture. NT antibody titer was expressed as the reciprocal of the dilution with no sign of CPE.

Immunofluorescent (IF) antibodies against N, F, and HN proteins

N, F, and HN genes were cloned from the KO.3 mumps Hoshino vaccine strain into pBluescript SK II(−) (Agilent Technologies, Santa Clara, CA, USA) using multi-cloning sites downstream of the T7 RNA promoter. 293T cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase, and plasmids were transfected with Mirus Superfect III (Invitrogen Life Technologies, Carlsbad, CA, USA) [20]. Transfected cells were fixed in preparation glasses and stored at −80°C. More than 80% of the cells were found to express N, F, and HN proteins after staining with anti-mumps polyclonal antibodies raised in rabbits. Serum samples were diluted at 1:10 and twofold serially diluted from 1:10 to 1:640. The serial dilutions were added to the wells on the glass slide and incubated at 37°C for 60 min.

Each well was then incubated with anti-human IgG labeled with fluorescein isothiocyanate (FITC) (FITC conjugated-goat anti-human polyvalent immunoglobulin IgG fraction of antiserum; Sigma–Aldrich, St Louis, MO,

USA) at 37°C for 40 min. FITC-positive cells were identified under a fluorescence microscope. A field with one or more FITC-positive cells, observed at a magnification of 1:40, was judged as positive. In addition, all these judgments were made by the same person.

Statistical analysis

Statistical analysis with t and χ^2 tests was performed using StatMate IV (ATMS, Tokyo, Japan).

Results

Relationship between NT and EIA antibody titers

Titers of NT and EIA antibodies against mumps virus were measured for 299 serum samples. The correlation between the two titers was very low ($R^2 = 0.30$) (Fig. 1), mainly because 67 of 255 EIA-positive samples (26.3%) were negative for NT. Only one of 188 samples (0.5%) was negative for EIA but positive for NT.

Comparison of EIA antibody with IF antibodies against F, HN, and N proteins

More than 80% of transfected cells were positive for the respective proteins in assays using hyperimmune serum. The correlations between EIA and IF antibody titers are shown in Fig. 2. The correlation coefficient between EIA and IF antibodies against F protein was 0.17 (Fig. 2a). That between EIA and IF antibodies against HN protein was 0.33 (Fig. 2b). Among 252 EIA-positive samples, 89 were negative for IF antibody against F protein, and 43 were also negative for the antibody against HN protein. In contrast, the correlation coefficient was 0.54 between EIA and IF

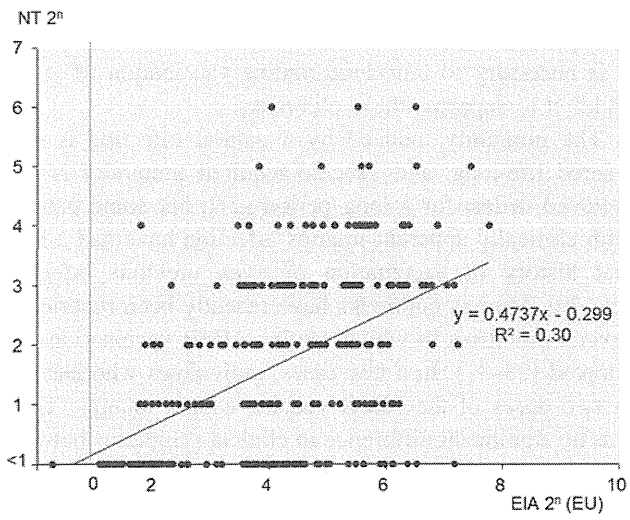

Fig. 1 Correlation between NT and EIA antibodies against mumps virus. NT antibody titers were assayed by 100% inhibition of CPE. EIA antibody titers were calculated by referring to standard positive sera supplied by the manufacturer. Both titers were expressed as 2^n. NT titers $\geq 2^{1.0}$ were regarded as positive. EIA $\geq 2^{2.0}$ were regarded as positive. EIA $<2^{0.0}$ was negative or uncertain

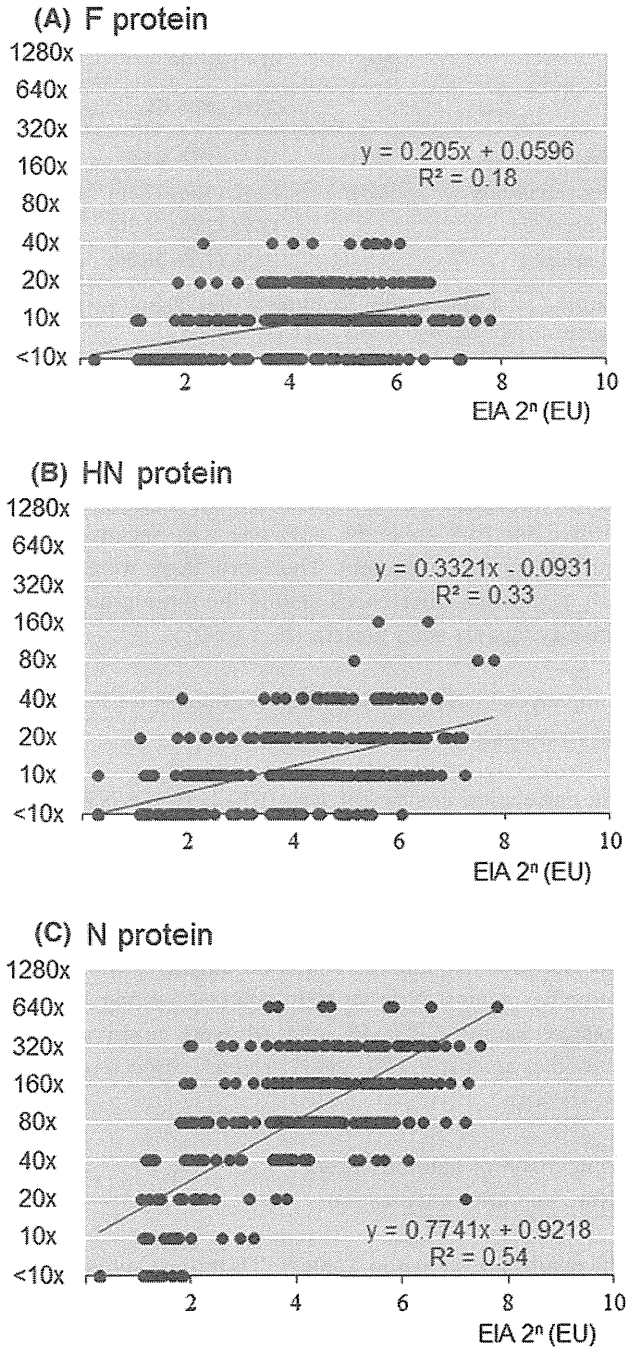

Fig. 2 Correlations between EIA and IF antibodies against three proteins. **a** Correlation between the EIA antibody and that against F protein. **b** Correlation between the EIA antibody and that against HN protein. **c** Correlation between the EIA antibody and that against N protein

Table 1 Serologic positive conversion of antibody titers against mumps before and after vaccination

	EIA	EIA titer (EU)	NT	NT titer ($\times 2^n$)	IF		
		GMT (95% CI)		GMT (95% CI)	Against F	Against HN	Against N
Before vaccination	0/31[a]	1.74 (1.15–2.65)	0/31[a]	0 (0–0)	1/31[a]	2/31[a]	11/31[a]
After vaccination	30/31[a]	12.12 (4.59–27.86)	14/31[a]	1.15 (0–2.34)	15/31[a]	19/31[a]	31/31[a]
NT-positive		13.92 (5.66–34.30)			14/14[a]	14/14[a]	14/14[a]
NT-negative		10.56 (4.29–26.00)			1/17[a]	5/17[a]	16/17[b]

Statistical evaluation was by use of the χ^2 test. Thirty-one subjects negative for EIA were immunized with mumps vaccine. Paired sera were obtained before and 4–6 weeks after vaccination

GMT geometric mean titer

[a] $P < 0.01$

[b] NS

antibodies against N protein (Fig. 2c). Among samples positive for EIA antibody, only one was negative for IF antibody against N protein. Therefore, there were statistically significant differences among the three groups of IF antibody against each protein ($P < 0.001$).

Comparison of NT antibody titers with those for F, HN, and N proteins

The correlation coefficient was 0.39 between NT and IF antibodies against F protein, 0.45 between NT and IF antibodies against HN protein, and 0.38 between NT and IF antibodies against N protein. Among 188 NT-positive samples, 39 and 19 were negative for IF antibody against F and HN proteins, but there was no negative result for IF antibodies against N protein. In contrast, among 111 NT-negative samples, 28, 48, and 84 were positive for IF antibodies against F, HN, and N proteins, respectively (data not shown).

Comparison of antibody titers before and after vaccination

The results are shown in Table 1. Thirty-one subjects were immunized with mumps vaccine and EIA, NT, and IF antibodies against N, F, and HN proteins were investigated before and after vaccination. Thirty out of 31 (96.8%) became EIA-positive and 14 (45.2%) became NT-positive. IF antibodies against F protein were positive in one subject before vaccination and in 15 after vaccination. Those against HN protein were detected in two before vaccination and in 19 after vaccination. Those against N protein were found in 11 before vaccination and in all after vaccination. The mean EIA titer was 13.92 EU for 14 subjects of the NT-positive group, and 10.56 EU for the NT-negative group. IF antibodies increased in one subject (5.9%) for F protein and five subjects (29.4%) for HN protein in the NT-negative group. Those against F and HN protein increased in all subjects with the positive response to NT ($P < 0.01$). All subjects in the NT-positive group and 16 subjects (94.1%) in the negative group had antibodies for N protein.

Discussion

In Japan, mumps, although an important infectious disease, is thought of a minor disease in comparison with measles and rubella. In most countries including the United States and European countries, implementation of a two-dose MMR vaccination scheme has dramatically reduced the number of mumps infections [21–23]. In Japan, MMR vaccine was administered from 1989 to 1993, but was discontinued because of an unexpectedly high incidence of aseptic meningitis [24, 25]. Since 1993, a monovalent mumps vaccine has been used but vaccine coverage is estimated to be less than 30% [26]. Therefore, outbreaks of mumps infection have often been found in schoolchildren. It is necessary to introduce routine vaccination of young children to suppress these outbreaks.

The immunity induced by a natural infection is considered life-long, and vaccine-acquired immunity is also believed to last for a long period [27], but some patients with clinically apparent mumps infection have had a clinical history of vaccination or even previous infection [28, 29]. Mumps outbreaks have recently been reported in several countries in which routine MMR vaccination was adopted [28–31]. In a few cases, individuals who had one or two doses of the MMR vaccine caught mumps. There was no significant difference in clinical symptoms between vaccinated and unvaccinated individuals in the case of a mumps outbreak in a summer camp in New York in 2005 [30]. In a mumps outbreak in Canada from 2009 to 2010 the effectiveness of one dose of the MMR vaccine ranged from 49.2 to 81.6% whereas for two doses it was estimated to be 66.3 to 88.0%; similar results have been reported in several studies [28]. Multistate outbreaks of mumps in

2006 showed two doses of the vaccine to be 76–88% effective, with attack rates not significantly different between one and two doses, and that immunity for mumps might decrease from 10 years after vaccination [31]. Also, the effectiveness of two doses of mumps vaccine declined from 98.8% in children 5–6 years of age to 86.4% in children 11–12 years of age [32]. The efficacy of live attenuated mumps vaccine was reported to be approximately 95% in phase III prelicensure serological responses [27]. Mumps virus is classified into 12 different genotypes (from A to L) on the basis of the sequence diversity of the SH gene, but it is thought to be serologically monotypic. Although antigenic differences might lead to reduced effectiveness, mumps vaccines have had high protective efficacy in outbreaks [31, 33]. With the exception of the Rubini strain vaccine, all available mumps vaccines worldwide, for example Jeryl-Lynn and Urabe Am9, have similar seroconversion rates and clinical efficacy and are regarded as acceptable for use in immunization programs [23]. Therefore, the age-related decrease in the vaccine's effectiveness seems to result from a waning of vaccine-acquired immunity. IgG-EIA and IgM-EIA antibody titers are used for diagnosis of mumps and to judge antibody prevalence in sero-epidemiological studies. EIA is superior in sensitivity, specificity, and simplicity, and suitable for large-scale surveillance. There is a problem with whether EIA antibody titers reflect protection against mumps infections.

In this study, a correlation between EIA and specific antibodies against mumps N protein was demonstrated, although there was a negative correlation of NT and IF antibodies against F and HN proteins. Many sera were negative for IF antibodies against F and HN proteins with high EIA titers, and the correlations between the EIA antibody and the antibodies against F and HN proteins were lower. From the results obtained for the serological responses for each antibody before and after vaccination, the EIA antibody became seropositive in 96.8% (30/31) of samples, but the seroconversion rate for the NT antibody was 45.2% (14/31). The IF antibody against N protein was found in all cases where the EIA antibody converted to positive. However, there were few cases where IF antibodies against F and HN proteins increased without any response in NT. Conversely, all sera with a positive response in NT were positive for IF antibodies against F and HN proteins. Thus, EIA antibody of mumps reflected the presence of antibodies against N protein. This shows that the development of EIA antibodies did not reflect protective immunity, because few antibodies against F and HN proteins were included in EIA antibodies. Several authors have reported that secondary vaccine failures were frequently observed in cases of natural mumps infections; IgM-EIA antibodies were detected, reflecting poor immunological memory of mumps infections [29, 34]. After measles virus infection, large amounts of antibodies were produced against N protein [35, 36]. The target of cell-mediated immunity is believed to be N protein. The presence of EIA reflects the immunological memory of N protein.

NT is the most reliable serological test for protective immunity, but has practical disadvantages. EIA has been widely used for serological diagnosis and immunological surveillance. Both serological methods have advantages and disadvantages, and each should be used taking into consideration its limitations.

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Review

Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) induced IgG1 and IgG4 antibody responses in young children

Tetsuo Nakayama[a,*], Takuji Kumagai[b], Ken J. Ishii[c,d], Toshiaki Ihara[e]

[a] Kitasato Institute for Life Sciences, Laboratory of Viral Infection, Tokyo, Japan
[b] Kumagai Pediatric Clinic, Sapporo, Japan
[c] National Institute of Biomedical Innovation, Laboratory of Adjuvant Innovation, Osaka, Japan
[d] Osaka University, Immunology Frontier Research Center, Laboratory of Vaccine Science, Osaka, Japan
[e] National Mie Hospital, Department of Pediatrics, Tsu, Mie Prefecture, Japan

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ABSTRACT

IgG subclass antibody responses are not fully understood. Alum-adjuvanted H5N1whole virion inactivated vaccine (WIV), a genetically reassortant vaccine seed strain originating from H5N1/A/Vietnam/1194/2004 and PR-8, induced significantly stronger antibody responses in neutralizing antibodies in children. In this report, IgG subclass antibody responses were investigated, and most serum samples were positive for IgG1 antibody before immunization. A significant response (more than 4-fold increase) of IgG1 antibody was observed in 67/193 (34.7%) and that of gG4 antibodies in 42/193(21.8%). Children <4 years of age showed a significant increase in IgG subclass antibodies but those ≥4 years showed lower responses. Alum- adjuvanted H5N1WIV induced an efficient immune response in young children especially <4 years.

Contents

1. Introduction

The 20th century saw three pandemics of influenza. The most devastating pandemic dated back to 1918, known as Spanish flu, and killed an estimated 40–50 million people, caused by H1N1 influenza virus transmitted through some animals not directly from an avian influenza virus [1]. Asian influenza A/H2N2 caused the second pandemic in 1957, and Hong Kong influenza A/H3N2 the third in 1968. After the 1968 pandemic, small local outbreaks were reported. Caused by H5N1, H7N7, or H9N2, they were considered to be from poultry. There was a regional outbreak of H5N1 in Hong Kong in 1997, and six of 18 patients died, causing a pandemic threat [2]. H5N1 is considered to be a target for pandemic vaccine, and WHO addressed sharing viruses and sequence information for a future pandemic vaccine development [3–5], and the development of an effective and safe vaccine is expected to mitigate the threat of a pandemic.

In Japan, alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originating from H5N1/A/Vietnam/1194/2004. In a clinical phase II/III trial in healthy adults, alum-adjuvanted WIV (HA protein: 15 μg) led to favorable immunogenicity (>70% sero-conversion rate in NT antibodies) without causing any serious systemic illnesses [6]. However, when it was administered to young infants and children at a reduced dose, 7.5 or 3 μg, a high body temperature (≥38.0 °C) was observed in >60% of recipients <7 years of age, but, unexpectedly, NT antibody titers were higher than those observed in the clinical trial in adults.

* Corresponding author at: Kitasato Institute for Life Sciences, Laboratory of Viral Infection, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail address: tetsuo-n@lisci.kitasato-u.ac.jp (T. Nakayama).

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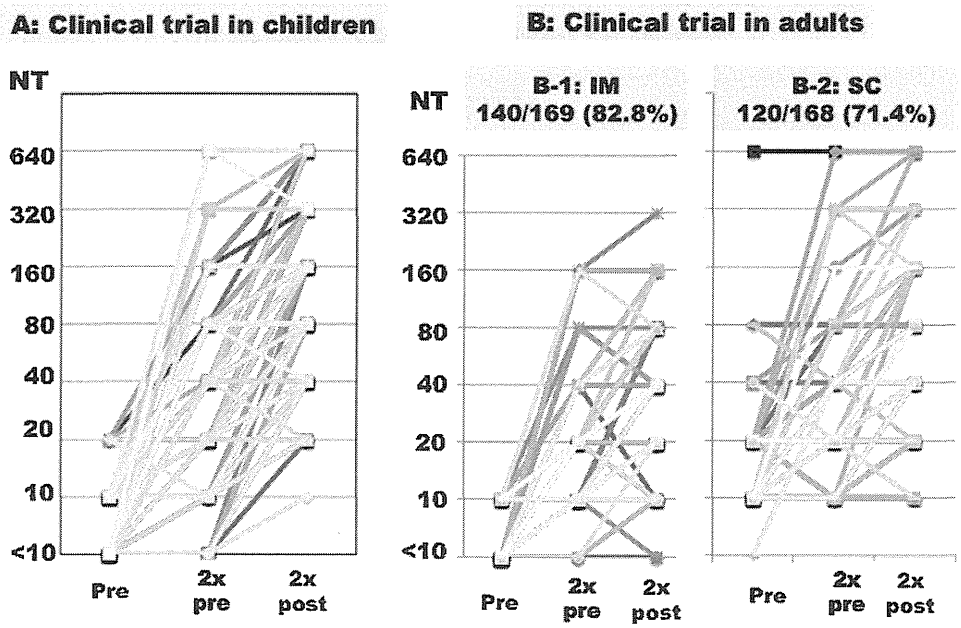

Fig. 1. Development of NT antibodies in clinical trials. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). The development of NT antibodies is shown in a clinical trial in children (A), in adults for intramuscular immunization (B-1), and for subcutaneous immunization (B-2). NT antibody titters are shown in *Y*-axis.

Functionally different IgG subclass antibody responses have been extensively investigated in mouse models, but human IgG subclass antibodies are not always functionally similar to the mouse. In mice, Th1 responses correlate with IgG2a, IgG2b and IgG3, regulated by the production of type I interferon (IFN), but in humans, IgG responses have not been strictly identified [7,8]. IgG1 is most abundant more than 50% of total IgG and IgG4, least abundant [9]. Human IgG1 reflected not only Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11]. Primary antibody responses require T-cell help through functionally different Th1 and Th2 cytokines secreted by antigen-presenting macrophages or dendritic cells [12,13]. Therefore, the analysis of IgG subclass antibody responses after vaccination provides supportive evidence of CD4-positive T cell functions for modulating acquired immunity. In this report, IgG subclass responses were investigated in children immunized with alum-adjuvanted H5N1 WIV.

2. Summary of alum-adjuvanted vaccine trials in adults and children

An alum-adjuvanted H5N1 WIV clinical study was conducted, involving 337 subjects aged 20–59 years. Two doses H5N1 vaccine were administered with an approximately 4 weeks interval. Serum samples were obtained just before the first dose, and just before and one month after the second dose. H5N1 vaccines induced poor immunogenicity when assayed by HI tests. The NT assay was carried out by micro-neutralization methods using homologous vaccine strain [14]. NT antibody responses against H5N1 in adults and children are shown in Fig. 1. In adult study, 337 subjects were enrolled and divided into two groups: 169 of intramuscular inoculation and 168 of subcutaneous inoculation. The results of NT response are shown in Fig. 1B. Sero-conversion was observed in 260/337 subjects (77%), demonstrating four-fold or higher responses after the second dose immunization: 140/169 (82.8%) in the intramuscular immunization group (Fig. 1B-1) and 120/168 (71.4%) in the subcutaneous immunization group (Fig. 1B-2). The incidence of a febrile reaction ≥37.5 °C was reported at 3% in an adult vaccination study [15]. Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. The results of NT antibodies are shown in Fig. 1A. All recipients became sero-converted in NT antibodies, but, unexpectedly, a high incidence of a febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 7 years. The incidence of a febrile reaction (≥38.0 °C) after vaccination declined with age: 5/5 (100%) in subjects less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years. Higher NT antibody titers seemed to be found in those with a higher body temperature after vaccination [15].

3. IgG subclass antibodies against H5N1

A quantitative enzyme immunoassay (EIA) was performed to detect IgG subclass antibodies against the H5N1 vaccine virus in 193 cases where informed consent was re-obtained. H5N1 WIV antigen was adjusted to 333 ng/ml in PBS (–) and wells of a 96-well plate were coated with 33 ng. Serial dilutions of serum samples were incubated, starting at 1:200 for IgG1, IgG2, and IgG4, and 1:20 for IgG3. HRP-conjugated monoclonal antibody against each human IgG1, G2, G3, and G4 was added and stained with o-Phenylenediamine enzyme substrate. The EIA titer was expressed as the reciprocal dilution of 100×2^n that gave two-fold OD in the negative control wells by linear regression assay. The results of IgG1 antibody responses in 193 children are shown in Fig. 2A, where informed consent was re-obtained for EIA assay. Many subjects possessed high levels of IgG1 antibodies ($\geq 100 \times 2^4$) before vaccination and did not demonstrate a significant immune response after vaccination. A significant IgG1 antibody response was observed in 67 (34.7%). The IgG1 antibody response was examined in 20 randomly chosen adults and high levels of IgG1 titer were noted just before immunization without any significant increase after the vaccination (Fig. 2B).

A significant increase in IgG2 antibodies was observed in 12 subjects (6.2%) and that in IgG3 antibodies in four (2.1%). The IgG4

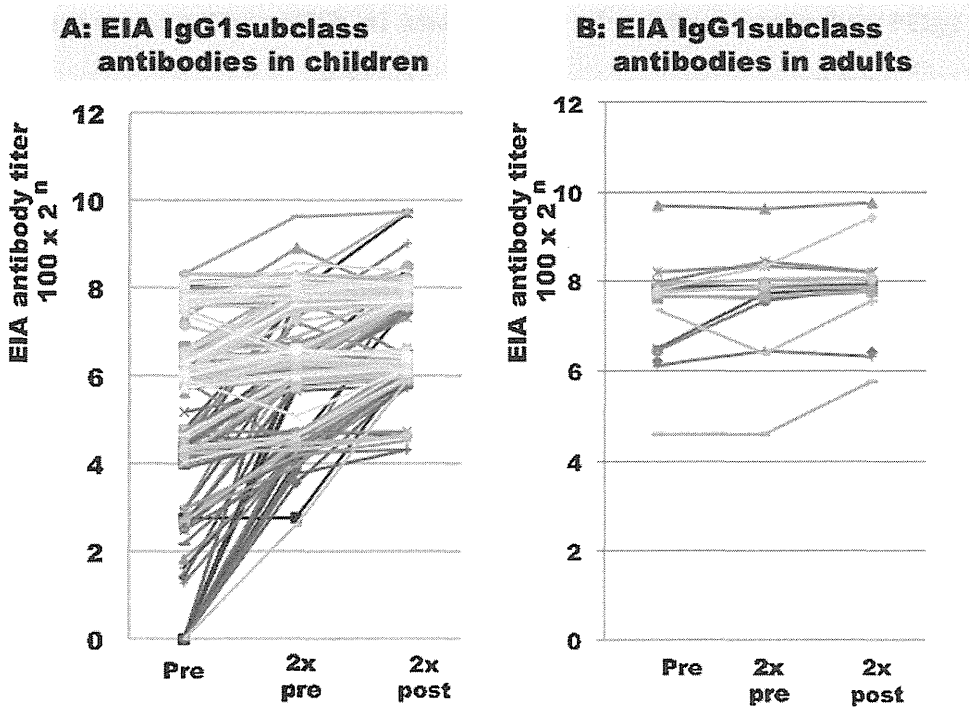

Fig. 2. IgG1 antibody responses in children and adults. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA antibody titers are shown in *Y*-axis for 100×2^n. EIA IgG1 subclass response in children is shown in panel A and that in adults in panel B.

response is shown in Fig. 3A. In 134 recipients, IgG4 antibody was negative before vaccination without a significant response after two dose vaccinations and 42 recipients (21.8%) showed positive responses. Competition assay was performed and the results of three sera are shown in Fig. 3B. Serial dilutions of antigen were mixed with serum samples and binding activity was examined. All serum samples became negative after competition and thus the IgG subclass assay was specific against H5N1 antigens.

4. IgG1 responses in different age groups

193 children were classified into four groups: aged <4 years (47 subjects), 4–6 years (42 subjects), 7–12 years (72 subjects), and ≥13 years (32 subjects). IgG1 EIA antibody responses are shown in Fig. 4 and Table 1. Among 47 subjects aged <4 years, 22 showed positive for IgG1 antibody prior to vaccination and 15 showed positive response after vaccination. Among the 25 infants in which no IgG1

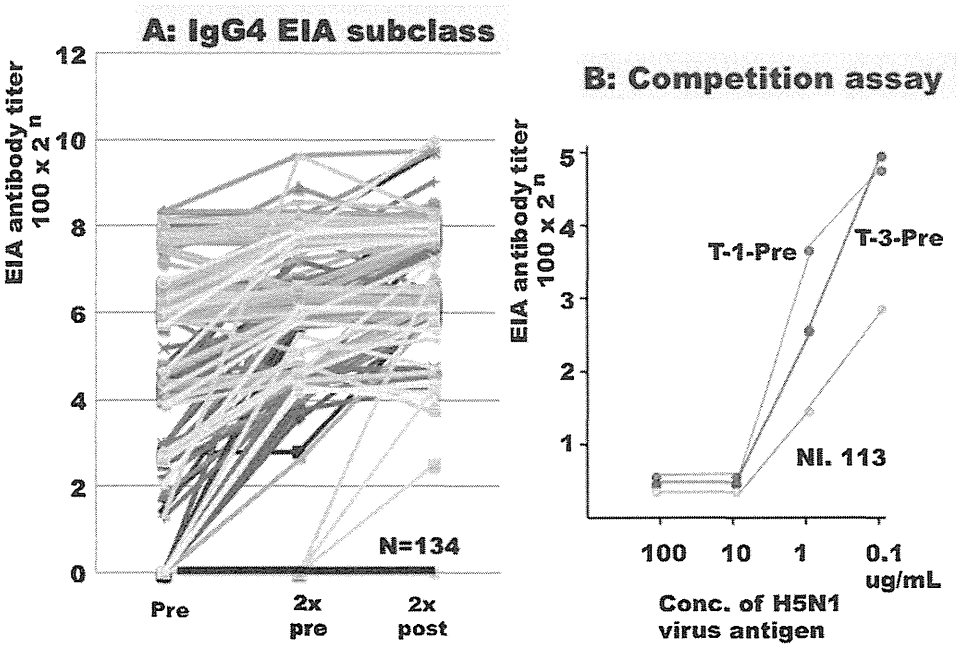

Fig. 3. IgG4 responses in children and the results of competition assay. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA IgG4 antibody titers are shown in *Y*-axis for 100×2^n in panel A. Among 193 subjects, 134 had no EIA responses in IgG4. The results of competition assay are shown in panel B. Serial 10-fold dilutions of H5N1 WIV antigen were mixed with serum samples. The EIA activity was examined.

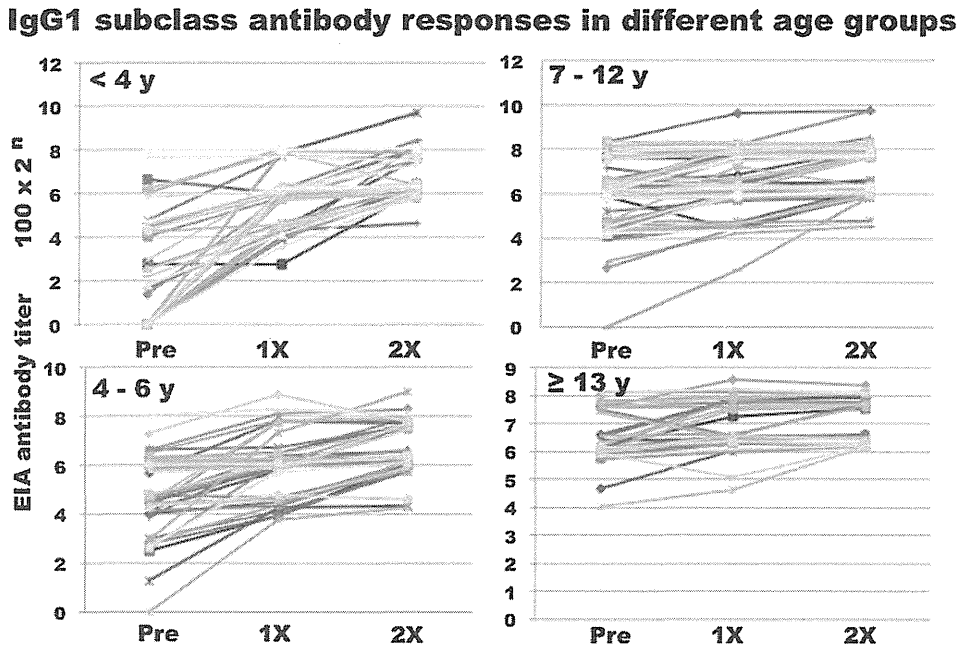

Fig. 4. Different IgG1 responses in different age groups: ≤4 years, 4–6 years, 7–12 years, and ≥13 years. Serum samples were obtained before the first dose (Pre) and before (1X) and four weeks after the second immunization (2X). IgG1 antibody titers are shown in *Y*-axis for 100×2^n.

EIA antibodies were detected before immunization, 17 showed positive after the first dose and the remaining 8 after the second dose. Of 42 subjects aged 4–6 years, 41 showed positive for IgG1 antibody before vaccination and 18 showed a significant response. One was negative pre-vaccination and sero- converted after the first dose. Of the 72 subjects aged 7–12 years, 71 were positive before vaccination and 7 showed a significant response. Among the 32 subjects aged ≥13 years, all tested positive before vaccination and only one showed a significant response. Most subject aged ≤4 years sero-converted, demonstrating four-fold or higher responses after the second immunization. Whereas, in subjects ≥7 years, IgG1 EIA antibody was detected before vaccination with a lower sero-conversion rate, similar to that observed in children ≥13 years and adults.

5. Discussion

Recent investigation on innate immunity has suggested that the development of acquired immunity against a specific antigen is modulated by the production of cytokines through functionally different Th1 and Th2 antigen-specific CD4-positive T lymphocytes [13]. The innate immune system consists of Toll-like receptors (TLRs), retinoic acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [16–18]. Investigation on cytokine production is essential for the better understanding of immune responses. In Japan, alum-adjuvanted H5N1 WIV has been licensed for adults but not for children. In a clinical trial of alum-adjuvanted H5N1 WIV in a pediatric group, a higher incidence of febrile reactions (≥38.0 °C) was observed with sufficient immune responses after vaccination. Cytokine productions were investigated in PBMCs obtained from non-vaccinated donors to know the reason behind these phenomena. H5N1WIV induced the higher levels of IFN-α, IL-6, IL-17, TNF-α, and MCP-1 than the control culture. With alum-adjuvanted H5 WIV, enhanced production of IL-1ß was demonstrated and IL-6 and TNF-α were produced similar to the levels obtained with H5N1 WIV [15]. WIV has genomic RNA that is recognized by TLR-7, inducing the production of IFN-α, which was essential for the antibody response in mice [19]. Inflammasome consists of NLRP3 and IL-1ß, IL-6, TNF-α, and IL-18 are induced in response to alum adjuvants through NLRP3 or other mechanisms. Alum-adjuvanted H5N1 WIV generated high titers of NT antibodies in young children, and, in this report, IgG subclass antibodies were investigated after immunization with alum adjuvanted H5N1 WIV.

IgG1 antibodies against H5N1 WIV antigens were detected in children ≥4 years of age and adult recipients before vaccination. The H5N1 influenza virus is not spread from human to human and has no history of large-scale outbreaks. The H5N1 WIV was a reassortant strain, whose envelop proteins, HA and NA were from H5N1/Vietnam/1194/2004 and remaining inner protein genes were from H1N1/PR8. Therefore, most subjects had IgG1 antibodies before vaccination. Approximately half of the recipients <4 years

Table 1
IgG1 EIA antibody responses in different age groups.

IgG1	<4 years (n = 47)	4–6 years (n = 42)	7–12 years (n = 72)	≥13 years (n = 32)
+++	22 (15)[a]	41 (18)[a]	71 (7)[a]	32 (1)[a]
– ++	17	1	1	0
– – +	8	0	0	0
– – –	0	0	0	0

+++: IgG1 antibody was positive before vaccination, one month after immunization of the first and second dose.
– ++: IgG1 antibody was negative before vaccination, and became positive after the first dose.
– – +: IgG1 antibody was negative before vaccination, and became positive after the second dose.
– – – IgG1 antibody was negative before vaccination, without antibody response after vaccination.
[a] Number of recipients with a significant responses.

had no detectable IgG1 antibodies before vaccination with a significant immune response, who did not experience H1N1 and H3N2 infections.

As for influenza infections, Garcon et al. [20] first reported high levels of IgG1 antibodies with lower amounts of IgG2 and IgG3 after immunization with different vaccine formulations; cold-adapted live recombinant, trivalent inactivated, and purified HA-conjugated vaccines to diphtheria toxoids. Hocart et al. [21] compared the subclass responses in natural infection with H3N2, and IgG1 levels in natural infection showed an 18-fold increase after infection and the other IgG subclasses, a 5- to 8-fold increase. The levels of IgG1 and IgG3 increased after immunization with live cold-adapted vaccines, and inactivated vaccines produced IgG1, IgG2, and IgG3 subclasses. IgG subclass responses were different from the vaccine formulations and also the increased levels of IgG1 differed with the serological status before vaccination. Stepanova et al. [22] reported different responses according to vaccine formulation and age. IgG1 and IgG4 responses were observed only in young adults immunized with the live influenza vaccine, the inactivated vaccine generating IgG1 and IgG3 in young adults, and IgG1 alone in the elderly. Human IgG1 reflected not only a Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11].

In this report, a significant IgG1 and IgG4 antibody responses were observed after immunization with alum-adjuvanted H5 WIV especially in young infants <4 years. It provided efficient immune response in young naïve infants. Considering the previous report that alum-adjuvanted vaccine induced inflammatory cytokines, including IFN-α, IL-1ß, IL-6, and TNF-α, they would modulate the expression of co-stimulatory molecules recognized by naïve CD4 helper T cells. Therefore, the IgG4 antibody response seems to be T cell-dependent, induced by innate immune impacts of WIV with alum adjuvant. But, it caused high incidence of febrile reactions, and efficient influenza vaccine formulation for priming in young infants is expected with low incidence of febrile reactions.

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Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) enhanced inflammatory cytokine productions

Tetsuo Nakayama[a,*], Yasuyo Kashiwagi[b], Hisashi Kawashima[b], Takuji Kumagai[c], Ken J. Ishii[d,e], Toshiaki Ihara[f]

[a] Kitasato Institute for Life Sciences, Laboratory of Viral Infection, Tokyo, Japan
[b] Tokyo Medical University, Department of Pediatrics, Tokyo, Japan
[c] Kumagai Pediatric Clinic, Sapporo, Japan
[d] National Institute of Biomedical Innovation, Laboratory of Adjuvant Innovation, Osaka, Japan
[e] Osaka University, Immunology Frontier Research Center, Laboratory of Vaccine Science, Osaka, Japan
[f] National Mie Hospital, Department of Pediatrics, Tsu, Mie Prefecture, Japan

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ABSTRACT

Alum-adjuvanted H5 whole virion inactivated vaccine (WIV) was licensed for adults in Japan but induced marked febrile reactions with significantly stronger antibody responses in children. In this study, the mechanisms behind the different responses were investigated. Lymphocytes were obtained from 25 healthy subjects who were not immunized with H5 vaccine, to examine the innate immune impact of the various vaccine formulations, analyzing the cytokine production profile stimulated with alum adjuvant alone, alum-adjuvanted H5 WIIV, plain H5 WIV, and H5 split vaccine. Alum adjuvant did not induce cytokine production, but H5 split induced IFN-γ and TNF-α. H5 WIV induced IL-6, IL-17, TNF-α, MCP-1, IFN-γ, and IFN-α. An extremely low level of IL-1β was produced in response to H5 WIV, and alum-adjuvanted H5 WIV enhanced IL-1β production, with similar levels of other cytokines stimulated with H5 WIV. Enhanced production of cytokines induced by alum-adjuvanted H5 WIV may be related to the higher incidence of febrile reactions with stronger immune responses in children but it should be further investigated why efficient immune responses with febrile illness were observed only in young children.

1. Introduction

In 2009, swine H1N1 influenza virus caused rapid global human-to-human transmission and was initially suspected as a new pandemic strain [1]. However, it actually emerged from swine influenza virus, which was first isolated in North America, genetically combined with human, swine, and avian genome compartments [2,3]. In this sense, pandemic A/H1N1 2009 was not a new pandemic strain [4,5]. Pre-existing antibody levels were reportedly low in young generations and most patients were young adults and children, not elderly [6]. A 2009 pandemic H1N1 vaccine seed was obtained after adaptation to egg, but the virus yield was poor in comparison with seasonal seeds. In Japan, egg-derived pandemic split vaccine was produced and introduced just after the peak of the outbreak. This pandemic raised several pressing issues: vaccine development, prompt supply and distribution, antigen saving, and vaccine efficacy to prepare for the unknown forthcoming pandemic.

In the 20th century, three pandemics of influenza occurred. The most devastating pandemic dated back to 1918, known as Spanish flu, caused by a highly pathogenic H1N1 influenza virus transmitted through some animals from avian pathogenic virus, estimated to have killed 40–50 million people [7]. In 1957, Asian influenza A/H2N2 caused the second pandemic, and Hong Kong influenza A/H3N2 appeared as the third pandemic in 1968. Seasonal influenza outbreaks or epidemics are caused by an antigenic drift of A/H1N1 or A/H3N2, whereas the pandemics appeared as antigenic shift, leading to new strains which are thought to be recombination with non-preexisting features of hemagglutinin (HA) and neuraminidase (NA) in human influenza viruses. After the 1968 pandemic of A/H3N2, several cases and small local outbreaks were reported, caused by new strains, H5, H7, or H9, and they were considered to be from poultry, and H5 is very close to human as a target for vaccine development [8–13]. There was a regional outbreak of H5 in Hong Kong in 1997, and six of 18 patients died, causing an H5 pandemic threat [9]. Sporadic H5 transmission on

* Corresponding author at: Kitasato Institute for Life Sciences, Laboratory of Viral Infection, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
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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poultry farms and in migratory birds has spread across Asia to the EU and Africa, and approximately 550 cases of human H5 infection have been reported since 2004, showing a high mortality rate of approximately 60%. Most cases have involved close and direct contact with poultry, with no definite case of human-to-human transmission [14]. There are several barriers to human-to-human transmission: receptor usage of HA protein, cleavage efficiency by cellular protease, and host factors. Now, H5 is very close to the human, and the primary strategy to prevent and control influenza pandemics is the development of an effective and safe vaccine to mitigate the uneasiness, uncertainty, and pandemic threat.

Split vaccine has been used for more than 40 years and H5 is known to be poorly immunogenic. A two-dose schedule of 90 μg split vaccine of H5/Vietnam/1203/2004 induced 57% seropositivity of HI ≥1:40, and 53% seropositivity of NT ≥1:40 without adjuvant [15]. The addition of alum adjuvant improved the immunogenicity and could reduce the antigen usage to 30 μg with a similar immunogenicity to plain split, 90 μg [16].

In Japan, alum-adjuvanted H5N1 whole inactivated virion (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004. In a clinical phase II trial in healthy adults, alum-adjuvanted 15 μg HA protein of WIV led to favorable immunogenicity (>70% sero-conversion rate in NT test) without demonstrating any serious systemic illnesses [17]. Whereas, when it was administered to young infants and children with a reduction in antigen doses, 7.5 or 3 μg, a high fever ≥37.5 °C was observed in over 60% of the recipients at less than six years of age, but, unexpectedly, NT antibody titers were higher than those observed in a clinical trial in adults. Recent detailed insights into the mechanisms of adjuvant effect on innate immunity and inflammasome have led to the better understanding of immunogenicity and immunotoxicity [18–20]. In this study, cytokine and chemokine responses were investigated to analyze the reason why a high incidence of febrile reactions was observed after the administration of alum-adjuvanted whole inactivated H5 vaccine to children.

2. Materials and methods

2.1. Study design and subjects

Twenty-five healthy subjects were enrolled in this study, aged 3 months to 59 years, who were not immunized with H5 vaccine. Among them, 20 subjects were under 20 years of age. The study design and protocol were discussed and approved by the ethical committee of Tokyo Medical University. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation through Ficoll-Paque™ Plus (GE Healthcare Bio-science, Uppssala, Sweden). They were adjusted to 1×10^6 cells in a 24-well plate in 1 ml of RPMI 1640 medium supplemented with 4% FBS and adequate antibiotics. They were stimulated with 100 μl of vaccine preparations or alum adjuvant alone.

2.2. Vaccine antigens

The NIBRG-14 strain, a genetically reassortant vaccine seed strain, originated from H5/A/Vietnam/1194/2004 and PR-8, was grown in MDCK and purified through zonal ultracentrifugation. Purified virus particles were inactivated by formalin treatment and used as whole inactivated vaccine (WIV). Alum-adjuvanted WIV was produced by adding alum adjuvant (1:1 mixture of Al phosphate and hydroxide) at a final alum concentration of 300 μg/ml. Purified virus particles were split by treatment with ether and Tween 80 and inactivated with formalin, and used as split vaccine material. Other strains were employed to compare the immunological responses: seasonal A/Brisbane/H1N1 and 2009 pandemic A/California/07/2009, produced by Kitasato Institute for Biologicals, Saitama. All vaccine materials were adjusted to 30 μg/ml HA protein concentration.

H5 WIV pandemic vaccine for clinical trial was produced from egg-derived WIV materials by Kitasato Institute for Biologicals, Saitama and Biken Intitutes, Kannonji.

2.3. Cytokine assay

Culture supernatants were harvested at 24 hr after stimulation with influenza vaccine materials and subjected to Bio-Plex Pro™ Human Cytokine Assay 17-plex, using Bio-Plex 200 (Bio-Rad, USA). The concentration of IFN-α was measured using an EIA kit (Verikine™ Human IFN-Alpha Serum Sample ELISA kit, pbl interferon, USA) and IL-1β and IL-6 were also measured using Quantikine Human IL-1β and Quantikine IL-6, respectively (R&D Systems, USA), following the instruction manual.

3. Results

3.1. Summary of alum-adjuvanted vaccine trial in children

An alum-adjuvanted H5N1 WIV clinical study was conducting involving 337 subjects aged 20–59 years. Two doses were given at 21–28 day intervals, and HI and NT antibodies were examined before immunization, just before the second dose, and one month after the second dose. NT antibodies became sero-converted in 260/337 (77%) in the15 μg group. No serious systemic adverse reaction was observed: febrile reaction ≥37.5 °C was reported in 3%. Alum-adjuvanted H5N1 WIV was licensed for stockpiling to prepare for a pandemic.

Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. 0.1 ml was given to those less than one year, 0.25 ml for those 1–6 years, and 0.5 ml for those over six years of age. Febrile illness ≥37.5 °C was observed in 203/374 (54%) after the first dose, but decreased to 33/367 (9.0%) after the second dose. Unexpectedly, a high incidence of febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 6 years and the incidence of febrile reaction (≥38 °C) after vaccination reduced by age: 5/5 (100%) in those less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years (Table 1).

NT titers after two-dose vaccination were compared in subjects who had a febrile reaction and those without febrile illness. The mean NT titer was $10 \times 2^{3.56 \pm 1.30}$ in those with febrile illness, being significantly higher than those without febrile illness, $10 \times 2^{2.76 \pm 1.26}$ ($p < 0.01$). Higher NT antibody titers seemed to be induced in those with a higher body temperature after vaccination (Table 2).

3.2. Cytokine induction by alum adjuvant

Alum adjuvant was prepared at the same concentration of 300 μg/ml. PBMCs were stimulated with 3 μg or 30 μg of

Table 1
Incidence of febrile reactions in different age groups.

	n	Fever+	≥38.0 °C
<1 year	5	5 (100%)	5 (100%)
1–3 years	92	68 (74%)	52 (57%)
4–6 years	90	57 (63%)	48 (53%)
7–12 years	134	63 (47%)	39 (29%)
≥13 years	53	10 (19%)	3 (6%)
Total	374	203 (54%)	147 (39%)

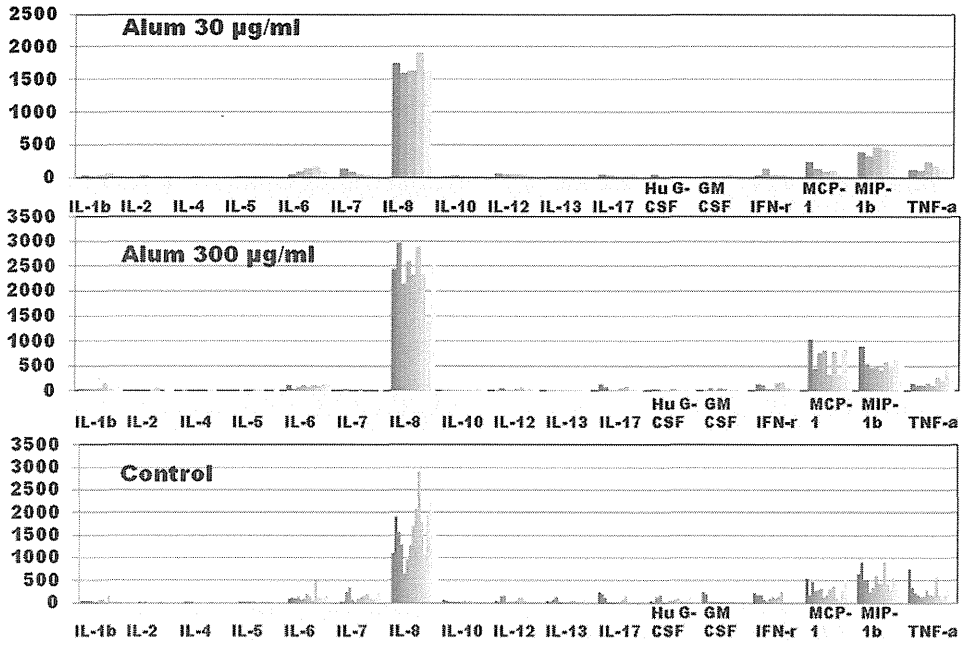

Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 μg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 μg/ml (1:10 dilution).

aluminum, and the results of cytokine profiles are shown in Fig. 1. Culture fluids were assayed using human 17plex. In control cultures of 25 subjects, IL-6, IL-7, IL-8, IFN-γ, MCP-1, MIP-1β, and TNF-α were produced at the baseline without any stimuli, and no additionally enhanced cytokine production was noted when stimulated with 30 μg alum adjuvant.

3.3. Cytokine production in response to different formulations of H5 influenza vaccines

H5 split materials were prepared and cytokine production profile was compared to those in response to the seasonal A/H1N1/Brisbane and A/H1N1/California/04/2009. IFN-γ was produced when stimulated with each split antigen, showing different levels of IFN-γ (Fig. 2). There was no significant difference in the other cytokine profiles among three split materials.

Alum-adjuvanted WIV, plain WIV, and the split formulation of the H5 vaccine antigen were adjusted to 30 μg/ml HA protein concentration. PBMC were stimulated with 3 μg of HA antigen. Through the analysis of 17 cytokines and chemokines, the productions of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 showed different profiles from control culture or when stimulated with aluminum alone. Results of cytokine profiles are shown in Table 3. IFN-γ and TNF-α were produced when stimulated with H5 split material. H5 WIV induced the higher production of IL-6, IL-17, TNF-α, and MCP-1 than control culture or those stimulated with Alum or H5 split materials. There was no increase in IL-1β production when stimulated with aluminium alone and H5 split antigen, but slightly higher levels of IL-1β production were observed in response to plain WIV. When stimulated with alum-adjuvanted WIV, the enhanced production of IL-1β was demonstrated and the other cytokines were produced similar to the stimulation with H5 WIV.

The 17-plex human cytokine assay demonstrates the cytokine profile and does not reflect the actual concentrations of the cytokines. As shown in Table 3, enhanced production of IL-1β was noted but IFN-α is not assayed in 17-plex kits. IL-1β, IL-6, and IFN-α were evaluated using EIA, and the results are shown in Fig. 3. IFN-α was produced when stimulated with WIV, and higher levels of IFN-α were demonstrated in subject numbers 21–25. In younger subjects less than one year of age (subject numbers 1–5), the enhanced production of IFN-α was shown in response to alum-adjuvanted WIV. A very low level of IL-1β was produced in response to WIV, and IL-1β production was enhanced when stimulated with alum-adjuvanted WIV. IL-6 was also produced in response to both WIV and alum-adjuvanted WIV, and alum-adjuvanted WIV enhanced the production of IFN-α, IL-1β, and IL-6. The production pattern of IFN-α in different age groups was similar to that of IL-6. IL-1β production profile was different from the others. Production of these cytokines seemed to be prominent in young infants at less than one year of age (subject Numbers 1–5) and adults (subject Numbers 21–25). Cytokine productions seemed to be different in each individual.

Table 2
Relationship between acute febrile reactions and antibody response.

	N	Mean ± SD[d]	95% C.I.	
Fever−	170	2.76 ± 1.26	2.58–2.95	
Fever+	200	3.56 ± 1.30	3.38–3.74	[a]
37.5–<38.0 °C	56	3.11 ± 1.27	2.77–3.45	
38.0–<39.0 °C	79	3.53 ± 1.32	3.24–3.82	[b]
≥39.0 °C	65	3.98 ± 1.17	3.70–4.27	[c]

[a] Mean NT titers were significantly different between subjects with febrile reactions after immunization and those without febrile reactions ($p < 0.01$).
[b] Significant difference was noted between NT titers in subjects with high body temperature ≥37.5–38.0 °C and in those with 38.0–39.0 °C ($p < 0.05$).
[c] Significant difference was noted between NT titers in subjects ≥37.5–38.0 °C and in those with ≥39 °C ($p < 0.01$).
[d] Mean titer of NT antibody expressed as 10×2^n.

4. Discussion

High-level immunogenicity is primarily required for a highly pathogenic pandemic, such as H5N1. Current split H5 was poor immunogenic and the WIV vaccine formulation has been reconsidered to have renewed merits concerning immunogenicity and cross-reaction [21–25]. Besides alum adjuvant, squalene oil emulsion adjuvants (MF59 and AS03) were used in H5 pandemic investigational split vaccines and induced

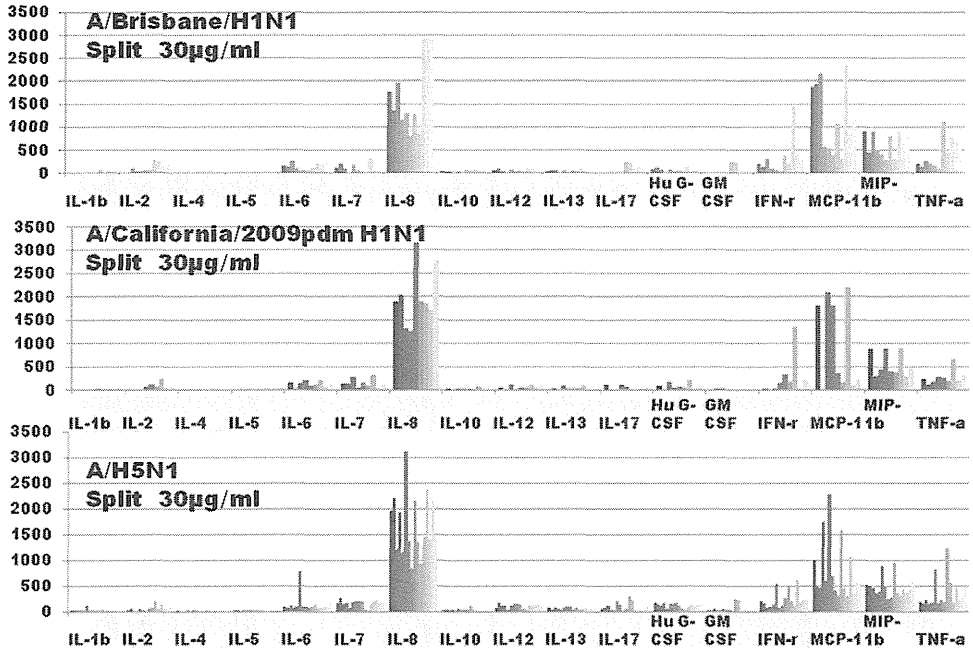

Fig. 2. *Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of 30 μg/ml of HA antigen, and PBMC were stimulated with 0.1 ml (3 μg/test).*

high-level immunogenicity with allowing for antigen saving, along with cross protective broad antibody responses [26,27]. This type of adjuvant was also applied for the 2009 pandemic vaccines, and resulted in efficient immunogenicity [23,24,28].

WIV was originally considered to induce high-level reactogenicity, and it was replaced by a split formulation in the 1960s [29–31]. H5 split vaccine was poorly immunogenic, and most European companies used oil emulsion adjuvants such as MF59 or AS03. Waddington et al. [25] reported the immunogenicity and reactogenicity of H1N1 pandemic vaccine comprising different formulations of AS03 oil-in-water emulsion adjuvanted and WIV in children at 6 months to 12 years of age. Seroconversion rates were nearly 98–99% in the AS03-adjuvanted vaccine group, but 80.6% at <5 years, and 95.9% at 5–12 years after immunization with WIV. An important finding was that WIV showed a strong age-dependent response in terms of immunogenicity, probably influenced by a past history of influenza infection. As for systemic adverse illness, febrile reaction was observed in approximately 10% of recipients aged <5 years, and in 3% of those aged 5–12 years after the administration of WIV. Wu et al. [21] reported that 5–15 μg of alum-adjuvanted H5 split vaccines were tolerated by children aged 3–11 years and 5–30 μg alum-adjuvanted split and 5 μg WIV vaccines were also tolerated by those aged 12–17 years. 10–15 μg of alum-adjuvanted split vaccine induced a 55% seroconversion and seroprotection rate in those aged 3–11 years, and 5 μg of alum-adjuvanted WIV induced a higher immunogenicity than 10 μg of adjuvanted split vaccine. When alum-adjuvanted WIV was used in young infants, a high incidence of febrile reactions (50–60%) was reported in a study in China although the number of recipients was very small [21].

In Japan, alum-adjuvanted WIV was licensed for adults but not for children. In a clinical trial of alum-adjuvanted WIV in a pediatric group, the incidence of febrile reactions (≥38 °C) after vaccination reduced by age: 100% in those less than one year, 50–60% in those 1–6 years, 29% in those 7–12 years, and 6% in those 13–19 years. The cytokine response was investigated in lymphocyte cultures stimulated with different H5 vaccine formulations to identify the reason for the immunogenicity and immunotoxicity of alum-adjuvanted H5 WIV. Cytokine production by PBMC was higher in young infants, but some teenagers and adults demonstrated a high-level cytokine response.

Many kinds of adjuvant have been developed, and they cause adverse reactions at the inoculation site or systemic reactions. Alum-based adjuvant was first approved for human use and continues to be widely used in many vaccines as an immuno-potentiator [29–31]. Two potential mechanisms are basically considered: (a) the formation of a depot from which the antigen is gradually released; (b) soluble antigen is converted to a particle form easily phagocytosed by antigen presenting cells (APC) such as dendritic cells or macrophages [31].

Recently, the stimulation on the innate immunity has been found to modulate the development of an acquired immune response through the production of cytokines [19,20]. The innate immune system consists of Toll-like receptors (TLRs), retinoic

Table 3
Production of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 when stimulated with Alum, H5 split, H5WIV and Alum adjuvanted H5 WIV.

	IL-1β	IL-6	IL-17	IFN-Y	TNF-α	MCP-1
Control	26.8 (13.3–40.3)	86.9 (46.4–127.3)	26.4 (13.3–39.5)	73.5 (45.7–101.3)	224.1 (148.4–299.9)	194.1 (120.8–267.4)
Alum	36.3 (21.6–51.0)	71.8 (50.7–92.9)	40.3 (26.1–54.5)	75.1 (56.6–93.7)	151.4 (114.4–188.4)	294.8 (154.5–435.0)
H5 split	21.6 (12.3–30.8)	145.4 (88.3–202.5)	69.3 (38.0–100.6)	182.3 (118.8–245.7)	328.5 (226.9–430.2)	544.3 (299.9–788.6)
H5WIV	50.1 (38.1–62.2)	503.6 (370.8–636.3)	180.0 (154.8–215.3)	354.4 (226.2–482.5)	843.4 (681.4–1005.4)	1452.5 (927.2–1977.8)
H5WIV + Alum	142.7 (63.0–22.4)	467.6 (306.3–628.8)	159.2 (133.5–185.0)	274.8 (169.0–380.5)	624.0 (424.3–823.7)	1023.2 (576.5–1469.9)

Lymphocytes were obtained from 25 healthy individuals who were not immunized with H5 vaccine. Mean values (pg/ml) are shown and ranges of 95% CI are in the parenthesis.

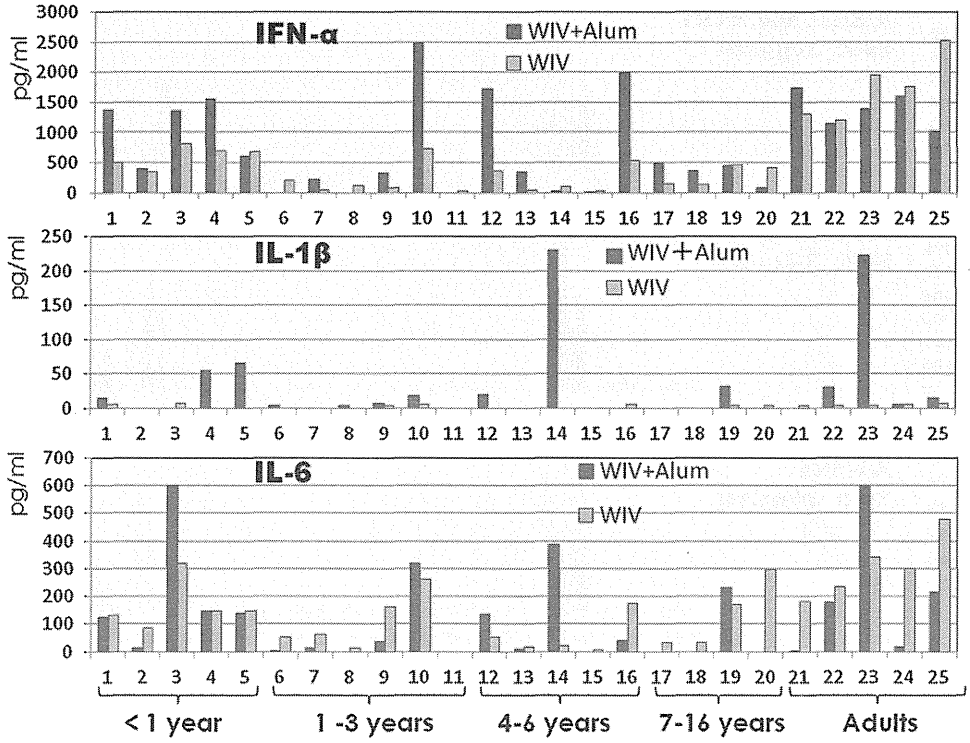

Fig. 3. IFN-α, IL-1β, and IL-6 production. IFN-α, IL-1β, and IL-6 were measured by EIA in PBMC cultures. PBMC were stimulated with H5 WIV and alum-adjuvanted WIV vaccine materials. Samples 1–5 were obtained from healthy individuals less than one year, those 6–11 from 1 to 3 years of age, those 12–16 from 4 to 6 years, those 17–20 from 7 to 16 years, and those 21–25 from adults. Black columns are cytokine productions stimulated with adjuvanted H5WI, and grey columns show those stimulated with H5 WIV.

acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [20,32–34]. Inflammasome consists of NLRP3, apoptosis-associated speck-like protein (ASC), which is thought to be an adaptor molecule of NLRP-3, resulting in the recruitment of caspase. It stimulates the production of inflammatory cytokines, IL-1β, IL-6, and IL-18 from proinflammatory molecules through the enzymatic activity of caspase [34]. Alum adjuvant induced cellular lysosomal damage or tissue damage and stimulated NLRP3 inflammasome through increased levels of uric acid caused by tissue damage [35,36]. The mechanisms of immunogenicity induced by Alum adjuvant have remained poorly understood regarding whether the stimulation of NLRP3 inflammasome is dispensable or not [37–39].

The activation of innate immunity increased antigen-specific adaptive immunity through TLRs induced by influenza vaccine without influencing NLRP3 inflammasome [40]. WIV influenza virus induced antigen-specific antibodies through the production of type I IFN involving the activation of TLR7 in mice [32,41]. Kuroda et al. [42] reported that alum induced LPS-primed macrophages to produce prostaglandin E2 (PGE2) and IL-1β. PGE_2 production was independent of NLRP3, ASC, and the caspase-1 inflammasome complex, and PGE_2 expression depended on cyclooxygenese (COX) and PGE synthase, regulated by spleen tryrosin kinase (Syk) and p38 MAP kinase in macrophages. PGE_2 was found to suppress Th1 responses with a reduced production of IL-2 and IFN-γ, but facilitated the differentiation of Th1 cells in the presence of IL-12 and, thus, cytokine species and their balance regulated PGE2 function on antibody production [18,42,43]. WIV and alum-adjuvanted WIV induced the production of the endogenous cytokines IL-1β, IFN-α, IL-6, and TNF-α, and they induced PGE2 in circumventricular organs through capillary fenestration, which is a well-known pyrogen [20,44].

WIV has genomic RNA that is recognized by TLR-7, inducing IFN-α [40]. In the clinical trial of alum-adjuvanted WIV, the incidence of febrile reactions (>38 °C) after vaccination reduced by age: 100% at less than one year, 50–60% at 1–6 years, 29% at 7–12 years, and 6% at 13–19 years. However, there was no comparative control group who received non-adjuvanted H5 plain WIV to discuss the incidence of febrile reactions. Cytokine production by PBMC was higher in young infants, some teenagers and adults in response to WIV. Enhanced productions of IFN-α, IL-1β, and IL-6 were demonstrated in very young subjects, and were suggested to be associated with a higher incidence of febrile reactions (immunotoxicity) and high immunogenicity (adjuvantogenicity). Cytokine profiles should be checked in serum from those who had high fever after immunization with alum-adjuvanted H5 WIV to observe the direct relationship between the enhanced cytokine level and febrile illness. Lymphocytes from adults also produced high levels of cytokines in response to alum-adjuvanted H5 WIV. Even though, sufficient immune responses were not observed in adults with lower incidence of febrile illness. It should be further investigated to clarify the different responsiveness to cytokines by aging.

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A possible relationship of natural killer T cells with humoral immune response to 23-valent pneumococcal polysaccharide vaccine in clinical settings

Tomomitsu Miyasaka[a,1], Tetsuji Aoyagi[b,1], Binei Uchiyama[c,2], Kazunori Oishi[d], Toshinori Nakayama[e], Yuki Kinjo[f], Yoshitsugu Miyazaki[f], Hiroyuki Kunishima[b], Yoichi Hirakata[b], Mitsuo Kaku[b], Kazuyoshi Kawakami[a,*]

[a] Department of Medical Microbiology, Mycology and Immunology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[b] Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[c] Division of Respiratory Diseases, Internal Medicine, Katta General Hospital, Shiroishi, Miyagi, Japan
[d] Laboratory of Clinical Research on Infectious Diseases, Department of Special Pathogens, Research Institute for Microbial Disease, Osaka University, Suita, Osaka, Japan
[e] Department of Medical Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan
[f] Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases, Tokyo, Japan

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ABSTRACT

Pneumococcal polysaccharide vaccine (PPV), a type-2 thymus-independent antigen, induces the activation of B cells by directly triggering their antigen receptors. Although this type of antigen generally does not undergo class switching from IgM to IgG, PPV has been known to induce IgG2 in vaccinated subjects, which suggests the possible involvement of certain innate immune lymphocytes supporting the activation of B cells and their class switching. In the present study, we addressed the possibility that natural killer (NK) T cells are involved in Ab production caused by PPV. We measured serum levels of IgG against pneumococcal capsular polysaccharides and the numbers of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ double negative (DN) invariant NKT (iNKT) cells and $CD3^+CD56^+$ NKT cells in the peripheral blood before and after PPV injection. IgG was increased after PPV injection, peaking at 4 weeks after injection in serotypes 6B, 19F and 23F and at 3 months in serotype 14. Low responders, whose serum concentrations of IgG peaked at less than double their original levels, constituted 16%, 13%, 13% and 16% of vaccinated subjects with regard to serotypes 6B, 14, 19F and 23F, respectively. A significant positive correlation was detected between an increase in DN iNKT cells and the elevation of anti-serotype 14 IgG; in serotype 19F, DN iNKT cells were more markedly increased in responders than in low responders. These results suggest that DN iNKT cells may be involved in IgG production caused by vaccination against pneumococcal capsular polysaccharides.

1. Introduction

Streptococcus pneumoniae is a major bacterial agent which causes community-acquired pneumonia as well as other invasive diseases, such as bacteremia and meningitis, which arise as complications of pneumonia in 15–30% of cases [1]. The incidence rate of pneumococcal bacteremia is 18 to 30 per 100,000 in the general population, but can be as high as 56 to 83 per 100,000, especially in people aged 65 years or over in the USA [2–5]. In Japan, pneumonia is the fourth leading cause of death, and *S. pneumoniae* is a leading causative agent of pneumonia, being detected in 23% of community-acquired pneumonia cases [6]. This bacterium is also frequently detected as an etiologic agent in secondary pneumonia arising as a complication of the flu [7–9]. Morens and co-workers have demonstrated that the majority of deaths in the 1918–1919 influenza pandemic resulted directly from secondary bacterial pneumonia caused by common upper respiratory-tract bacteria, among which *S. pneumoniae* was most frequently detected in autopsy lung samples [10].

To prevent these pneumococcal diseases, 23-valent pneumococcal polysaccharide vaccine (PPV) is used for people aged 65 years or older and younger people with certain risk factors such as chronic cardiopulmonary diseases [11]. PPV is a type 2 thymus-independent (TI-2) antigen, which does not require helper T cells for the activation of B cells [12]. While thymus-dependent (TD) antigens activate B cells *via* engagement of CD40 by CD40L

* Corresponding author at: Department of Medical Microbiology, Mycology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai-shi, Miyagi 980-8575, Japan. Tel.: +81 22 717 7946; fax: +81 22 717 7910.
E-mail address: kawakami@med.tohoku.ac.jp (K. Kawakami).
[1] TM and TA contributed equally to this study.
[2] Present address: Department of Respiratory Diseases, Miyagi Cardiovascular and Respiratory Center, Kurihara, Miyagi, Japan.

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during cognate interaction with helper T cells, TI-2 antigens directly trigger surface immunoglobulin for the activation of B cells [13]. These different types of antigen produce distinct humoral immune responses: TD-antigens undergo class switching from IgM to IgG, which causes affinity maturation of Ab and induces memory B cell response; TI-2 antigens, on the other hand, do not [14]. Although PPV is a TI-2 antigen, Barrett and Ayoub [15] have found that it induces the restriction of production of IgG2 specific for pneumococcal polysaccharides. Snapper and co-workers [16] have reported that interferon (IFN)-γ contributes to Ab class switching to IgG3 in mice, which corresponds to IgG2 in humans, after PPV administration [15]. These findings suggest that a certain group of innate immune cells may be involved in the activation of B cells and Ab class switching caused by PPV.

Natural killer (NK) T cells, which express both αβ T cell antigen receptors and NK cell markers, have been identified as a novel lymphocyte population that acts in the innate stages of immune responses [17]. A major subset of NKT cells is the invariant NKT (iNKT) cells, which possess an extremely limited repertoire with antigen receptors consisting of Vα14-Jα18 in mice and Vα24-Jα18 in humans [18]. These cells recognize glycolipid antigens, such as α-galactosylceramide (α-GalCer), in the context of CD1d molecules on dendritic cells [19], which leads to the rapid production of IFN-γ and IL-4 [20,21]. iNKT cells are concentrated in the thymus, liver and bone marrow in mice [20,22] and occur at a rate of approximately 0.05% in human peripheral blood [23]. In our previous studies using a mouse model [24], iNKT cells were observed to play a critical role in neutrophilic inflammatory responses to and host defense against pneumococcal infection through production of IFN-γ. Interestingly, Kobrynski and co-workers have demonstrated that Ab production after PPV injection was completely abrogated in mice lacking iNKT cells [25]. These earlier observations raised the possibility that iNKT cells may contribute to Ab production and class switching caused by the administration of PPV.

In the present study, to address this possibility in a clinical setting, we analyzed the relationship between serum concentrations of Ab against pneumococcal capsular polysaccharides and the number of $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ double negative (DN) iNKT cells in the peripheral blood of subjects who received PPV administration. We found that DN iNKT cell counts increased, and that this increase was positively correlated with the production of IgG against a certain serotype of *S. pneumoniae*.

2. Materials and methods

2.1. Subjects

Fifty-five outpatients with chronic respiratory diseases were vaccinated with 0.5 ml of PPV (Pneumovax®, Banyu Pharmaceutical Co., Tokyo, Japan), intramuscularly, at the Department of Respiratory Diseases, Katta General Hospital, Shiroishi-shi, Miyagi, Japan between July 2006 and August 2008 after giving informed consent. The PPV23 contained 25 μg each of 23 different types of pneumococcal polysaccharide antigen (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). Serum samples were collected prior to vaccination and at 2 weeks, 4 weeks, 3 months, 6 months and 1 year after vaccination. The average age of these subjects was 74.4 years (range 61–88 years); 67.3% of subjects were male, 43.6% were smokers, and 10.9% were receiving glucocorticoid therapy. The most common chronic respiratory diseases among these patients were chronic obstructive pulmonary disease, bronchial asthma, bronchiectasis and old pulmonary tuberculosis (Table 1). This study was approved by the institutional ethics committees of Tohoku University, Sendai, Japan (#2005-233) and Katta General Hospital. We also paid the utmost attention to ensure that personal information was handled in compliance with our institutions' guidelines.

Table 1
Clinical characteristics of all subjects ($n = 55$).

	Number (%)
Males	37 (67.3)
Smoking	24 (43.6)
Alcohol abuse	1 (1.8)
Underlying diseases	
COPD	20 (36.4)
Bronchial asthma	13 (23.6)
Bronchiectasis	2 (3.6)
Old pulmonary tuberculosis	6 (10.9)
Chronic cardiovascular diseases	5 (9.1)
Immunosuppressive conditions	1 (1.8)
Chronic renal failure	1 (1.8)
Chronic liver diseases	1 (1.8)
Diabetes mellitus	8 (14.5)
Treatment with glucocorticoids	6 (10.9)
Home oxygen therapy	6 (10.9)

Mean age (yr) ± SD = 74.4 ± 6.6.

2.2. Measurement of anti-pneumococcal capsular polysaccharide Ab

Serotype-specific antibodies against 6B, 14, 19F and 23F (American Type Culture Collection, Manassas, VA, USA) were measured by means of a third-generation Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [26] after absorption of non-specific antigens to cell wall polysaccharide (CWP: Statens Serum Institute, Copenhagen, Denmark) and serotype 22F (American Type Culture Collection). In brief, microtiter plates (MICROLON: Greiner Bio-One, Frickenhausen, Germany) were coated individually with 100 μl of a polysaccharide antigen: either 5 μg/ml of 6B, 2.5 μg/ml of 14, 5 μg/ml of 19F or 2.5 μg/ml of 23F, in PBS. After five hours of incubation at 37 °C, these plates were stored at 4 °C until use, which occurred within 6 months. Prior to testing, the sera from our patients and U.S. anti-pneumococcal reference serum [89-SF: kindly provided by Dr. Milan S. Blake (Food and Drug Administration, Silver Spring, MD, USA)] were also stored at −80 °C. Serum samples and 89-SF were diluted with an absorption buffer of 0.05% Tween-20 PBS to 1:50 and 1:100, respectively, and incubated at room temperature for 30 min. Next, serial two-fold dilution of these sera to 1:51200 were performed arbitrarily; the resulting solutions were added to the wells and incubated at 37 °C for 1 h. After the microtiter plates were washed, a detection antibody, consisting of AP-conjugated goat anti-human IgM or IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:2000, was added to each well. *p*-nitro phenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA) was dissolved with 1 mol/l of diethanolamin (Sigma–Aldrich) to a concentration of 1 mg/ml as a substrate solution. Then, after the plates were washed again, this substrate was added to the wells and incubated at room temperature. Sodium hydroxide was added at 3 M to stop the enzyme reaction, and the absorbance values were detected at 405 nm as well as at 600 nm for reference. The concentrations of IgM and IgG Abs were calculated on the basis of a reference standard based on the 89-SF absorbance and expressed as μg/ml.

2.3. Flow cytometric analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were collected from patients before vaccination and at 2 weeks, 4 weeks, 3 months and 6 months after vaccination. After Fc receptors on the cell surface were blocked, PBMCs were stained with FITC-anti CD3 [Clone: UCHT1 (eBioscience, San Diego, CA, USA)] and PE-anti CD56 [Clone:

B159 (BD Biosciences, Franklin Lakes, NJ, USA)] mAbs and PE-α-galactosylceramide (α-GalCer)-conjugated CD1d tetramer. These cells were also stained with APC-anti-CD4 and -CD8 mAbs [Clones: RPA-T4 and RPA-T8 (eBioscience), respectively]. Isotype control IgG (eBioscience) for each Ab and PE-α-GalCer-unconjugated CD1d tetramer were used as references. Flow cytometric analysis was performed using a Cytomics FC500 cytometry system (Beckman Coulter, Fullerton, CA, USA). The number of NKT cells (/μl) was calculated as follows: white blood cell (WBC) counts (100/μl) × % of lymphocytes in WBC/100 × % of NKT cells in lymphocytes. The WBC counts and % of lymphocytes were measured in blood samples collected from the patients during routine examinations.

2.4. Statistical analysis

Ab concentrations in sera, fold increases after vaccination and number of NKT cells in peripheral blood are expressed as geometric means. The concentrations of serum Ab and degrees of change in NKT cell counts during the first 2 weeks after vaccination were compared between responders and low responders using the Mann–Whitney *U*-test. The concentrations of serum Ab between pre- and peak levels were compared using Wilcoxon *t*-test. The correlation between the degree of change from pre-vaccination to peak levels of anti-pneumococcal IgG and the degree of change in NKT cell counts during the first 2 weeks post-vaccination was tested using Spearman's correlation test. A p value less than 0.05 was considered significant.

3. Results

3.1. Serum levels of anti-pneumococcal Ab after vaccination

Initially, we measured the concentrations of IgM anti-pneumococcal Ab against serotypes 6B, 14, 19F and 23F in 15 subjects at various time intervals after pneumococcal vaccination. As shown in Fig. 1A, the pre-vaccination levels of IgM Ab were 0.91, 0.59, 1.04 and 0.26 μg/ml for serotypes 6B, 14, 19F and 23F, respectively, and these levels were not altered during the six months post-vaccination.

Next, we measured the concentrations of IgG anti-pneumococcal Ab against the same serotypes in 55 subjects. As shown in Fig. 1B, in contrast to IgM Ab, IgG Ab began to increase during the second week, reached its peak at the fourth week for serotypes 6B, 19F and 23F and at the third month for serotype 14, then decreased one year after vaccination. For all the serotypes, the peak values were significantly higher than the values measured before vaccination (1.60 *vs.* 4.53, 3.04 *vs.* 12.87, 2.98 *vs.* 7.73 and 1.69 *vs.* 6.32 μg/ml for serotypes 6B, 14, 19F and 23F, respectively). One year post-vaccination, IgG levels had decreased from the peak levels by 12.3%, 37.8%, 25.2% and 41.5% for serotypes 6B, 14, 19F and 23F, respectively.

3.2. Responders and low responders

The individuals who received PPV administration were divided into two groups based on their responsiveness, *i.e.* responders and low responders. Here, we defined responders as individuals whose peak IgG levels were more than twice their IgG levels before vaccination, and low responders as individuals whose serum IgG concentrations were less than 2 μg/ml before vaccination and whose peak IgG levels were less than twice their IgG levels before vaccination. By these definitions, 62%, 62%, 45%, and 65% of the 55 vaccinated individuals were responders, and 16%, 13%, 13%, and 16% were low responders, with regard to the serotypes 6B, 14, 19F and 23F, respectively. As shown in Table 2, for all serotypes, peak IgG levels were significantly higher than IgG levels measured before

Table 2
Serotype-specific antibody levels in responders and low responders.

Serotype	Time point	Geometric mean concentrations (μg/ml) (95% CI)			Geometric mean increase from pre-vaccination to peak concentration (*n*-fold) (range)		
		All subjects (n = 55)	Responders[a]	Low responders[b]	All subjects (n = 55)	Responders[a]	Low responders[b]
6B	Pre	1.60 (1.20–2.14)	1.08 (0.76–1.52)	1.33 (1.10–1.60)			
	Peak	4.53 (3.44–5.95)**	4.56 (3.04–6.84)**	2.02 (1.57–2.59)*#	2.83 (0.89–78.89)	4.24 (2.00–78.89)	1.52 (0.11–1.92)
14	Pre	3.04 (1.96–4.70)	2.20 (1.38–3.50)	0.74 (0.35–1.60)			
	Peak	12.87 (8.46–19.59)**	21.14 (13.88–32.19)**	1.11 (0.52–2.37)$	4.24 (0.68–120.18)	9.61 (2.11–120.18)	1.49 (0.86–1.93)
19F	Pre	2.98 (2.20–4.03)	2.04 (1.41–2.95)	1.06 (0.86–1.30)			
	Peak	7.73 (5.70–10.49)**	11.23 (7.55–16.72)**	1.51 (1.18–1.93)$	2.60 (0.81–49.48)	5.51 (2.03–49.48)	1.43 (1.09–1.90)
23F	Pre	1.69 (1.18–2.43)	1.43 (0.95–2.15)	0.61 (0.35–1.06)			
	Peak	6.32 (4.18–9.55)**	8.96 (5.44–14.76)**	0.83 (0.49–1.40)$	3.73 (0.97–60.62)	6.25 (2.03–60.62)	1.36 (0.97–1.67)

*p < 0.05, **p < 0.01, compared with pre-vaccination level; #p < 0.05, $$p$ < 0.01, compared with peak level in responders.
[a] Responders are 34, 33, 26 and 36 subjects for serotypes 6B, 14, 19F and 23F, respectively.
[b] Low responders are 9, 8, 10 and 9 for serotypes 6B, 14, 19F and 23F, respectively.