ントが含まれていないことから，不活化全粒子ワクチンの臨床応用への動きもある。安全性の面からもワクチンの詳細な作用機序の解明，または安全なアジュバントの開発が，安全にかつ効果的にワクチンを接種するためには必要となってくる。

おわりに

これまでに多くの自然免疫受容体が同定され，それらの機能解析が行われてきた。実際に，自然免疫研究の発展に伴いワクチンやアジュバントの作用機序が明らかとなりつつある。それと同時に，アジュバントの重要性も示されてきた。これまではアラムアジュバントが主流であったが，今後は各ワクチンに適したアジュバントの開発が行われるであろう。その際に，ワクチン，アジュバントともに作用機序が明らかとなっていることは安全性の面から非常に重要である。現在，不活化全粒子ワクチンからコンポーネントワクチンへと需要がシフトしている。こういった背景からも，新規ワクチン開発におけるアジュバントの重要性はいうまでもない。さらなる免疫学の発展とともに，効果的でより安全なワクチン，アジュバントが開発されることを期待する。

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

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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 WIV, 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

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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%)

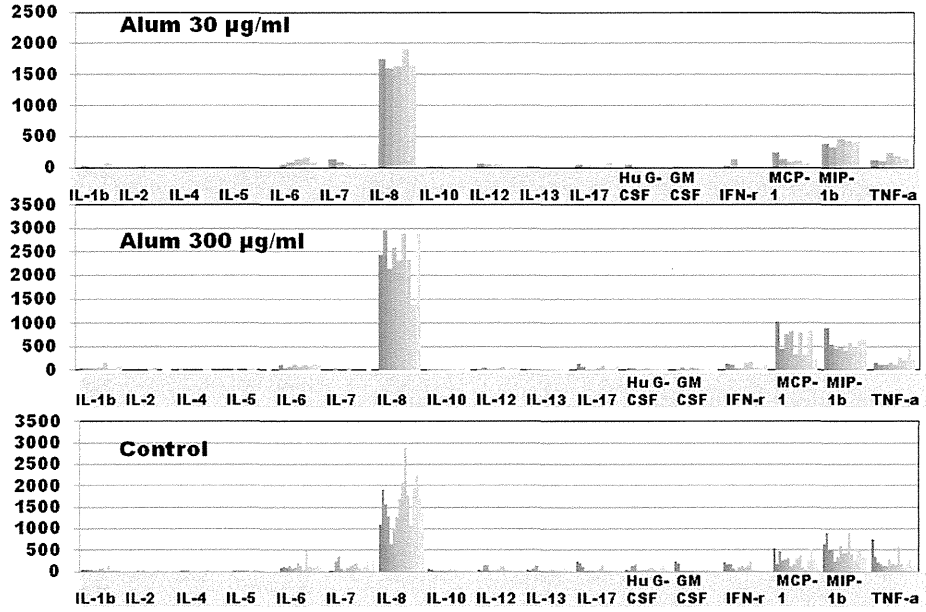

Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 μg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 μg/ml (1:10 dilution).

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

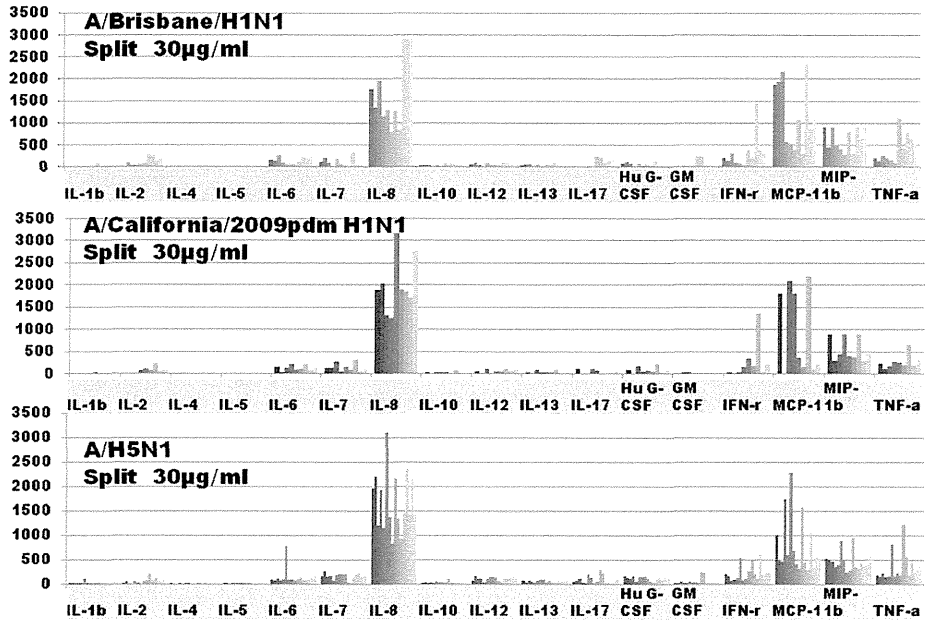

Fig. 2. Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of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

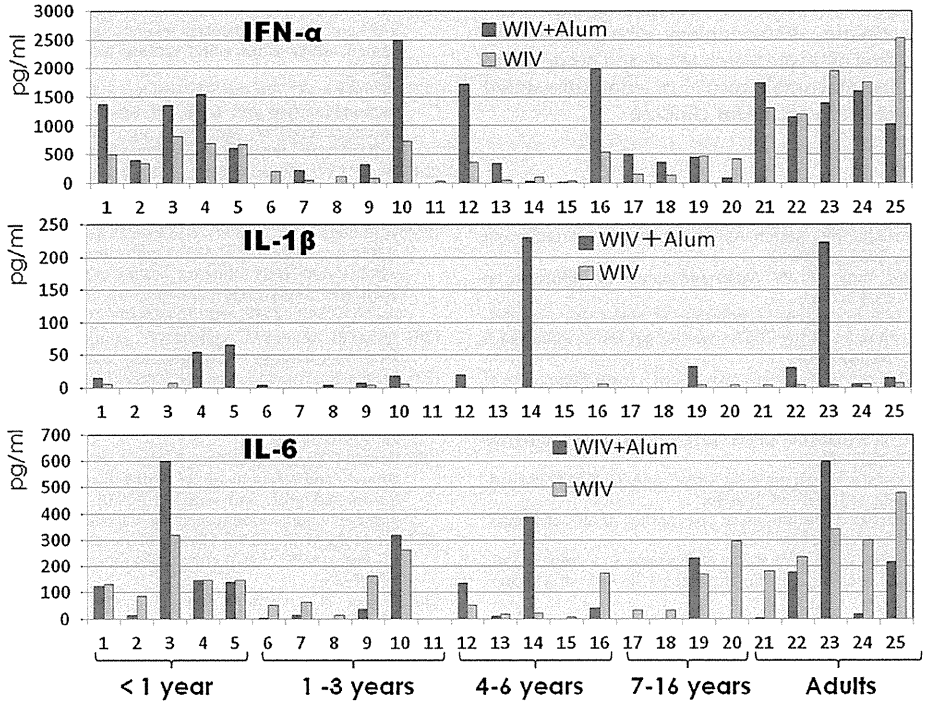

Fig. 3. IFN-α, IL-1β, and IL-6 production. IFN-α, IL-1β, and IL-6 were measured by EIA in PBMC cultures. PBMC were stimulated with H5 WIV and alum-adjuvanted WIV vaccine materials. Samples 1–5 were obtained from healthy individuals less than one year, those 6–11 from 1 to 3 years of age, those 12–16 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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Review

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

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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.

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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.

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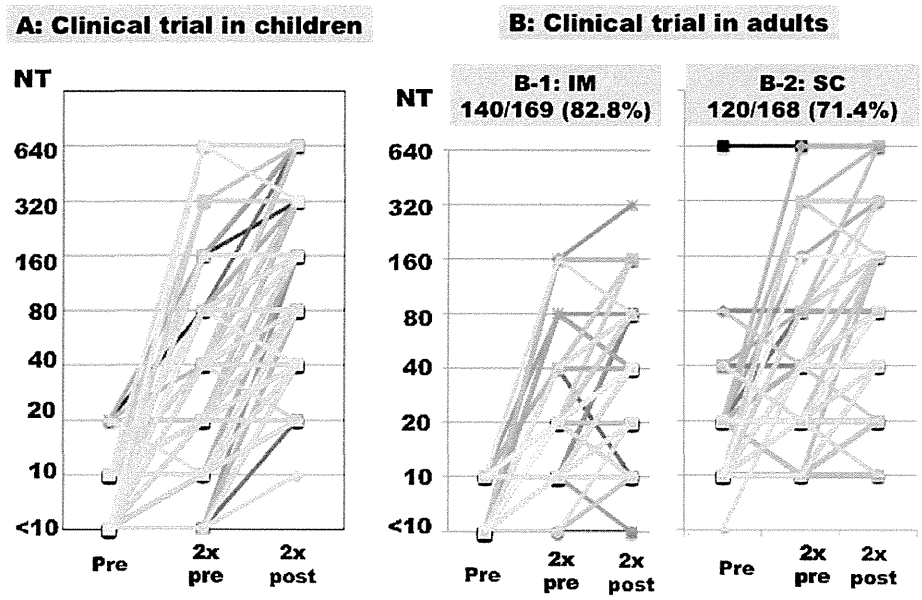

Fig. 1. Development of NT antibodies in clinical trials. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). The development of NT antibodies is shown in a clinical trial in children (A), in adults for intramuscular immunization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

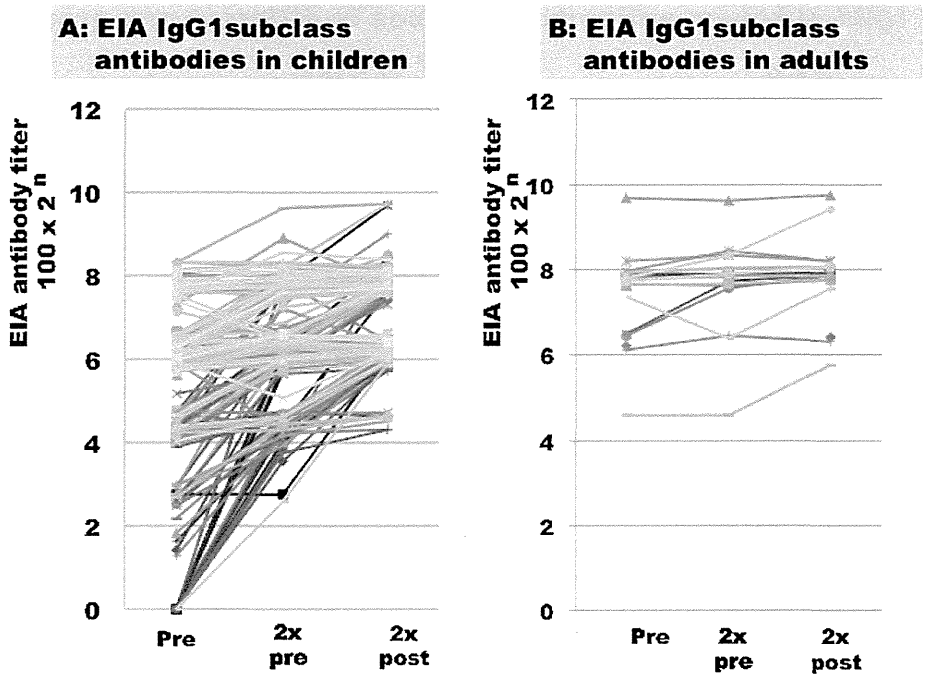

Fig. 2. IgG1 antibody responses in children and adults. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA antibody titers are shown in *Y*-axis for 100×2^n. EIA IgG1 subclass response in children is shown in panel A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

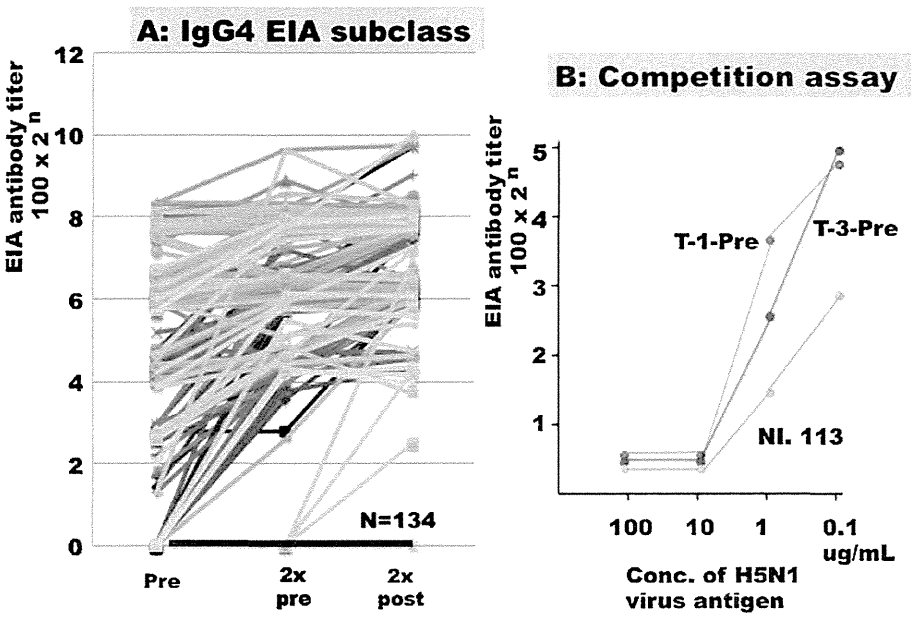

Fig. 3. IgG4 responses in children and the results of competition assay. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA IgG4 antibody titers are shown in *Y*-axis for 100×2^n in panel A. Among 193 subjects, 134 had 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.

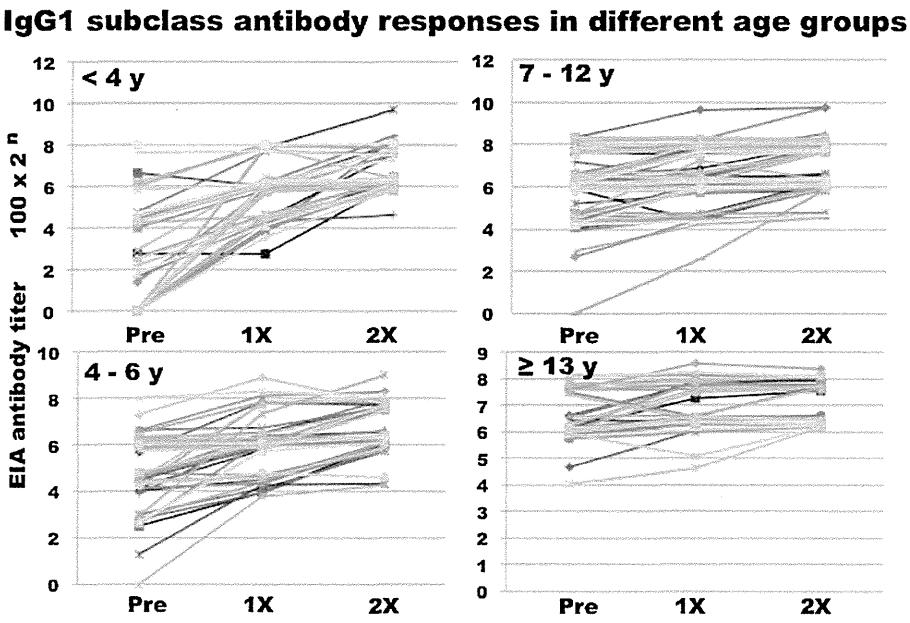

Fig. 4. Different IgG1 responses in different age groups: ≤4 years, 4–6 years, 7–12 years, and ≥13 years. Serum samples were obtained before the first dose (Pre) and before (1X) and four weeks after the second immunization (2X). IgG1 antibody titers are shown in *Y*-axis for 100×2^n.

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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Rapid Communication

Detection of pandemic influenza A (H1N1) 2009 virus RNA by real-time reverse transcription polymerase chain reaction

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Key words influenza A(H1N1), pandemics, real-time, reverse transcription polymerase chain reaction.

The pandemic influenza A(H1N1) 2009 (A(H1N1)pdm09) virus emerged in Mexico in April 2009 and spread rapidly worldwide.[1] The virus caused severe illness, including pneumonia, which led to hospitalization.[2,3] The high-risk groups for A(H1N1)pdm09 include pregnant women, children <5 years old, elderly people, and people with chronic diseases, such as bronchial asthma and diabetes.[3,4] The virus isolation method is widely used but it occasionally takes several days to identify typical cytopathic effects. Reverse transcription polymerase chain reaction (RT–PCR) is the method of choice for laboratory confirmation of A(H1N1)pdm09. The advantage of real-time RT–PCR is that it allows quantitative assessment of the number of virus gene copies in clinical samples. In the present study, we developed a real-time RT–PCR assay for detecting A(H1N1)pdm09 virus RNA in clinical samples.

Methods

Patients and specimen collection

From August to December 2009, specimens were collected from patients with influenza-like illness at hospitals in Kyoto City for laboratory confirmation. Informed consent was obtained from all patients or their guardians. Sixty-eight nasal swabs were collected from hospitalized patients at 12–48 h after onset of fever, before administration of antiviral drugs. These patients required hospitalization because of their clinical symptoms, such as fever, cough and dyspnea, and all recovered within 7 days. The nasal swab specimens were suspended in 2 mL Eagle's Minimal Essential Medium (EMEM) supplemented with 10% of bovine plasma albumin (BPA). Sixteen nasal swabs for the rapid influenza diagnostic test (RIDT) were collected from outpatients and suspended in 320 μL diluent supplied with a commercially available RIDT (ESPLINE Influenza A & B-N, FUJIREBIO, Tokyo, Japan). The RIDT was performed according to the manufacturer's instructions. Two drops of each RIDT diluent suspension were dripped into the sampling area of the RIDT device. A reaction was confirmed by the appearance of blue lines, including reference and influenza A or B lines, in the sampling area after 15 min. When a blue reference line and a blue line at either of the A or B positions was observed, the sample was judged positive for influenza A or B. The remainder of 16 influenza-A-positive RIDT diluents was used for real-time RT–PCR. The median age of the hospitalized patients and outpatients was 7.5 years (range: 3 months–75 years). Two patients were admitted to intensive care units and required mechanical ventilation due to respiratory failure. Two hundred microliters of bronchial aspirate specimens was collected on day 18 after fever onset from a 59-year-old patient and on days 3, 5 and 11 after fever onset from a 61-year-old patient. Nasal swab specimens were not collected from these patients. Bronchial aspirate specimens were suspended in 2 mL EMEM supplemented with 10% BPA. The nasal swabs, diluent suspensions for RIDT and bronchial aspirates were kept at 4–8°C at the hospitals at which they were collected, and transported to the Kyoto City Institute of Health and Environmental Science in a transport box at 4–8°C within 24 h of sample collection. Three types of specimens, nasal swabs, bronchial aspirates and diluent suspensions for the RIDT were used for RNA extraction. Thirty-eight nasal swabs suspended in EMEM were used for virus isolation. The samples were kept at 4–8°C until RNA extraction and inoculation for virus isolation.

Virus isolation

Madin-Darby canine kidney (MDCK) cells were inoculated with 100 μL EMEM and incubated in 24-well plates at 33.5°C. When cytopathic effects were observed, the supernatants were harvested, and the virus subtypes were identified using a hemagglutination inhibition test.

Real-time RT–PCR

RNA was extracted from 140 μL of each sample with the QIAamp Viral RNA kit (Qiagen, Tokyo, Japan). RNA was suspended in 60 μL AVE buffer and stored at −80°C. Real-time RT–PCR for detection of A(H1N1)pdm09 virus RNA was performed as described previously.[5] The following primers and TaqMan probe for the hemagglutinin (H) gene of

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A(H1N1)pdm09 virus were used: forward, AGCAATTGAG CTCAGTGTCATCA (H gene position 359–381) and reverse, TGGGCCATGAACTTGTCTTG (H gene position 424-405) and probe FAM-AAAGGTTTGAGATATTCC-BHQ1(H gene position 386–403). RT–PCR was performed with QuantiTect Probe RT–PCR Kit (Qiagen) in a total volume of 25 μL. This included 12.5 μL 2× master mix, 2.0 μL 10 μM primers, 0.5 μL 10 μM probe, 0.25 μL QuantiTect RT-Mix, 0.1 μL RNase inhibitor (20 U/μL), 5 μL RNA template, and 2.65 μL RNAase-free water. Reverse transcription and amplification were performed at 50°C for 30 min, followed by 45 cycles of 94°C for 15 s and 54°C for 75 s using an ABI prism 7500 (Applied Biosystems [ABI], Tokyo, Japan). The threshold cycle (Ct) is the cycle in which the fluorescence intensity of a sample reaches the fluorescent-intensity threshold. The Ct value for each sample was determined using ABI 7500 v2.0.3 software, and a standard curve was then computed from the Ct values of the diluted standards. The absolute quantities of the unknown samples were then extrapolated based on their Ct values.

Synthetic RNA preparation

The H gene of a clinical isolate of A(H1N1)pdm09 virus (A/Kyoto/01/2009 [H1N1], DNA Data bank of Japan [DDBJ], accession number, AB675659) was amplified using primers based on flanking regions of the primer sets used for real-time RT–PCR (forward, AGAAAAGAATGTAACAGTAACACACT CTGT, and reverse, AATGTAGGATTTGCTGAGCTTTGG). DNA fragments were used to produce synthetic RNA transcripts for internal standards in real-time RT–PCR. The PCR products (H gene position 111–549, 439 bp) were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Tokyo, Japan). Sequencing confirmed that cloned DNA was identical to the H gene of A(H1N1)pdm09. Plasmid DNA was cleaved by *Xba*I and *Hin*dIII and subcloned into pGEM-3Zf(+). The pGEM-3Zf(+) vector contains T7 RNA polymerase promoters flanking a cloning region, including restriction sites for *Xba*I and *Hin*dIII. The cleaved DNA (H gene position 148–481) was inserted into the cloning region downstream of a T7 promoter from the 5′ to 3′ direction. The linear DNA templates were transcribed into RNA transcripts using the T7 RiboMax Express Large Scale RNA Production System (Promega, Tokyo, Japan). T7 RNA polymerase synthesized single-strand RNA from the template downstream of the promoter. The DNA template was removed with DNase following the transcription reaction. Concentration of synthetic RNA stock was determined by spectrophotometry at 260 nm. The final RNA copy number per 1 μL of the synthetic RNA stock was determined from the concentration of RNA and molecular weight. Ten serial dilutions of synthetic RNA containing 10–10^7 copies/μL were used as standard RNA for real-time RT–PCR.

Results

The copy number of synthetic A(H1N1)pdm09 virus RNA was 15.5 $\log_{10}$ RNA copies/μL. Serial 10-fold diluted synthetic RNA was subjected to real-time RT–PCR (Fig. 1). The detection limit was 10 copies of standard RNA per reaction tube. The concentration of standard RNA was strongly correlated with the Ct of 1–7 copies $\log_{10}$ RNA/μL. The standard curve had a slope of −3.632 ± 0.12 and a very high correlation coefficient (0.99 ± 0.01). Clinical samples from patients were tested using real-time RT–PCR. Data are shown as the copy number of virus RNA per 1 mL of EMEM or RIDT diluent. A(H1N1)pdm09 virus RNA was detected in 61 of 68 nasal swabs suspended in EMEM, all 16 RIDT diluent suspensions, and all four bronchial aspirates. These real-time RT–PCR-positive patients were considered to be laboratory-confirmed cases. The copy number of A(H1N1)pdm09 virus RNA in the clinical samples from the laboratory-confirmed cases is shown in Table 1. Thirty-four real-time RT–PCR-positive and four negative samples were subjected to virus isolation.

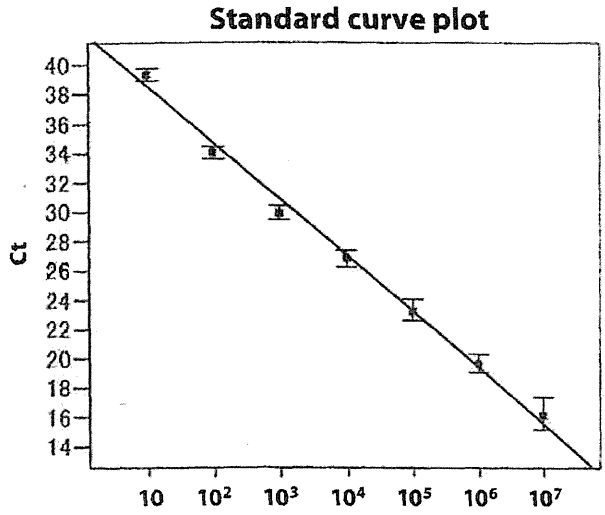

Fig. 1 Standard curve generated by real-time reverse transcription polymerase chain reaction. Standard pandemic influenza A(H1N1)2009 virus RNA was serially diluted from 10^7 to 10^1 copies/μL and amplified. Ct values were plotted against copy number. Quantity, copy number of influenza A(H1N1)2009 virus per reaction tube. Data indicate the mean ± SD Ct value from four experiments.

Table 1 Copy number of pandemic influenza A(H1N1)2009 virus RNA in clinical samples

Samples	*n*	Copy number (Log_{10} RNA copies/ml)[§]	
		Mean	Range
Nasal swabs (in 2 mL of EMEM)[†]	61	5.8	4.2–9.1
Nasal swabs (in RIDT diluent)[‡]	16	8.1	5.9–9.9
Bronchial aspirates (in 2 mL of EMEM)[†]	4	4.6	4.2–5.0

[†]Nasal swabs and bronchial aspirates were suspended in 2 mL EMEM. [‡]Nasal swabs were suspended in 320 μL diluent supplied with a commercially available RIDT. [§]Data indicate the copy number of virus RNA per 1 mL EMEM or RIDT diluent. EMEM, Eagle's Minimal Essential Medium; RIDT, rapid influenza diagnostic test.

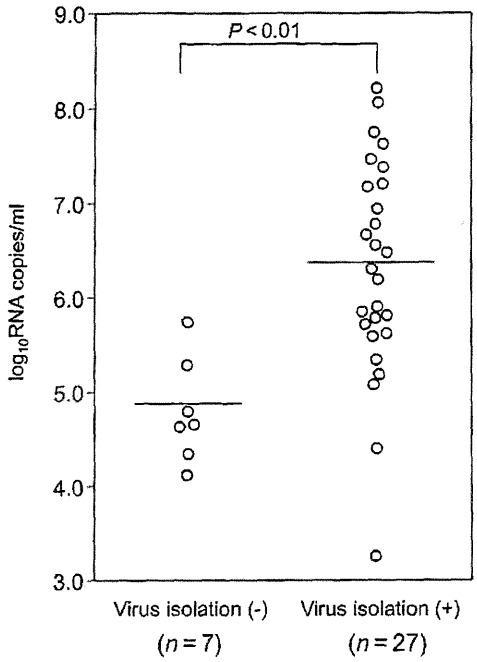

Fig. 2 Comparison of copy number of pandemic influenza A(H1N1)2009 virus RNA between virus-isolation-positive and -negative samples. Data indicate the copy number of virus RNA per 1 mL Eagle's Minimal Essential Medium. $P < 0.01$ (Mann–Whitney *U*-test).

A(H1N1)pdm09 virus was not isolated from four real-time RT–PCR-negative samples. Of 34 real-time RT–PCR-positive samples, the copy number of A(H1N1)pdm09 in virus-isolation-positive and -negative samples is shown in Figure 2. A(H1N1)pdm09 virus was isolated from 27 samples. The mean copy number of A(H1N1)pdm09 virus RNA in virus-isolation-positive and -negative samples was 6.3 and 4.8, respectively. The mean copy number of A(H1N1)pdm09 virus RNA in virus-isolation-positive samples was significantly higher than that of -negative samples ($P < 0.01$, Mann–Whitney *U*-test).

Discussion

We developed real-time RT–PCR for detecting A(H1N1)pdm09 virus RNA and found that the detection limit was 10 copies per reaction tube using synthetic standard RNA. The primers for real-time RT–PCR were designed for amplification of the negative strand of influenza virus RNA. The synthetic standard RNA was successfully amplified using one-step RT–PCR kit, suggesting that negative-strand RNA was synthesized. We demonstrated the copy number of A(H1N1)pdm09 virus RNA in clinical samples, including nasal swabs and bronchial aspirates suspended in EMEM from hospitalized patients, and nasal swabs suspended in RIDT diluent from outpatients. The nasal swabs and bronchial aspirates were suspended in different volumes of EMEM or RIDT diluent. The sampling procedure, such as the swabbing site in the nasal cavity, the swabbing time and the strength of swabbing, might have varied between the physicians performing the procedure, which might have affected the viral load attached to the swab. Therefore, it is difficult to assess the correlation between viral load and clinical severity.

Studies on the changes in viral load of patients with A(H1N1)pdm09 using real-time RT–PCR have been reported.[6,7] Clearance of A(H1N1)pdm09 viral load is slow in patients with acute respiratory distress syndrome.[8] However, the sampling procedures were not reported in these studies. A more prolonged period of viral shedding was observed in immunosuppressed patients, such as those undergoing hematopoietic stem cell transplantation.[9] Monitoring viral load throughout the clinical course is considered useful for predicting disease severity and evaluating effectiveness of antiviral therapy. To compare the viral load of patients using real-time RT–PCR assay, it is important that technical variations in sampling are minimized as much as possible.

It has been reported that real-time RT–PCR is a highly sensitive procedure for detecting A(H1N1)pdm09 when compared with conventional virus isolation methods.[9] Influenza virus RNA is detected from virus-isolation-negative samples by real-time RT–PCR.[10] As shown in the present study, the mean copy number of A(H1N1)pdm09 virus RNA in virus-isolation-positive samples was significantly higher than that of negative samples. We detected A(H1N1)pdm09 virus RNA in seven virus-isolation-negative samples. These samples were kept at 4–8°C until inoculation, however, samples might have lost their infectivity during the transportation or test procedures. These results suggest that the presence of infectious virus in the clinical samples is an important factor for virus isolation.

The present study shows that real-time RT–PCR can detect A(H1N1)pdm09 virus RNA from clinical samples containing viable and nonviable virus.

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インフルエンザワクチン
——その特徴と効果
Influenza vaccine：Its features and effectiveness

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◎現在，季節性インフルエンザ対策に使われているワクチンの剤型はスプリットワクチンであり，プライミング効果は劣るものの，優れたブースティング効果が認められている．注射で接種したワクチンで誘導される免疫の主体は血中IgG抗体であり，粘膜に滲み出ることで感染防御に働いている．赤血球凝集抑制抗体40倍は50%の成人の発症を予防する抗体価である．IgG抗体は変異したインフルエンザウイルスに対する反応がIgA抗体や細胞性免疫よりも劣る欠点がある．インフルエンザワクチンの効果が低いのは小児と高齢者である．今シーズン(2011/12)から小児への免疫原性を高めるために，接種量が世界標準量に増加した．アメリカでは高齢者の免疫原性を高めるために，成人接種量の4倍量のヘマアグルチニンを含むインフルエンザワクチンが使用されている．インフルエンザ発症予防のためには，高い血中抗体価を誘導しておくことが大切である．

Key word：インフルエンザ，インフルエンザワクチン，スプリットワクチン，免疫原性，パンデミック

インフルエンザウイルスは，核蛋白の抗原性からA型，B型，C型に分類される．A型インフルエンザウイルスの自然宿主はカモであり，多くの鳥類や哺乳類に感染するが，B型とC型はヒトのウイルスである．突然の高熱，頭痛，筋肉痛，関節痛などのインフルエンザ様症状(influenza like illness：ILI)を示すのはA型とB型である．B型はヤマガタ系とビクトリア系に大別される．

A型インフルエンザウイルスにはエンベロープ上に2種類の構造蛋白〔ヘマグルチニン(HA)とノイラミニダーゼ(NA)〕が存在する．HAは16種類，NAは9種類あるため，A型インフルエンザウイルスは理論上144種類の亜型が存在する．現在ヒトの間で流行している亜型はA(H1N1)とA(H3N2)である．2009年にパンデミックを起こしたウイルスはA旧ソ連型と免疫原性が大きく異なるA(H1N1)亜型である．毎年ヒトの間では規模の大小はあるもののA(H1N1)，A(H3N2)，B型が流行してILIを発症させるため，毎年のインフルエンザワクチン接種が勧められている．本稿では，インフルエンザワクチンの特徴と効果について解説する．

インフルエンザワクチンの剤型(表1)

インフルエンザワクチンには毎年のインフルエンザ流行に備える季節性インフルエンザワクチンと，現在ヒトの間で流行しているA型インフルエンザウイルス亜型と異なる亜型が出現したときに備えて準備しているプロトタイプワクチンとがある．わが国で現在使用している季節性インフルエンザワクチンの剤型は発育鶏卵で増殖したインフルエンザウイルス全粒子を精製，不活化した後，接種局所の副反応や発熱に関与しているエンベロープをエーテル処理で取り除いたスプリットワクチンである．感染防御抗原(「サイドメモ1」参照)であるHAを分離精製していることからHAワクチンともよばれている．皮下注射で接種する．

スプリットワクチンは全粒子ワクチンに比べプライミング効果は劣るが，ブースティング効果は認められている(「サイドメモ2」参照)．現在カイ

表 1　インフルエンザワクチンの剤型と免疫効果

剤型	各インフルエンザウイルスのHA量(μg/dose)	免疫効果	
		プライミング	ブースティング
季節性インフルエンザワクチン			
不活化ワクチン			
全粒子ワクチン	15	＋	＋
スプリットワクチン			
通常量ワクチン	15	±	＋
高用量ワクチン*	60	±	＋＋
皮内接種用ワクチン*	9	±	＋
サブユニットワクチン†	開発中	−？	＋
ビロゾーマルワクチン†	15	＋	＋
生ワクチン	15	＋＋	＋
プロトタイプワクチン(不活化ワクチン)			
全粒子ワクチン	15	＋	＋
アルミアジュバント加全粒子ワクチン	15	＋＋	＋
スクワレン系アジュバント加スプリットワクチン	15/7.5‡	＋＋＋	＋＋

HA量：インフルエンザワクチンに含まれる各コンポーネントのHA(ヘマグルチニン)量.
*：日本では認可されていないが，アメリカで認可されている.
†：サブユニットワクチンは培養細胞でHA蛋白を増幅させて製造されたワクチン，ビロゾーマルワクチンはビロゾームにHAとノイラミニダーゼ(NA)を付着させたワクチンでヨーロッパで使用.
‡：MF59を用いているノバルティスのHA量は15μg/dose，AS03を用いているグラクソスミスクラインのHA量は7.5μg/dose.

コ由来細胞にHA遺伝子を挿入して増生させたHAを精製したサブユニットワクチンの開発が行われている[1]．サブユニットワクチンのプライミング効果も不十分である.

皮内接種は少ない抗原量で皮下接種や筋肉接種と同等の免疫原性が認められている．アメリカでは2011/12シーズンから，スプリットワクチンを用いた皮内接種用インフルエンザワクチンが認可された[2]．接種抗原量は9μgである．0.1 mL皮内接種する.

アメリカやロシアでは経鼻接種するインフルエンザ生ワクチンが使用されている．3種類の温度変異株(「サイドメモ3」参照)を親株とし，HAとNAをそのシーズンのワクチン株に組み換えて製造する．インフルエンザの免疫がない小児に接種すると感冒様症状が出現するリスクが高く，成人

サイドメモ1　感染防御抗原

感染防御抗原とは，ウイルスが細胞に感染するときに中心的役割を果たすウイルス蛋白のことであり，これらの蛋白に対する抗体が感染防御の中心的役割を担っている．インフルエンザウイルスのヘマグルチニン(HA)，B型肝炎ウイルスのHBs抗原，麻疹ウイルスのH蛋白とF蛋白などが代表である.

サイドメモ2　プライミングとブースティング

免疫に関与する細胞群として抗原提示細胞，免疫未熟細胞，免疫記憶細胞，免疫実行細胞がある．抗原提示細胞は生体に入った異物を認識し，免疫未熟細胞に情報を提示する細胞群で，樹状細胞，Langerhans細胞などのマクロファージ系の細胞である．免疫情報の提示を受けた免疫未熟細胞(Tho細胞，Bo細胞)は，成熟して免疫記憶細胞(Th_1細胞，Th_2細胞，B細胞)になる．免疫記憶細胞は免疫実行細胞(形質細胞，キラーT細胞)を誘導し抗体を産生させる．免疫記憶細胞をおよび免疫実行細胞を誘導することをプライミングといい，誘導された免疫実行細胞の数を増加させ，免疫を高めることをブースティングという．生ワクチンでは一度に大量の免疫実行細胞を誘導することができるが，不活化ワクチンでは2回以上接種してまずプライミングし，4〜6カ月後以降にブースティングする．一度誘導された免疫記憶細胞は消失しないので，免疫記憶細胞が誘導されていると4〜6カ月後以降ならばいつでもブースティングが認められる．なお，キラーT細胞は生きたウイルスでないと誘導できない.

表 2　インフルエンザワクチンと麻疹ワクチンの比較

項目	インフルエンザ	麻疹
発症させるウイルス	A(H1N1)，A(H3N2)，B	1種類
ウイルスの変異	連続変異しやすい	ゆっくりと変異
	不連続変異あり	不連続変異なし
感染症の病態	局所性ウイルス感染症	全身性ウイルス感染症
感染予防	sIgA 抗体，血中抗体，CMI	血中抗体，CMI*
発症予防抗体価		
50%予防	HI 抗体 40 倍	
90%予防	HI 抗体 160 倍	120 mIU/mL†
感染予防抗体価	発症予防抗体価と同じ	800 mIU/mL†
ワクチン		
剤型	スプリットワクチン(HA)‡	生ワクチン
抗体価の半減期	半年	約 3 年
接種後の発症予防抗体価	≧70%**	≧95%
接種回数	毎年 1 回	生涯 2 回

sIgA：分泌型 IgA，CMI：細胞性免疫，HI：赤血球凝集抑制，HA：ヘマグルチニン．
*：麻疹では，CMI は感染からの回復に重要な役割を果たしている．
†：国際単位，≧120 mIU/mL は中和抗体で≧4 倍，≧750 mIU/mL は中和抗体で≧32 倍．
‡：エーテル処理によりウイルスの立体構造をこわし，HA を分離精製している．
**：ヨーロッパ医薬品庁が定めるインフルエンザワクチン評価基準における抗体陽性率，HI 抗体 40 倍以上の割合．

ではスプリットワクチンと比べてブースティング効果が劣っている．なお，生ワクチンでは分泌型 IgA(sIgA)抗体と細胞性免疫が賦活されるため，ウイルスの変異に対する対応力が優れている[3,4]．アメリカでは 2〜49 歳が接種対象者である．

多くの人が免疫をもたない新型インフルエンザウイルス対策用にわが国で準備されているプロトタイプワクチンの剤型は，発育鶏卵で増殖させたウイルス全粒子をアルミアジュバントと反応させたワクチンである(アルミアジュバント加全粒子ワクチン)[5]．現在培養細胞で増殖させたウイルスを用いたプロトタイプワクチンの開発が行われている．

一方，ヨーロッパではスプリットワクチンにスクワレン系アジュバントを加えたインフルエンザワクチンを，アメリカでは Vero 細胞で増殖させたインフルエンザウイルスを用いた全粒子ワクチンをプロトタイプワクチンとして準備している．いずれのワクチンもプライミング効果とブースティング効果，さらにブースティングによる交差免疫の誘導が認められている．

サイドメモ 3

温度変異株(ts mutant)

一般にヒトに感染するウイルスは 37℃で効率よく増殖し，高温(39℃)でも比較的よく増殖する．温度変異株とは野生株と比べて高温での増殖が低下した株であり，麻疹ワクチン株である AIK-C 株が代表である．多くの生ワクチン株は温度変異性をもっている．インフルエンザ生ワクチンの製造に使用される 3 種類の親株(ワクチン製造の鋳型になる株)はいずれも上気道の温度である 33℃で増殖効率がよく，37℃では増殖効率が劣る温度変異株である．

インフルエンザの病態と発症予防(表 2)

インフルエンザは気道にウイルスが感染して症状が出現する局所性ウイルス感染症であり，ウイルス血症は認められない．局所性ウイルス感染症でワクチンが開発されているのはインフルエンザだけである．

インフルエンザの発症予防および回復には，sIgA 抗体，血中 IgG 抗体，細胞性免疫が関与している．血中 IgG 抗体はほぼ同じ濃度が気道粘膜に滲み出る．血中赤血球凝集抑制(hemagglutination inhibition：HI)抗体が高いほど，発症予防効果が優れている．全身性ウイルス感染症の発症予防レベルは 90%以上のヒトの発症を予防する抗