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H．知的財産権の出願・登録状況

1．特許取得（出願）

1) 特許公開２００５－９７２６７「粘膜免疫誘導アジュバントを含む新規ワクチン」

2) 特許公開２００９－２４２３６７「混合免疫賦活剤を含む新規ワクチン」

2．実用新案登録

なし

# Ⅱ．研究成果の刊行に関する一覧表

# 研究成果の刊行に関する一覧表

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# Ⅲ. 研究成果の刊行物・別刷

# Cross-Protection against H5N1 Influenza Virus Infection Is Afforded by Intranasal Inoculation with Seasonal Trivalent Inactivated Influenza Vaccine

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***Background.*** Avian H5N1 influenza A virus is an emerging pathogen with the potential to cause substantial human morbidity and mortality. We evaluated the ability of currently licensed seasonal influenza vaccine to confer cross-protection against highly pathogenic H5N1 influenza virus in mice.

***Methods.*** BALB/c mice were inoculated 3 times, either intranasally or subcutaneously, with the trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season. The vaccine included A/NewCaledonia/20/99 (H1N1), A/NewYork/55/2004 (H3N2), and B/Shanghai/361/2002 viral strains and was administered together with poly(I):poly($C_{12}U$) (Ampligen) as an adjuvant. At 14 days after the final inoculation, the inoculated mice were challenged with either the A/HongKong/483/97, the A/Vietnam/1194/04, or the A/Indonesia/6/05 strain of H5N1 influenza virus.

***Results.*** Compared with noninoculated mice, those inoculated intranasally manifested cross-reactivity of mucosal IgA and serum IgG with H5N1 virus, as well as both a reduced H5N1 virus titer in nasal-wash samples and increased survival, after challenge with H5N1 virus. Subcutaneous inoculation did not induce a cross-reactive IgA response and did not afford protection against H5N1 viral infection.

***Conclusions.*** Intranasal inoculation with annual influenza vaccine plus the Toll-like receptor–3 agonist, poly(I):poly($C_{12}U$), may overcome the problem of a limited supply of H5N1 virus vaccine by providing cross-protective mucosal immunity against H5N1 viruses with pandemic potential.

In 1997, people in the Hong Kong area became infected with a highly pathogenic avian influenza A virus, H5N1, apparently before that virus adapted to a mammalian species [1–3]. Of the 18 patients who developed respiratory disease, 3 died. The World Health Organization has reported 168 deaths for 278 cases of laboratory-confirmed infection with H5N1 avian influenza, in Southeast Asia, Europe, and Africa, between January 2003 and March 2007. In some instances, human-to-human transmission of the H5N1 virus appears to have occurred [4], suggesting that this virus has the potential to cause an influenza pandemic [5]. Furthermore, an H5N1 virus (A/Hanoi/30408/2005) resistant to oseltamivir was isolated from a Vietnamese girl [6], and H5N1 viruses isolated from individuals in Hong Kong in 1997 were found to be resistant to interferon and tumor-necrosis factor–$\alpha$ [7]. The development of anti-H5N1 vaccines is thus a priority in efforts to prevent a human pandemic of H5N1 influenza.

We recently have shown that the combination of poly(I:C), a synthetic double-stranded RNA, and intranasal vaccine (split-product virus vaccine of either

Received 25 December 2006; accepted 24 April 2007; electronically published 5 October 2007.

Potential conflicts of interest: none reported.

Financial support: Japan Society for the Promotion of Science (grant-in-aid 07205 to T.I.); Ministry of Health, Labor and Welfare (grant H19 trans 002 to H.H.); Research on Health Sciences Focusing on Drug Innovation (grant KH51045 to H.H.).

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**The Journal of Infectious Diseases 2007;196:1313–20**



0022-1899/2007/19609-0009$15.00

DOI: 10.1086/521304

strain A/PuertoRico/8/34 or strain A/HongKong/156/97) protects mice against infection with avirulent A/PuertoRico/8 or highly pathogenic H5N1 (A/HongKong/483/97) influenza virus [8, 9]. poly(I:C), however, has a poor safety profile. poly(I):poly($C_{12}$U) (Ampligen) is structurally similar to double-stranded RNA and has exhibited a safe profile in double-blind placebo-controlled phase 2/3 clinical trials [10–12], in which it has been administered, in >75,000 intravenous doses (average dose, 400 mg), to humans. Our preliminary observations indicated that, as an adjuvant, Ampligen also has a protective effect against A/PuertoRico/8 and H5N1 influenza viruses. Furthermore, intranasal inoculation with either a formalin-inactivated H5N1 vaccine or an adenovirus vector–based influenza vaccine protected mice against lethal and heterologous H5N1 virus [13–15]. In 2003, Takada et al. [16] reported that intranasal inoculation with a formalin-inactivated virus vaccine (strain H1N1, H1N2, H3N1, H3N2, H5N4, or H9N2) at high doses protected mice against infection with heterologous A/HongKong/483/97 (H5N1) virus. These findings led us to examine whether intranasal inoculation with both Ampligen and a trivalent inactivated influenza vaccine—A/NewCaledonia/20/99 (H1N1), A/NewYork/55/2004 (H3N2), and B/Shanghai/361/2002—prepared for the 2005–2006 season protected mice against challenge with lethal and heterologous H5N1 virus.

In the present report, we demonstrate that intranasal inoculation with the current trivalent inactivated influenza vaccine combined with Ampligen as a mucosal adjuvant elicited protective immunity against both an H5N1 strain (A/HongKong/483/97) isolated in 1997 and more-recent H5N1 isolates (A/Vietnam/1194/04 and A/Indonesia/6/05) and that it significantly improved the survival rate after challenge with H5N1 virus. The results of our study suggest that the cross-protective immunity induced by such vaccination is mediated by a mucosal immune response, most likely by secretory IgA antibodies specific for influenza-virus proteins.

## MATERIALS AND METHODS

***Mice.*** Female BALB/c mice 6–8 weeks old were purchased from Japan SLC and were kept under specific-pathogen–free conditions.

***Viruses.*** The wild-type strains A/HongKong/483/97 (H5N1), A/Vietnam/1194/04 (H5N1), and A/Indonesia/6/2005 (H5N1) were used in the present study. The A/HongKong/483/97 virus [17], isolated from patient with fatal influenza, was prepared in Mardin-Darby canine kidney (MDCK) cells without any special step for adaptation to mice. The Vietnam/1194/04 virus and the Indonesia/6/05 virus were propagated in 10-day–old embryonated chicken eggs, for 2 days at 37°C. These viruses were stored at −80°C, and virus titers were quantified by plaque assay using MDCK cells.

***Vaccine and adjuvant.*** The trivalent inactivated influenza vaccine (split-product virus vaccines, hemagglutinin [HA] vaccine) prepared for the 2005–2006 season and including A/NewCaledonia/20/99 (H1N1), A/NewYork/55/2004 (H3N2), and B/Shanghai/361/2002 was purchased from Kitasato Institute (Saitama, Japan). poly(I):poly($C_{12}$U) (Ampligen) was provided by Hemispherx Biopharma.

***Inoculation and viral challenge.*** Mice were anesthetized with diethyl ether and were inoculated 3 times (at consecutive intervals of 3 and 2 weeks), either intranasally or subcutaneously, with 1 μg of the trivalent vaccine (0.33 μg of each vaccine strain) with or without 10 μg of Ampligen. According to a modification of a procedure described elsewhere [18–20], each mouse (5–10 mice/group) was anesthetized and then subjected to infection by inoculation with 4 μL of H5N1 virus suspension (1000 pfu) in PBS into both nostrils (2 μL/nostril) at 14 days after the final inoculation (1000 pfu corresponds to ~63 times the 50% mouse lethal dose for the A/Vietnam H5N1 strain in the 10-μL infection model). All animal experiments were performed in accordance with the Guides for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of NIID; infection with H5N1 virus was performed under Biosafety Level 3 containment and was approved by NIID.

***Measurement of virus titers and anti-vaccine antibodies.*** From mice euthanized while anesthetized with chloroform, nasal-wash and serum samples were collected for measurement of virus titers and vaccine-specific antibodies. ELISA for determination of the levels of specific IgA and IgG antibodies was performed as described elsewhere [19], with plates coated either with the trivalent vaccine (split-product virus vaccines, HA vaccine) used for vaccination (table 1) or with a formalin-inactivated H5N1 virus vaccine (NIBRG14) [21] derived from a recombinant avirulent avian virus that contains modified HA and neuraminidase (NA) from the highly pathogenic avian influenza strain A/Vietnam/1194/04 and other viral proteins from influenza virus A/PuertoRico/8/34 (H1N1) (table 2). Before the hemagglutination inhibition (HI) tests were performed, receptor-destroying enzyme II (Denka Seiken) was added to the red blood cell–treated serum samples overnight at 37°C, to inactivate nonspecific hemagglutination inhibitors, followed by incubation for 1 h at 56°C, to inactivate receptor-destroying enzyme. HI tests were performed according to the microtiter method of Sever [22]. Virus titers were measured by a plaque assay using MDCK cells, as described elsewhere [23, 24].

***Antigen-specific T cell response.*** Antigen-specific T cell responses were measured as described elsewhere [25]. The spleen was removed from mice 10 days after the third vaccination. T cells were purified from a single-cell suspension by depletion of $CD11b^+$, $CD45R^+$, $DX5^+$, and $Ter\text{-}119^+$ cells, by use of a

**Table 1. Titers of antibodies specific for the trivalent vaccine.**

| Vaccine | | | | End point–dilution titer, means ± SEs[a] | | |
|---|---|---|---|---|---|---|
| Trivalent vaccine, μg | Ampligen, μg | Route | Virus strain | Vaccine-specific IgA in nasal wash | Vaccine-specific IgG in serum | HI titer in serum[b] |
| 1 | 10 | in | A/NewCaledonia (H1N1) | 64 | 12,800 | 20 |
| | | | A/NewYork (H3N1) | 64 | 19,200 ± 6400 | 20 |
| | | | B/Shanghai | 32 | 19,200 ± 6400 | 10 |
| 1 | ... | in | A/NewCaledonia (H1N1) | ND | ND | <10 |
| | | | A/NewYork (H3N1) | ND | ND | <10 |
| | | | B/Shanghai | ND | ND | <10 |
| ... | 10 | in | A/NewCaledonia (H1N1) | ND | ND | <10 |
| | | | A/NewYork (H3N1) | ND | ND | <10 |
| | | | B/Shanghai | ND | ND | <10 |
| 1 | 10 | sc | A/NewCaledonia (H1N1) | ND | 204,800 | 320 |
| | | | A/NewYork (H3N1) | ND | 204,800 | 320 |
| | | | B/Shanghai | ND | 102,400 | 160 |
| 1 | ... | sc | A/NewCaledonia (H1N1) | ND | 204,800 | 160 |
| | | | A/NewYork (H3N1) | ND | 204,800 | 160 |
| | | | B/Shanghai | ND | 204,800 | 160 |
| ... | 10 | sc | A/NewCaledonia (H1N1) | ND | ND | <10 |
| | | | A/NewYork (H3N1) | ND | ND | <10 |
| | | | B/Shanghai | ND | ND | <10 |
| ... | ... | ... | A/NewCaledonia (H1N1) | ND | ND | <10 |
| | | | A/NewYork (H3N1) | ND | ND | <10 |
| | | | B/Shanghai | ND | ND | <10 |

**NOTE.** At 0, 3, and 5 weeks, mice were immunized with 1 μg of trivalent vaccine and 10 μg of Ampligen, as indicated. Nasal-wash and serum samples were collected 2 weeks after the final immunization. HI, hemagglutination inhibition; in, intranasal; ND, not detected; sc, subcutaneous.

[a] Data are for 5 mice. These titers were achieved by diluting the samples until optical-density values reached background levels.

[b] HI titers against homologous viruses in serum were measured at 2 weeks after the final immunization and are expressed as reciprocals of the highest dilution that completely inhibits hemagglutination of 4 hemagglutinin units of virus.

magnetic cell-sorting system (Miltenyi Biotec). For preparation of antigen-presenting cells, splenocytes from normal BALB/c mice were depleted of CD90 (Thy1.2)–positive cells and were irradiated at 2000 cGy. Purified splenic T cells ($1 \times 10^5$ cells/well) were cultured for 4 days with irradiated antigen-presenting cells ($5 \times 10^5$ cells/well) in the absence or presence of viral antigens (at concentrations of 0.1 μg/mL and 1 μg/mL). The concentration of interferon (IFN)–γ in culture supernatants was then measured by use of ELISA for the mouse cytokine (Biosource International). T cell proliferation was monitored by the addition of [$^3$H]thymidine (18.5 kBq/well) (ICN Biomedicals) 8 h before the cells were harvested. The cells were harvested onto a 96-well microplate bonded with a GF/B filter (Packard Instruments). Incorporated radioactivity was calculated by a microplate scintillation counter (Packard Instruments). Data are means ± SDs of values from 2 independent experiments, each performed with T cells from 5 mice/group.

***Statistics.*** All data are presented as means ± SEs or means ± SDs, and experimental groups were compared by use of Student's *t* test for paired observations. $P < .05$ was considered to be statistically significant.

## RESULTS

***Ampligen's effects as a mucosal adjuvant for seasonal influenza vaccine.*** To evaluate the efficacy of Ampligen as a mucosal adjuvant for the trivalent inactivated influenza vaccine, we examined the IgA and IgG responses to each of the 3 component strains—A/NewCaledonia (H1N1), A/NewYork (H3N2), and B/Shanghai—in mice inoculated intranasally or subcutaneously with 1 μg of the vaccine with or without 10 μg of Ampligen. A high concentration of IgA antibodies to HA was apparent in the nasal-wash samples from mice inoculated intranasally with both the trivalent vaccine and Ampligen (table 1); in contrast, a mucosal IgA response was not observed either in mice inoculated intranasally with the trivalent vaccine without Ampligen or in mice inoculated subcutaneously with the trivalent vaccine with or without Ampligen. Compared with