

Protection Against Influenza Virus Infection by Intranasal Administration of Hemagglutinin Vaccine With Chitin Microparticles as an Adjuvant

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Chitin in the form of microparticles (chitin microparticles, CMP) has been demonstrated to be a potent stimulator of macrophages, promoting T-helper-1 (Th1) activation and cytokine response. In order to examine the mucosal adjuvant effect of CMP co-administered with influenza hemagglutinin (HA) vaccine against influenza infection, CMP were intranasally co-administered with influenza HA vaccine prepared from PR8 (H1N1) virus. Inoculation of the vaccine with CMP induced primary and secondary anti-HA IgA responses in the nasal wash and anti-HA IgG responses in the serum, which were significantly higher than those of nasal vaccination without CMP, and provided a complete protection against a homologous influenza virus challenge in the nasal infection influenza model. In addition, CMP-based immunization using A/Yamagata (H1N1) and A/Guizhou (H3N2) induced PR8 HA-reactive IgA in the nasal washes and specific-IgG in the serum. The immunization with A/Yamagata and CMP resulted in complete protection against a PR8 (H1N1) challenge in A/Yamagata (H1N1)-vaccinated mice, while that with A/Guizhou (H3N2) and CMP exhibited a 100-fold reduction of nasal virus titer, demonstrating the cross-protective effect of CMP and influenza vaccine. It is suggested that CMP provide a safe and effective adjuvant for nasal vaccination with inactivated influenza vaccine. **J. Med. Virol. 75:130–136, 2005.** © 2005 Wiley-Liss, Inc.

KEY WORDS: influenza; chitin microparticles; nasal vaccine; adjuvant; IgA

INTRODUCTION

Effectiveness and safety are important issues to be considered in the development of a vaccine. The mucosal immune system is usually the first immunological barrier against influenza virus infections [Mestecky and McGhee, 1987]. The respiratory tract mucosa is the primary site of infection and the immunological compartment where the host immune system attacks the influenza virus. Secretory IgA antibodies are major effectors providing a front-line defense against influenza viruses in the respiratory tract mucosa [Shvartsman and Zykov, 1976; Underdown and Schiff, 1986; Murphy, 1994]. The influenza virus causes annual epidemics of influenza, largely due to the selection of new variants with mutations in the surface hemagglutinin (HA). The surface HA determines the antigenic properties of the virus and combines with sialic acid residues on epithelial cells during cell attachment [Renegar and Small, 1994]. Inactivated vaccines against the influenza virus are administered parenterally to induce serum anti-HA IgG antibodies that are highly protective against homologous virus infection, but are less effective against heterologous virus infection [Renegar and Small, 1994; Murphy and Webster, 1996]. In contrast, a large number of studies have shown that the mucosal immunity

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acquired by natural infection, which is largely due to the secreted form of IgA (s-IgA) in the respiratory tract, is more effective and provides greater cross-protection against different virus strains than the systemic immunity induced by parenteral vaccines in human [Clements et al., 1983; Couch and Kasel, 1983; Johnson et al., 1986; Murphy and Clements, 1989; Renegar and Small, 1994] and mice [Liew et al., 1984; Underdown and Schiff, 1986]. In this regard, induction of s-IgA in the RT has a great advantage in protecting against unpredictable epidemics of influenza.

It has been demonstrated that intranasal immunization with an inactivated vaccine together with cholera toxin B (CTB) subunits containing a trace amount of holotoxin (cholera toxin B*, CTB*) induces not only s-IgA with strong cross-protection against infection by virus variants of the same subtype in the upper respiratory tract, but also serum IgG with weak cross-protection against variant virus infection in the lower respiratory tract of mice [Tamura et al., 1988, 1992a,b, 1994b]. These findings were consistent with previous reports [Ramphal et al., 1979; Kris et al., 1985; Nedrud et al., 1987]. Although CTB* is an effective adjuvant for enhancing production of s-IgA, it has some adverse side effects such as producing excessive nasal discharge in humans. Adjuvants which are as effective as CTB* and safe for human use are in great demand for clinical application in nasal vaccination.

Chitin (a natural polysaccharide of *N*-acetyl-D-glucosamine) consisting of microparticles (1–20 μm in diameter) is one of the candidates for an immune enhancing adjuvant, because it can be derived from safe non-microbial sources such as shrimp, crab, and lobster. Chitin is non-allergenic, biodegradable, and biocompatible. Chitin-derived products are now used widely in the medical, veterinary, cosmetic, health supplement, and environmental industries [Okamoto et al., 1993; Strong et al., 2002]. Chitin is also a major component of fungal spores and induces a T-helper-1 (Th1) response. The innate immune system of the lung is well adapted for the clearance of airborne spores largely through phagocytosis by macrophages. This process involves secretion of IL-12 and IL-18 from the macrophages, which enhances Th1 immune responses [Strong et al., 2002]. It has been reported that the intranasal application of chitin microparticles (CMP) results in elevation of Th1 cytokines, including IL-12, IFN- γ , and TNF- α [Schaffner et al., 1982; Strong et al., 2002], and stimulation of a nasal-associated lymphoid tissue by CMP provides a bridge between the innate and adaptive immune systems [Strong et al., 2002]. Chitosan which is the partially deacetylated form of chitin has been used as a vaccine adjuvant due to its muco-adhesive properties, and has been shown to enhance antibody responses to mucosally delivered vaccine antigens [Bacon et al., 2000].

In this study, the mucosal adjuvant activity of CMP was studied when they were intranasally administered with inactivated influenza HA vaccine. It is also demonstrated that nasal CMP-based vaccine resulted in

cross-protective immune responses against homologous and heterologous influenza variants.

MATERIALS AND METHODS

Hemagglutinin (HA) Vaccines and Influenza Viruses

HA vaccines (split-product virus vaccines) were prepared from the family Orthomyxoviridae, genus *Influenzavirus A,B*, species *influenzavirus A* including A/Puerto Rico/8/34 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Guizhou/54/89 (A/Guizhou, H3N2) and *influenzavirus B*, B/Ibaraki/2/85 (B/Ibaraki) strains according to the method of Davenport et al. [1964] at the Kitasato Institute (Saitama, Japan). These viruses were grown in the allantoic cavities of 10–11-day fertile chicken eggs, purified and disintegrated with ethyl ether. The vaccine contains all the proteins from the virus particle; however, the major component of the vaccine is HA molecules (about 30% of the total protein). The virus, family Orthomyxoviridae, genus *Influenzavirus A,B*, species *influenzavirus A*, A/Puerto Rico/8/34 (A/PR8; H1N1) used for the challenge experiment was adapted to mice by subculturing 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day fertile chicken eggs.

Adjuvants

CTB subunits containing a trace amount of holotoxin (CTB*) was prepared by adding 0.1% of holotoxin to CTB (Sigma, St. Louis, MO). The CMP were prepared by sonication of dissolved purified chitin (Sigma-Aldrich, Poole, UK) in sterile, endotoxin-free phosphate-buffered saline (PBS). The sonicated chitin particles were collected by centrifugation, washed with 70% (v/v) ethanol, and washed five times with sterile PBS to remove soluble chitin. The diameters of CMP were compared to those of standardized beads, which were 1 and 20 μm in diameter (Polysciences, Inc., Warrington, PA) by flow cytometry (FACS) analysis. The diameters of 98% of the CMP were smaller than 20 μm and 33% were less than 1 μm in size. The sterility of CMP was confirmed by plating onto agar plates, demonstrating no colony formation on the plates. The concentration of endotoxin of CMP solution was examined by a *Limulus* Amebocyte Lysate Assay (Bio Whittaker, Wokingham, UK) and was shown to be less than 1 EU/ml.

Immunization and Infection With Influenza Virus in Mice

Female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan), aged 6–8 weeks at the time of immunization, were used in all experiments. All animal experiments were carried out in accordance with the Guides for Animal Experiments performed at NIID and approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases.

Five mice for each experimental group were anesthetized by diethyl ether and immunized primarily by dropping 5 μ l of PBS containing either 1 μ g of HA vaccines with 10 or 100 μ g of CMP, or 1 μ g of CTB* into the nostrils. The second immunization was carried out at 4 and 6 weeks later from the primary immunization (three-dose immunization protocol).

According to a modified procedure of Yetter et al. [1980] and Tamura et al. [1996, 1998], each mouse was anesthetized and infected intranasally by dropping 1.2 μ l of PBS containing a virus suspension with 1×10^2 PFU of mouse-adapted PR8 virus into each nostril. As 1.2 μ l of the virus suspension remained in the local nasal area, the initial viral infection was limited to the nose area.

Measurement of the Virus Titer and Anti-PR8 HA Antibodies of the Samples From the Infected Mice

After complete anesthesia with chloroform, the mice were killed. Serum and nasal wash were collected from the mice for measurement of the virus titer and antibodies against PR8 HA. The levels of IgA and IgG antibodies against HA molecules purified from the A/PR8 viruses were determined by ELISA as described previously [Tamura et al., 1996]. Briefly, ELISA was conducted sequentially from the solid phase (EIA plate; Costar, Cambridge, MA) with a ladder of reagents consisting of the following: first, HA molecules purified from the A/PR8 virus according to the procedure of Phelan et al. [1980]; second, nasal wash or serum; third, goat anti-mouse IgA antibody (α -chain specific anti-IgA antibody; Amersham Biosciences, Piscataway, NJ), or goat anti-mouse IgG antibody (γ -chain-specific anti-IgG antibody; Amersham), or anti-mouse IgG1 and IgG2a (BD Pharmingen, San Diego, CA) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, MD); and finally, *p*-nitrophenylphosphate. Absorbance was measured at 405 nm using an ELISA reader. A twofold serial dilution of either purified HA-specific IgA or HA-specific monoclonal IgG (160 ng/ml) was used as a standard as described previously [Asahi et al., 2002]. The binding kinetics of the standard HA-specific monoclonal IgG was comparable to purified HA-specific IgG from immunized mice. The IgA and IgG antibody concentrations of unknown specimens were determined from the standard regression curve constructed for each assay with the programmed SJeia Autoreader (model er-8000; Sanko Jun-yaku, Tokyo Japan).

The titers of the subclasses of IgG antibodies against HA molecules were also determined by ELISA. Antibody-positive cut-off values were set at the mean \pm 2 SD for pre-immune sera. The antibody titer determined by ELISA was expressed as the highest serum dilution giving a positive reaction. HA-specific monoclonal IgG1 and normal mouse serum were used as controls. The HA-specific monoclonal antibody was recognized exclusively

by anti-mouse IgG1 antibody, but not by anti-mouse IgG2a antibody (Fig. 2).

The virus titer was measured as follows: each 200 μ l of serial 10-fold dilutions of the nasal wash was inoculated into Madin–Darby canine kidney (MDCK) cells in a six-well plate grown in Dulbecco's modified minimum essential medium supplemented with 10% of fetal calf serum. After 1 hr incubation, each well was overlaid with 2 ml of agar medium according to the method described by Tobita et al. [1975]. The number of plaques in each well was counted at 2 days after inoculation. The experiments were repeated three to five times and the results combined. The data were represented as the mean \pm SD.

Statistics

Comparisons between experimental groups were made by Student's *t*-test, and $P < 0.05$ was considered as significant.

RESULTS

Antibody Response to HA and Protection Against Virus Infection in Mice Immunized Intranasally With the HA Vaccine With Chitin Microparticles as an Adjuvant

The mucosal adjuvant efficacy of CMP for influenza HA vaccine was studied. The antibody responses against PR8 HA molecules were examined in mice immunized intranasally with PR8 vaccine together with different amounts (10 or 100 μ g) of CMP or 1 μ g of CTB* and boosted twice at 4 and 6 weeks after the initial immunization. The secondary anti-PR8 HA IgA antibody responses in the nasal washes and anti-PR8 HA IgG Ab responses in the serum in the immunized mice are shown in Figure 1. The adjuvant effect of CMP was enhanced with an increase of the amount of CMP (Fig. 1). The concentration of s-IgA collected from the nasal wash was more than 100 ng/ μ l with an average of 140 ng/ μ l when the mouse was immunized with 10 μ g of CMP, and inoculation of 100 μ g of CMP with vaccine induced an increase to over 300 ng/ μ l of the concentration of s-IgA in the nasal mucosa.

Meanwhile, high levels of serum anti-HA IgG responses were induced in mice given 10 μ g of CMP with vaccine. The serum IgG responses seemed to parallel the s-IgA response in the nasal wash after immunization with 10 or 100 μ g of CMP as adjuvants (Fig. 1). This suggests that intranasal administration of CMP with influenza HA vaccine could induce s-IgA in the nasal area as well as serum IgG.

The IgG subtypes after inoculation of CMP with the HA vaccine were examined (Fig. 2). The IgG2a titer was dramatically increased along with the increase in the amount of CMP (from 10 to 100 μ g). This result was consistent with the observation that intranasal application of CMP enhanced Th1 cytokines such as IL-12, INF- γ , and TNF- α [Strong et al., 2002].

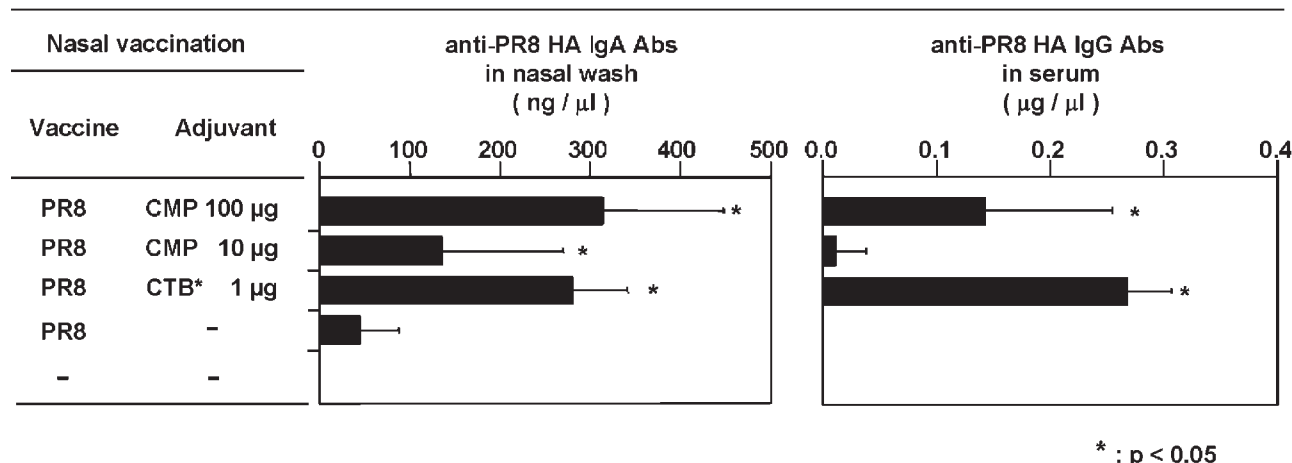


Fig. 1. Anti-PR8 hemagglutinin (HA)-specific IgA in the nasal wash and IgG in the serum from BALB/c mice immunized with intranasal vaccine with 10 or 100 μg of chitin microparticles (CMP), or 1 μg of cholera toxin B* (CTB*) as an adjuvant according to the three-dose regimen. The nasal wash and serum samples were collected at 2 weeks after the third immunization. The antibody titers of five mice from each group were measured by ELISA. The range of anti-HA IgA titers were 187.7–540.0 ng/μl (100 μg of CMP with PR8), 24.9–268.8 ng/μl (10 μg of CMP with PR8), 202.7–347.0 ng/μl (1 μg of CTB* with PR8), and

0–150.8 ng/μl (PR8 alone), respectively. The anti-HA IgA titer was not detected in the non-treated group. The range of anti-HA IgG titers were 0.0–312.1 μg/μl (100 μg of CMP with PR8), 0.0–57.86 μg/μl (10 μg of CMP with PR8), 223.5–327.2 μg/μl (1 μg of CTB* with PR8), respectively. The anti-HA IgG titer was not detected in the group treated with PR8 alone nor in the non-treated group. Each column represents mean ± SD. *P < 0.05 versus the value for the group with non-immunized mice (Student's *t*-test).

Protective Efficacy Against Live Influenza Virus Challenge

The protective effect of intranasal administration of HA vaccine with CMP against influenza viral infection was studied. In control mice, virus titers were 10^{2.9} PFU/ml in the nasal wash at 3 days after infection with 1.2 μl of influenza virus (100 PFU) in each nostril (Fig. 3). The mice immunized with HA vaccine without CMP adjuvant showed no protective effect compared with the control mice (Fig. 3). The mice immunized with HA vaccine together with 10 or 100 μg of CMP showed complete protection against the viral challenge infection in a

manner similar to the CTB*-treated group (Fig. 3). Thus, intranasal administration of HA vaccine with CMP adjuvant protected the mice against influenza virus infection. This protective effect was consistent with the enhancement of s-IgA and IgG antibody responses after inoculation of HA vaccine with CMP (Fig. 1).

Cross-Protective Effect of Influenza HA Vaccine With Chitin Microparticles as an Adjuvant

To characterize the cross-protective effects of CMP-based intranasal influenza vaccination against variant subtypes of influenza viruses, each group of mice was immunized intranasally with various vaccines (3 μg) together with CMP (100 μg) and boosted 4 and 6 weeks later. At 3 days post infection with A/PR8 (H1N1)

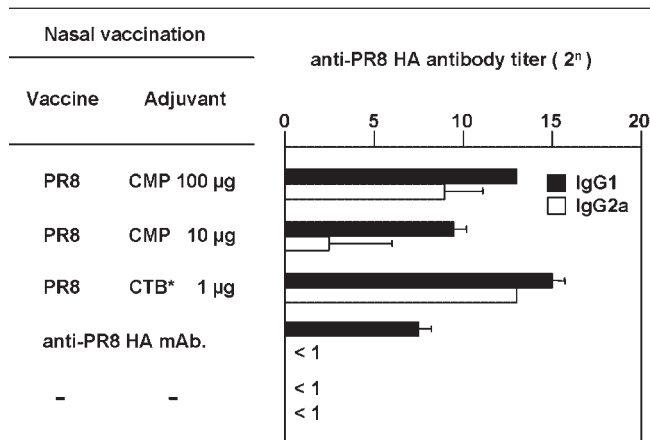


Fig. 2. Anti-PR8 HA-specific subtype of IgGs from BALB/c mice immunized with intranasal vaccine with 10 or 100 μg of CMP, or 1 μg of CTB* as an adjuvant according to the three-dose regimen. The same samples used in Figure 1 were analyzed for IgG subtypes by using IgG1- and IgG2a-specific monoclonal antibodies. HA-specific monoclonal IgG1 and normal mouse serum were used as controls. The antibody titers of five mice from each group were measured by ELISA. Each column represents mean ± SD.

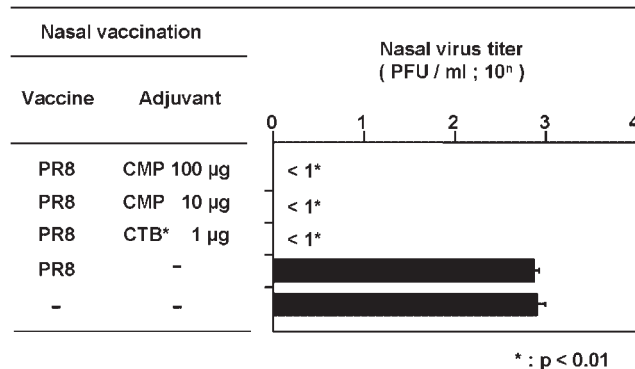


Fig. 3. Virus titers of the nasal washes from mice immunized with intranasal vaccine with 10 or 100 μg of CMP, or 1 μg of CTB* as an adjuvant according to the three-dose regimen. The mice were intranasally infected with 100 PFU of PR-8 influenza virus 2 weeks after the final immunization. The nasal washes were collected at 3 days after the virus challenge. The virus titer was measured by a plaque assay. Each column represents mean ± SD (n = 5). *P < 0.01 versus the value for the group with non-immunized mice (Student's *t*-test).

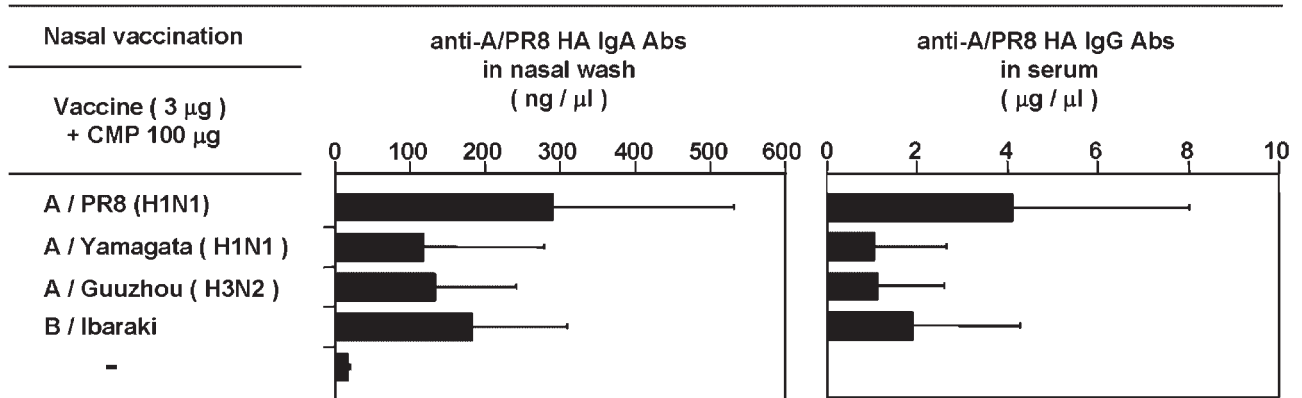


Fig. 4. Cross-protective antibody responses against PR8 HA in mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guuzhou (H3N2), and B/Ibaraki vaccine with 100 μg of CMP as an adjuvant. The range of anti-A/PR8 HA IgA titers were 87.7–631.9 ng/μl (A/PR8), 19.9–356.8 ng/μl (A/Yamagata), 76.5–331.9 ng/μl (A/Guuzhou), 46–346.32 ng/μl (B/Ibaraki), and 12.8–18.7 ng/μl (non-treated), res-

pectively. The range of anti-A/PR8 HA IgG titers were 1.0–9.5 μg/μl (A/PR8), 0.0–3.4 μg/μl (A/Yamagata), 0.3–3.3 μg/μl (A/Guuzhou), and 0.1–5.3 μg/μl (B/Ibaraki), respectively. The anti-A/PR8 HA IgG titer was not detected in the non-treated group. Each column represents mean ± SD (n = 5).

influenza virus performed at 2 weeks after final immunization, high s-IgA antibody responses (>200 ng/ml) in the nasal washes and high IgG antibody responses (>1 μg/ml) in the serum were observed in the mice immunized with A/PR8 virus (Fig. 4), and complete protection against the A/PR8 virus challenge was also observed (Fig. 5).

Immunization with the A/Yamagata (H1N1) nasal vaccines induced relatively low levels of nasal anti-A/PR8 HA s-IgA and serum IgG (Fig. 4), yet resulted in complete protection against challenge with 100 PFU of A/PR8 virus in 1.2 μl/nostril (Fig. 5). The mice immunized with A/Guuzhou (H3N2) virus vaccine with CMP showed similarly low responses of A/PR8 HA-reactive s-IgA (<0.1 μg/ml) in the nasal wash and IgG (<1 μg/ml) in the serum as those immunized with A/Yamagata (H1N1) virus, although this group of mice still exhibited the ability to eliminate virus compared to control (100-fold reduction) after A/PR8 virus challenge (Fig. 5). Almost no protective effect was observed in mice

intranasally immunized with B/Ibaraki vaccine, which demonstrated similar levels of A/PR8 HA-reactive s-IgA in the nasal wash and IgG in the serum as those immunized with A/Yamagata virus. These data indicate the production of cross-protective immune responses by intranasal vaccination with CMP adjuvant against homologous or heterologous virus infection in the upper respiratory tract, and the cross-reactive response was dependent on the virus strain.

DISCUSSION

Effective immunization strategies to protect against influenza virus infection involve the induction of mucosal immune responses at the nasal mucosal epithelium, which is the initial target of virus infection. To achieve effective protection against influenza infection at the mucosa, bacterial toxin-derived adjuvants such as CTB or heat-labile enterotoxin of *Escherichia coli* have been administered in conjunction with immunization [Tamura et al., 1988, 1989a,b, 1994a; Komase et al., 1998; Hagiwara et al., 1999]. Efforts to reduce the toxicity of the bacterial toxin-derived adjuvants have been carried out by using mutant toxins [Hagiwara et al., 1999, 2001] or by reducing the total amount of CTB required by adding a trace amount (0.1%) of holotoxin [Tamura et al., 1995]. Although the attenuated bacterial toxins are safe experimentally for pre-clinical animal models, it is still somewhat problematic for administration in human vaccination. An effective and safe adjuvant for intranasal vaccination in humans will be of great value. In this study, a new adjuvant system for intranasal vaccination without bacterial toxins or their derivatives is described.

CMP as an adjuvant is comparable to CTB* in enhancing anti-HA antibodies when administered intranasally with vaccine. Nasal immunization of vaccine and CMP exhibited not only an increase in mucosal s-IgA, but also a high titer of anti-HA IgG in the serum, and provided complete protection against viral infection at a level

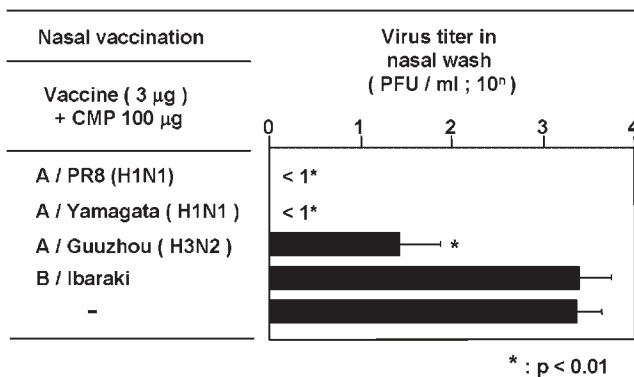


Fig. 5. Virus titers of nasal wash from mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guuzhou (H3N2), and B/Ibaraki vaccine with CMP as an adjuvant. The nasal washes were collected at 3 days after the virus challenge. The virus titer was measured by a plaque assay. Each column represents mean ± SD (n = 5). *P < 0.01 versus the value for the group with non-immunized mice (Student's *t*-test).

comparable to CTB* (Fig. 3). Thus, CMP seem to be an effective adjuvant for a nasal influenza vaccine.

The advantage of the nasal route of vaccination for influenza is the induction of s-IgA at the mucosal epithelium, which elicits cross-protective immunity more effectively than serum IgG [Tamura et al., 1992a]. In fact, we have observed the cross-protective effect of CMP combined with vaccine against various strains of influenza virus, including A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki. The PR8 HA-reactive secretory IgA in the nasal wash and serum IgG were detected in the mice immunized with the same H1N1 virus strain, A/Yamagata, and a complete protective effect against viral challenge was observed in this group. In addition, immunization of the H3N2 strain, A/Guizhou, produced a similar level of secretory IgA and serum IgG as those of A/Yamagata, and exhibited a modest viral protection effect which was a 100-fold reduction of viral titer compared to that of the non-treated group. Thus, production of cross-reactive s-IgA caused homologous and heterologous protection against viral infection, although other mechanisms might be involved in addition to cross-reactive protection.

Although the detailed mechanism of the adjuvant effect of CMP is still unclear, intranasal application of small doses (10–100 µg) of CMP has been shown to result in elevation of Th1 cytokines, such as IL-12, IFN-γ, and TNF-α, and reduction in IL-4 production during allergen challenge [Strong et al., 2002]. It has been reported that immunization with chitin increased Th1 responses in spleen cells, delayed-type hypersensitivity reactions, and serum IgG2a levels along with decreases of Th2 responses [Shibata et al., 2001]. Furthermore, oral administration of chitin has been reported to decrease IgE levels and lung eosinophil numbers, and inhibit Th2 cytokine response [Shibata et al., 2000]. The Th1 immunostimulatory properties induced by vaccine and CMP were affirmed by the enhancement of IgG2a response in a CMP dose-dependent manner (Fig. 2).

It is necessary for the development of a prophylactic vaccine that both vaccine and adjuvant are safe for use in humans. CMP, which are already used widely as medical supplements [Okamoto et al., 1993; Strong et al., 2002], might provide an alternative to microbial-derived adjuvants such as CTB. An intranasal vaccination protocol consisting of an influenza HA vaccine with CMP is described as an effective and safe adjuvant in a three-dose vaccination strategy to vaccinate against influenza. Further studies are needed to determine if such a nasal vaccine would be effective in humans.

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