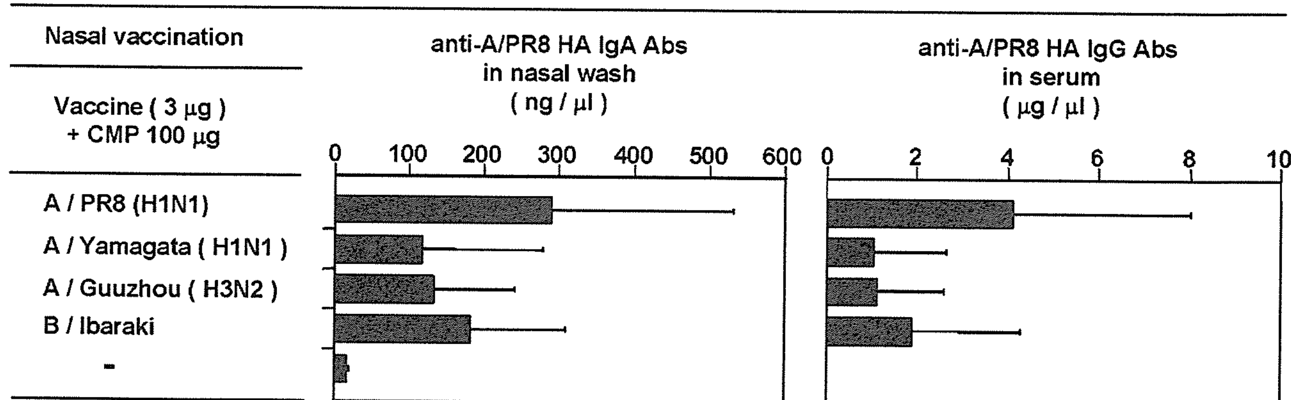

Fig. 4. Cross-protective antibody responses against PR8 HA in mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki vaccine with 100 μg of CMP as an adjuvant. The range of anti-A/PR8 HA IgA titers were 87.7–631.9 ng/μl (A/PR8), 19.9–356.8 ng/μl (A/Yamagata),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

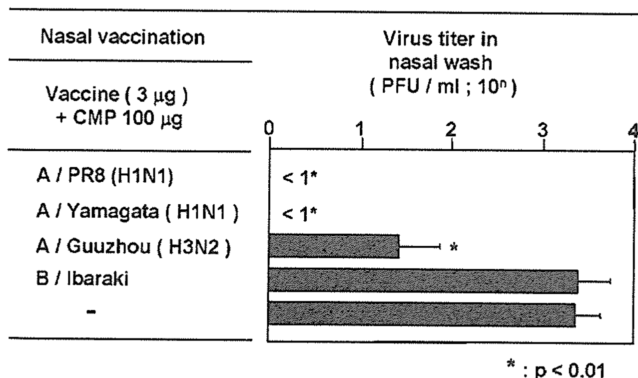

Fig. 5. Virus titers of nasal wash from mice intranasally immunized with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki vaccine with CMP as an adjuvant. The nasal washes were collected at 3 days after the virus challenge. The virus titer was measured by a plaque assay. Each column represents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

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

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 secretary 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.

REFERENCES

Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, Shimada S, Nanno M, Matsuoka Y, Ohwaki M, Iwakura Y, Suzuki Y, Aizawa C, Sata T, Kurata T, Tamura S. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J Immunol 168:2930–2938.

Bacon A, Makin J, Sizer PJ, Jabbal-Gill I, Hinchcliffe M, Illum L, Chatfield S, Roberts M. 2000. Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens. Infect Immun 68:5764–5770.

Clements ML, O'Donnell S, Levine MM, Chanock RM, Murphy BR. 1983. Dose response of A/Alaska/6/77 (H3N2) cold-adapted reassortant vaccine virus in adult volunteers: Role of local antibody in resistance to infection with vaccine virus. Infect Immun 40:1044–1051.

Couch RB, Kasel JA. 1983. Immunity to influenza in man. Annu Rev Microbiol 37:529–549.

Davenport FM, Hennessy AV, Brandon FM, Webster RG, Barrett CD, Jr., Lease GO. 1964. Comparisons of serologic and febrile responses in humans to vaccination with influenza a viruses or their hemagglutinins. J Lab Clin Med 63:5–13.

Hagiwara Y, Komase K, Chen Z, Matsuo K, Suzuki Y, Aizawa C, Kurata T, Tamura S. 1999. Mutants of cholera toxin as an effective and safe adjuvant for nasal influenza vaccine. Vaccine 17:2918–2926.

Hagiwara Y, Iwasaki T, Asanuma H, Sato Y, Sata T, Aizawa C, Kurata T, Tamura S. 2001. Effects of intranasal administration of cholera toxin (or *Escherichia coli* heat-labile enterotoxin) B subunits supplemented with a trace amount of the holotoxin on the brain. Vaccine 19:1652–1660.

Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. 1986. Immunity to influenza A virus infection in young children: A comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J Infect Dis 154:121–127.

Komase K, Tamura S, Matsuo K, Watanabe K, Hattori N, Odaka A, Suzuki Y, Kurata T, Aizawa C. 1998. Mutants of *Escherichia coli* heat-labile enterotoxin as an adjuvant for nasal influenza vaccine. Vaccine 16:248–254.

Kris RM, Asofsky R, Evans CB, Small PA, Jr. 1985. Protection and recovery in influenza virus-infected mice immunosuppressed with anti-IgM. J Immunol 134:1230–1235.

Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 14:350–356.

Mestecky J, McGhee JR. 1987. Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv Immunol 40:153–245.

Murphy BR. 1994. Mucosal immunity to viruses. In: Ogra PL, Lamm ME, McGhee JR, Mestecky J, Strober W, Biendnstock J, editors. Handbook of mucosal immunology. San Diego: Academic. 333p.

Murphy BR, Clements ML. 1989. The systemic and mucosal immune response of humans to influenza A virus. Curr Top Microbiol Immunol 146:107–116.

Murphy BR, Webster RG. 1996. Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B, Straus SE, editors. Fields virology. Philadelphia: Lippincott-Raven. pp 1397–1445.

Nedrud JG, Liang XP, Hague N, Lamm ME. 1987. Combined oral/nasal immunization protects mice from Sendai virus infection. J Immunol 139:3484–3492.

Okamoto Y, Minami S, Matsuhashi A, Sashiwa H, Saimoto H, Shigemasa Y, Tanigawa T, Tanaka Y, Tokura S. 1993. Application of polymeric *N*-acetyl-D-glucosamine (chitin) to veterinary practice. J Vet Med Sci 55:743–747.

Phelan MA, Mayner RE, Bucher DJ, Ennis FA. 1980. Purification of influenza virus glycoproteins for the preparation and standardization of immunological potency testing reagents. J Biol Stand 8:233–242.

Ramphal R, Cogliano RC, Shands JW, Jr., Small PA, Jr. 1979. Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis. Infect Immun 25:992–997.

Renegar KB, Small PA, Jr. 1994. Passive immunization: Systemic and mucosal. In: Ogra PL, Lamm ME, McGhee JR, Mestecky J, Strober W, Biendnstock J, editors. Handbook of mucosal immunology. San Diego: Academic. 347p.

Schaffner A, Douglas H, Braude A. 1982. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. J Clin Invest 69:617–631.

Shibata Y, Foster LA, Bradfield JF, Myrvik QN. 2000. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. J Immunol 164:1314–1321.

Shibata Y, Honda I, Justice JP, Van Scott MR, Nakamura RM, Myrvik QN. 2001. Th1 adjuvant *N*-acetyl-D-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. Infect Immun 69:6123–6130.

Shvartsman YS, Zykov MP. 1976. Secretory anti-influenza immunity. Adv Immunol 22:291–330.

Strong P, Clark H, Reid K. 2002. Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in murine models of allergy. Clin Exp Allergy 32:1794–1800.

Tamura S, Samegai Y, Kurata H, Nagamine T, Aizawa C, Kurata T. 1988. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. Vaccine 6: 409–413.

Tamura S, Kurata H, Funato H, Nagamine T, Aizawa C, Kurata T. 1989a. Protection against influenza virus infection by a two-dose regimen of nasal vaccination using vaccines combined with cholera toxin B subunit. Vaccine 7:314–320.

Tamura SI, Samegai Y, Kurata H, Kikuta K, Nagamine T, Aizawa C, Kurata T. 1989b. Enhancement of protective antibody responses by cholera toxin B subunit inoculated intranasally with influenza vaccine. Vaccine 7:257–262.

Tamura S, Ito Y, Asanuma H, Hirabayashi Y, Suzuki Y, Nagamine T, Aizawa C, Kurata T. 1992a. Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. J Immunol 149:981–988.

Tamura SI, Asanuma H, Ito Y, Hirabayashi Y, Suzuki Y, Nagamine T, Aizawa C, Kurata T, Oya A. 1992b. Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. Eur J Immunol 22:477–481.

Tamura S, Asanuma H, Tomita T, Komase K, Kawahara K, Danbara H, Hattori N, Watanabe K, Suzuki Y, Nagamine T, et al. 1994a. *Escherichia coli* heat-labile enterotoxin B subunits supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine. Vaccine 12:1083–1089.

Tamura S, Yamanaka A, Shimohara M, Tomita T, Komase K, Tsuda Y, Suzuki Y, Nagamine T, Kawahara K, Danbara H, et al. 1994b. Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. Vaccine 12:419–426.

Tamura S, Ishihara K, Miyata K, Aizawa C, Kurata T. 1995. Mechanism of enhancement of the immune responses to influenza vaccine with cholera toxin B subunit and a trace amount of holotoxin. Vaccine 13:339–341.

Tamura S, Miyata K, Matsuo K, Asanuma H, Takahashi H, Nakajima K, Suzuki Y, Aizawa C, Kurata T. 1996. Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. J Immunol 156:3892–3900.

Tamura S, Iwasaki T, Thompson AH, Asanuma H, Chen Z, Suzuki Y, Aizawa C, Kurata T. 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. J Gen Virol 79:291–299.

Tobita K. 1975. Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. Med Microbiol Immunol (Berl) 162:23–27.

Tobita K, Sugiura A, Enomote C, Furuyama M. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med Microbiol Immunol (Berl) 162:9–14.

Underdown BJ, Schiff JM. 1986. Immunoglobulin A: Strategic defense initiative at the mucosal surface. Annu Rev Immunol 4:389–417.

Yetter RA, Lehrer S, Ramphal R, Small PA, Jr. 1980. Outcome of influenza infection: Effect of site of initial infection and heterotypic immunity. Infect Immun 29:654–662.

JOURNAL OF VIROLOGY, Mar. 2005, p. 2910–2919
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.5.2910–2919.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.
Vol. 79, No. 5

Synthetic Double-Stranded RNA Poly(I:C) Combined with Mucosal Vaccine Protects against Influenza Virus Infection

Takeshi Ichinohe,[1,2] Izumi Watanabe,[1,2] Satoshi Ito,[1,2] Hideki Fujii,[3] Masami Moriyama,[4] Shin-ichi Tamura,[5] Hidehiro Takahashi,[1] Hirofumi Sawa,[6] Joe Chiba,[2] Takeshi Kurata,[1] Tetsutaro Sata,[1] and Hideki Hasegawa[1]*

Department of Pathology, National Institute of Infectious Diseases, Musashimurayama-shi,[1] Department of Immunology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku,[3] and Department of Microbiology and Immunology, School of Medicine, Keio University, Shinano-machi, Shinjyuku-ku,[4] Tokyo, Department of Biological Science and Technology, Tokyo University of Science, Yamazaki, Noda, Chiba,[2] Laboratory of Prevention of Viral Diseases, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka,[5] and Laboratory of Molecular & Cellular Pathology, 21st Century COE, Program for Zoonosis Control, Hokkaido University School of Medicine, and CREST, JST, Kita-ku, Sapporo,[6] Japan

Received 13 July 2004/Accepted 21 October 2004

The mucosal adjuvant effect of synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid [poly(I:C)] against influenza virus was examined under intranasal coadministration with inactivated hemagglutinin (HA) vaccine in BALB/c mice and was shown to have a protective effect against both nasal-restricted infection and lethal lung infection. Intranasal administration of vaccine from PR8 (H1N1) with poly(I:C) induced a high anti-HA immunoglobulin A (IgA) response in the nasal wash and IgG antibody response in the serum, while vaccination without poly(I:C) induced little response. Intracerebral injection confirmed the safety of poly(I:C). In addition, we demonstrated that administration of poly(I:C) with either A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine conferred complete protection against PR8 challenge in this mouse nasal infection model, suggesting that poly(I:C) possessed cross-protection ability against variant viruses. To investigate the mechanism of the protective effect of poly(I:C), mRNA levels of Toll-like receptors and cytokines were examined in the nasal-associated lymphoid tissue after vaccination or virus challenge. Intranasal administration of HA vaccine with poly(I:C) up-regulated expression of Toll-like receptor 3 and alpha/beta interferons as well as Th1- and Th2-related cytokines. We propose that poly(I:C) is a new effective intranasal adjuvant for influenza virus vaccine.

When developing a vaccine, both prophylactic effectiveness and safety should be considered. It has been reported that the respiratory tract (RT) mucosal immune system is usually the first immunological barrier against influenza virus infection (16, 17, 36) and a primary site of influenza virus infection. The influenza virus causes annual epidemics of influenza by altering the antigenic properties of its surface hemagglutinin (HA), which is involved in binding of sialic acids to the surface of susceptible cells (23). Inactivated vaccines against the influenza virus have been administered parenterally to induce serum anti-HA immunoglobulin G (IgG) antibodies (Abs) that are highly protective against homologous virus infection but are less effective against heterologous virus infection (19, 23). In contrast, a number of studies have shown that the mucosal immunity acquired by natural infection, which is mainly due to the secreted form of IgA (s-IgA) in the RT, is more effective and cross-protective against virus infections than systemic immunity induced by parenteral vaccines in humans (4, 5, 11, 18, 23) and mice (15, 36). In this regard, induction of s-IgA at the RT has a great advantage in protecting against unpredictable epidemics of influenza.

* Corresponding author. Mailing address: Department of Pathology, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-6572. E-mail: hasegawa@nih.go.jp.

We have demonstrated that intranasal immunization with an inactivated vaccine together with cholera toxin B subunits (CTB) containing a trace amount of holotoxin (CTB*) induces not only s-IgA with strong cross-protection against infection by variant viruses belonging to the same subtype in the upper RT but also serum IgG with weak cross-protection against variant virus infection in the lower RT in mice (26, 30–32). These findings were consistent with those of previous reports (13, 20, 22). Although CTB* is an effective adjuvant to produce s-IgA, it has some side effects, such as nasal discharge in humans. Several attempts to reduce the side effects have been carried out by introduction of mutations in CTB (8) or using physiological adjuvants, such as complement component C3d (37). Therefore, there is a need for an adjuvant that is both as effective as CTB* and safe for human use for the clinical application of intranasal influenza virus vaccine.

Double-stranded RNA (dsRNA) acts as a molecular mimic associated with viral infection, because most viruses produce dsRNA during their replication (10). It has also been shown that mammalian Toll-like receptor 3 (TLR3) recognizes dsRNA and activates the NF-κB (1) pathway, resulting in activation of alpha/beta interferon (IFN-α/β), which enhances the primary antibody response against subcutaneous immunization of soluble materials (14). The adjuvant activity of IFN-α/β seems to play an important role in bridging the gap between innate and adaptive immunity (14).

In the present study, we demonstrated that the mucosal adjuvant activity of intranasal administration of synthetic dsRNA polyriboinosinic polyribocytidylic acid [poly(I:C)] with inactivated influenza virus HA vaccine induced cross-protective immune responses against homologous and heterologous variant influenza virus infection.

MATERIALS AND METHODS

HA vaccines and influenza viruses. HA vaccines (split-product virus vaccines) were prepared from influenza viruses, including A/Puerto Rico/3/334 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Beijing/262/95 (A/Beijing; H1N1), A/Guizhou/54/89 (A/Guizhou; H3N2), B/Ibaraki/2/85 (B/Ibaraki), B/Aichi/5/88 (B/Aichi), and B/Yamagata/16/88 (B/Yamagata) strains according to the method of Davenport et al. (6) at the Kitasato Institute (Saitama, Japan). These viruses were grown in allantoic cavities from 10- to 11-day-old fertile chicken eggs, purified, and disintegrated with ethyl ether. The vaccine contained all proteins from virus particles. However, the major component of the vaccine was HA molecules (about 30% of total protein). The PR/8 virus used for the challenge experiments was adapted to mice by subculturing 148 times in ferrets, 596 times in mice, and 73 times in 10-day-old fertile chicken eggs.

Preparation of adjuvants. Cholera toxin B subunits containing a trace amount of holotoxin were prepared by adding 0.1% CT (holotoxin) to CTB obtained from Sigma (St. Louis, Mo.). Synthetic double-stranded RNA poly(I:C) was kindly provided by Toray Industries, Inc. (Kamakura, Kanagawa, Japan). Heat-denatured double-stranded RNA poly(I:C), which was boiled at 95°C for 5 min and cooled immediately on ice, was used as a negative control.

Immunization with vaccine and virus challenge. Female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan), age 6 to 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 with diethyl ether and immunized primarily by dropping 5 μl of phosphate-buffered saline (PBS) containing 1 μg of HA vaccine with 0.1 to 10 μg of poly(I:C) into each nostril. Four weeks later, they were reimmunized in the same manner with or without the same adjuvant.

According to a modification of the procedure of Yetter and coworkers (27, 29, 38), each mouse was anesthetized and infected by intranasal administration of 1 μl of PBS containing virus suspension with 1,000 PFU of mouse-adapted PR8 virus into each nostril. As 1 μl of the virus suspension remained in the local nasal area and could not enter the lung tissue, the initial viral infection was limited to the nasal area. To examine cross-reactivity of poly(I:C) treatment against variant influenza virus subtypes, the mice were immunized intranasally with various vaccines (1 μg) together with poly(I:C) (3 μg) and boosted with each vaccine alone 4 weeks later. Two weeks after the second immunization, the immunized mice were challenged by upper RT infection with the A/PR8 virus, and 3 days later nasal wash and serum specimens were collected for virus and Ab titration.

Measurement of the virus titer and anti-PR8 HA antibodies. Serum, nasal wash fluid, and bronchoalveolar wash fluid were collected for measurement of virus titer and antibodies against PR8 HA from mice that were sacrificed under anesthesia with chloroform. The levels of IgA and IgG Abs against HA molecules purified from the A/PR8 viruses were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (29). Briefly, ELISA was conducted sequentially from the solid phase (EIA plate; Costar, Cambridge, Mass.) 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. (21); second, nasal wash fluid, bronchoalveolar wash fluid, or serum; third, either goat anti-mouse IgA Ab (α-chain specific; Amersham) or goat anti-mouse IgG antibody (γ-chain-specific anti-IgG antibody; Amersham), or anti-mouse IgG1 and IgG2a (BD Pharmingen, San Diego, Calif.) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, Md.); and fifth, *p*-nitrophenylphosphate. The chromogen produced was measured by determining the absorbance at 405 nm with an ELISA reader. A twofold serial dilution of either purified HA-specific IgA (320 ng/ml) or HA-specific monoclonal IgG (160 ng/ml) was used as a standard as described previously (2). The binding kinetics of the standard HA-specific monoclonal IgG were comparable to purified HA-specific IgG from immunized mice. In the IgG subtype assay, 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. No HA-specific IgG1 or IgG2a was detected in normal mouse serum. To examine whether poly(I:C) is effective for protection against influenza virus-induced lethal pneumonia, mice were challenged with a lethal dose (1,000 PFU in 20 μl) of PR8 virus at 2 weeks after the second immunization. The survival rate of the mice was estimated at 2 weeks after the viral challenge. The viral titer of the lung wash fluid was examined 3 days after the challenge (see Fig. 2). The antibody concentrations of unknown specimens were determined from the standard regression curve constructed for each assay with a programmed SJeia Autoreader (model er-8000; Sanko Jun-yaku, Tokyo, Japan).

Virus neutralization by antisera was determined as previously described (3). Briefly, virus was mixed with inactivated antisera from naive or vaccinated mice at 37°C for 1 h, and the mixture was added to Madin-Darby canine kidney (MDCK) cells in a total volume of 200 μl. The neutralizing capacity of antisera was measured by comparing the reduction in the number of infected cells per sample to sera from age-matched naive mice. Inhibition of virus was assessed by the additional reduction in infectivity beyond the background of naive-mouse antisera. Inhibition was measured by 50% inhibition of virus infection (beyond nonspecific inhibition). Samples were run in duplicate assays on the same day and averaged, and data are presented as the average per group.

The virus titer was measured as follows: aliquots of 200 μl of serial 10-fold dilutions of the nasal wash fluid were inoculated into MDCK cells in six-well plates. After a 1-h incubation, each well was overlaid with 2 ml of agar medium according to the method of Tobita and coworkers (34, 35). The number of plaques in each well was counted at 2 days after inoculation. All of the experiments were repeated independently at least three times. The data are presented as means ± standard deviations (SD).

RNA isolation, cDNA synthesis, and real-time PCR. The expression of TLR3 and TLR4 in nasal-associated lymphoid tissues (NALTs) in vaccinated or influenza virus-infected mice was examined. Mice were inoculated with influenza virus or intranasally administered influenza virus HA vaccine with or without poly(I:C). The NALTs were collected sequentially up to 72 h after administration. The mRNA levels of TLR3 and TLR4 in the NALTs were measured by real-time quantitative PCR after reverse transcription. Total RNA was extracted from the NALTs in mice by using an SV-Total RNA isolation kit (Promega, Madison, Wis.) and cDNA was synthesized using Omniscript reverse transcriptase (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions.

Real-time quantitative PCR was performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, Calif.) with a QuantiTect Probe PCR kit (QIAGEN), TaqMan probes (Applied Biosystems), and primers (Sigma Genosys, Ishikari, Japan) listed in Table 1 designed with Primer Express (Applied Biosystems). The system uses two dye layers to detect the presence of target and control sequences. The FAM (6-carboxyfluorescein) dye layer yields results for quantification of the cytokine target mRNA. The cytokine and TLR targets examined were IFN-α, IFN-β, IFN-γ, interleukin-4 (IL-4), IL-6, IL-12 p40, TLR-3, and TLR-4. PCR was carried out in a volume of 20 μl; initial denaturation at 50°C for 2 min and 95°C for 15 min was followed by 45 cycles of 94°C for 15 s and 60°C for 1 min. For each sample, PCR was performed in duplicate. cDNA levels were determined using the standard curve of cycle thresholds. All data obtained were normalized to the β-actin cDNA level.

Effects of intracerebral injection of poly(I:C). Seven-week-old BALB/c female mice were injected intracerebrally with poly(I:C) or CTB* at various doses [0.25, 2.5, or 25 μg for poly(I:C) and 2.5, 10, or 25 μg for CTB*] in 25 μl of PBS, and their mortality and body weights were monitored for 7 days. PBS was used as a negative control.

Antigen-specific T-cell response. Antigen-specific T-cell responses were measured as previously described (24). Spleens were harvested from mice 1 week after the boost vaccination. After the preparation of a single cell suspension, T cells were purified by depletion of $CD11b^+$ (Mac-1), $CD45R^+$ (B220), $DX5^+$, and Ter-119^+ cells by using a magnetic cell sorting system (MACS; Miltenyi Biotec, Bergisch, Germany). To prepare antigen-presenting cells, normal BALB/c mouse splenocytes were depleted of CD90 $(Thy1.2)^+$ cells by MACS and irradiated at 2,000 cGy.

Purified T cells taken from the spleen (10^5 cells/well) were cultured with irradiated antigen-presenting cells (5×10^5 cells/well) in the presence or absence of PR8 vaccine (0.1, 1, or 10 μg/ml). Four days after the cultivation, the level of cytokine concentration in the culture supernatant was measured by ELISA using a mouse IFN-γ immunoassay kit (Biosource International, Camarillo, Calif.) according to the manufacturer's instruction. T-cell proliferation was monitored by the incorporation of [^{3}H]thymidine (18.5 kBq/well; ICN Biomedicals, Costa Mesa, Calif.) added 8 h prior to cell harvest. The cells were harvested on a 96-well microplate bonded with a GF/B filter (Packard Instruments, Meriden, Conn.). Incorporated radioactivity was counted by a microplate scintillation counter (Packard Instruments).

TABLE 1. Primers for quantitative PCR[a]

Target	Sequences
TLR3	
Forward	CCC AGC TCG ATG TTT CCT ACA
Reverse	CAG GCT TGG GAG ATA GGA GAA G
Probe	CAA CCT CCA TGA TGT CGG CAA CGG
TLR4	
Forward	CAT GGA ACA CAT GGC TGC TAA
Reverse	GTA ATT CAT ACC CCT GGA AAG GAA
Probe	CTA TAG CAT GGA CCT TAC CGG GCA GAA GG
IFN-α	
Forward	CTG CTA GTG ATG AGC TAC TGG TCA A
Reverse	GGG TCA AGG CTC TCT TGT TCC T
Probe	CTG CTC CCT AGG ATG TGA CCT GCC TCA
IFN-β	
Forward	GCT CCT GGA GCA GCT GAA TG
Reverse	TCC GTC ATC TCC ATA GGG ATC T
Probe	TCA ACC TCA CCT ACA GGG CGG ACT TC
IFN-γ	
Forward	AGC CAG ATT ATC TCT TTC TAC CTC AGA
Reverse	GCA ATA CTC ATG AAT GCA TCC TTT
Probe	CAG GCC ATC AGC AAC AAC ATA AGG GTC
IL-4	
Forward	CGC CAT GCA CGG AGA TG
Reverse	CGA GCT CAC TCT CTG TGG TGT T
Probe	TGC CAA ACG TCC TCA CAG CAA CGA
IL-6	
Forward	CCA GAA ACC GCT ATG AAG TTC CT
Reverse	CAC CAG CAT CAG TCC CAA GA
Probe	TCT GCA AGA GAC TTC CAT CCA GTT GCC
IL-12p40	
Forward	AGC TCG CAG CAA AGC AAG AT
Reverse	TGG AGA CAC CAG CAA AAC GA
Probe	TGT CCT CAG AAG CTA ACC ATC TCC TG
β-Actin	
Forward	CAC CGA TCC ACA CAG AGT ACT TG
Reverse	CAG TGC TGT CTG GTG GTA CCA
Probe	CAG TAA TCT CCT TCT GCA TCC TGT CAG CAA

[a] Probes labeled with FAM (5′) and TAMRA (6-carboxytetramethylrhodamine) (3′).

Histopathological examination. Excised lung, brain, and nasal tissues were fixed with 10% neutral-buffered formalin, and nasal tissue was decalcified in the EDTA solution. After fixation, tissues were embedded in paraffin by conventional methods and stained with hematoxylin and eosin (H&E).

Statistics. Comparisons between the groups were made with the *t* test for paired observations, and a *P* of <0.05 was considered significant.

RESULTS

Intranasal immunization of HA vaccine with poly(I:C) protects against influenza virus infection. To estimate the mucosal adjuvant efficacy of poly(I:C) for influenza virus HA vaccine, the antibody response against PR8 HA molecules was examined in mice immunized intranasally with 1 μg of PR8 vaccine with different amounts (0 to 10 μg) of poly(I:C). The mice received the primary immunization with vaccine and poly(I:C) at 6 weeks before infection, and secondary immunization was performed with either vaccine with poly(I:C) or vaccine alone at 2 weeks prior to infection (Fig. 1). The highest concentration of anti-HA IgA Ab in the nasal wash fluid was observed in animals given repeated immunization with 1 μg of vaccine and 10 μg of poly(I:C). The concentration of anti-HA IgA Ab of mice immunized with vaccine and 1 μg of poly(I:C) twice was similar to that of mice that received primary immunization with vaccine and 3 μg of poly(I:C) followed by secondary immunization with vaccine alone.

The concentration of anti-HA IgG in the serum was also measured in the same experimental groups. The highest level of anti-HA IgG was also observed in mice that were inoculated twice with vaccine and 10 μg of poly(I:C). The serum IgG responses seemed to parallel the IgA response in the nasal wash. Nasal IgA and serum IgG levels were markedly low in mice immunized with 1 μg of denatured poly(I:C) (Fig. 1). The antibody titers of IgG1 and IgG2a in mice immunized with HA vaccine and poly(I:C) were comparable to those in mice inoculated with CTB* combined with HA vaccine (data not shown).

Next, we examined the protective effect of intranasal administration of HA vaccine with poly(I:C) against influenza virus infection. In control mice, virus titers were $10^{3.1}$ PFU/ml in the nasal wash fluid at 3 days after infection with 1,000 PFU of influenza virus (Fig. 1). Mice immunized with HA vaccine alone showed no protective effect in the nasal wash compared with controls (Fig. 1). On the other hand, mice immunized with vaccine with either 1 μg of poly(I:C) twice or 3 μg of poly(I:C) once at the first immunization showed complete protection against viral infection (Fig. 1). In mice immunized with vaccine with either 0.1 or 1 μg of poly(I:C), significant reductions in virus titers in the nasal wash fluid were observed compared with mice immunized with vaccine alone. Thus, intranasal administration of HA vaccine with poly(I:C) adjuvant protected mice against influenza virus infection, and the protective effect of poly(I:C) was not observed after denaturation. The results were consistent with production of IgA and IgG antibodies.

We next investigated whether poly(I:C) is effective for protection against influenza virus-induced lethal pneumonia in mice. At 2 weeks after lethal virus challenge (40 50% lethal doses [LD_{50}] of the A/PR8 virus), no mice survived in the nonimmunized group or the groups immunized by various influenza B virus with poly(I:C), which showed marked body weight reduction (Fig. 2A and B). In contrast, no death occurred in the group of mice immunized with various amounts of poly(I:C) without body weight loss (Fig. 2A and B). The viral titer of the lung wash fluid of the nonimmunized group and the groups immunized by various influenza B virus with poly(I:C) was around $10^{6.2}$ PFU/ml, while those of the mice vaccinated with either poly(I:C) or CTB* were below the level of detection (Fig. 2A). We next examined the pathological findings of the lungs in the group immunized with A/PR8 (Fig. 2C) and B/Aichi (Fig. 2D). The lung specimens of mice immunized with A/PR8 vaccine followed by 40 LD_{50} A/PR8 virus infection demonstrated bronchi and alveoli without any inflammatory change at 8 days after the virus challenge. In the mice immunized with B/Aichi vaccine followed by the same amount of A/PR8 virus, marked pneumonia with destruction of bronchial mucosa and interstitial infiltration of inflammatory cells was recognized in the lung specimen at 8 days after the challenge. A similar pathological change was observed in the group immunized by various influenza B virus with poly(I:C) (data not shown). Thus, it seems that the lung virus titer and the

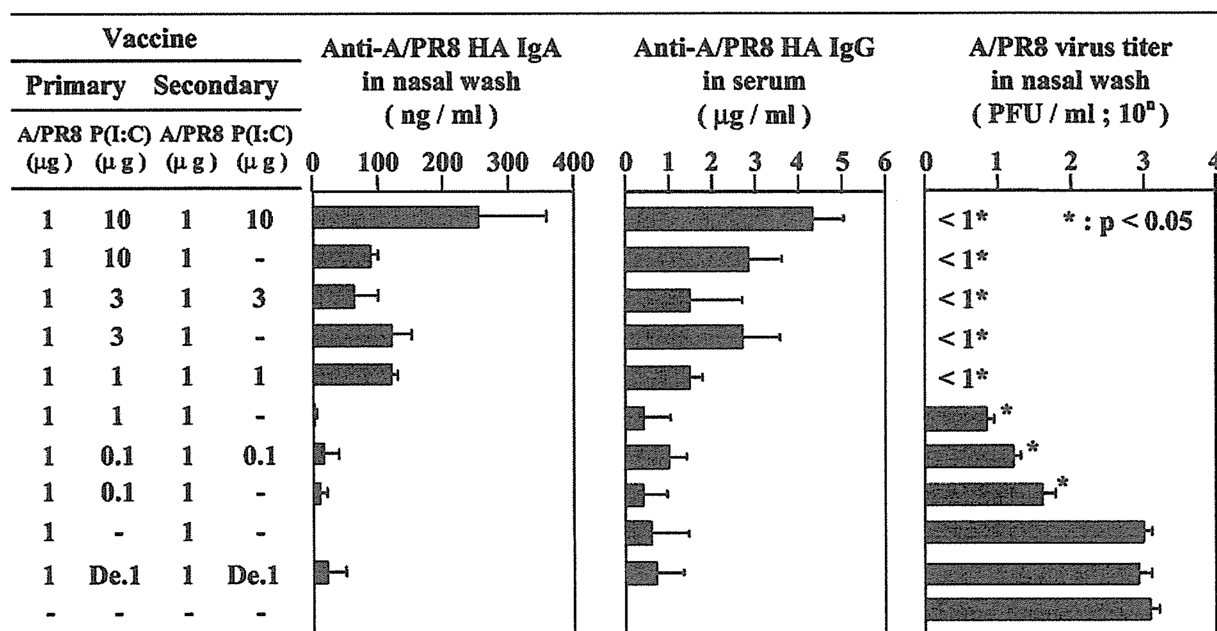

FIG. 1. Anti-PR8 HA-specific antibody titer and PR8 virus titer. Anti-PR8 HA-specific IgA and IgG responses in BALB/c mice that received primary intranasal immunization with 0.1 to 10 μg of poly(I:C) as an adjuvant. Secondary immunization was performed 4 weeks after primary immunization with or without the adjuvant. The nasal wash and serum samples were collected 2 weeks after the second immunization. The antibody titers of five mice from each group were measured by ELISA. The same groups of mice were infected intranasally with 1,000 PFU of PR8 influenza virus at 2 weeks after the second immunization. Nasal wash fluids were collected 3 days after virus challenge. The virus titers were measured by plaque assay. De, heat-denatured poly(I:C). Each column represents the mean ± SD. The virus titers were statistically compared to those of nonimmunized mice.

pathological change were well correlated, suggesting that the complete inhibition of lung virus titer by intranasal vaccination with poly(I:C) reflects complete protection against lethal influenza virus pneumonia.

Cross-protective effect of influenza virus HA vaccine with poly(I:C). We next characterized the cross-protective effect of intranasal vaccination with poly(I:C) against various influenza virus subtypes. Mice received primary immunization with 1 μg of various vaccines with 3 μg of poly(I:C) and secondary immunization with vaccine alone. Both the IgA antibody titer (>200 ng/ml) in the nasal wash fluid and IgG antibody (>1 μg/ml) in the serum were markedly high in mice immunized with A/PR8 virus vaccine, resulting in the disappearance of PR8 virus in the nasal wash fluid (Fig. 3A). Immunization with A/Beijing (H1N1) and A/Yamagata (H1N1) vaccines induced relatively high levels of nasal IgA and serum IgG against A/PR8 HA and also conferred complete protection against A/PR8 virus (Fig. 3A). Mice immunized with A/Guizhou (H3N2) virus vaccine showed low responses of A/PR8 HA-reactive IgA in the nasal wash fluid and IgG in the serum (Fig. 3A), resulting in low protective efficiency against A/PR8 virus challenge (Fig. 3A). Next, the neutralization activity to A/PR8 virus was examined in vitro by using the sera from the same group of the mice. The mice immunized by A/PR8 vaccine had neutralized antibody against A/PR8 (1:256); however, no neutralizing activity was recognized in the sera from the mice vaccinated by A/Beijing (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B influenza viruses (Fig. 3A). To examine the cross-protective effects of poly(I:C) combined influenza virus vaccine to lethal influenza virus challenge, the mice immunized with vaccine from various strains including A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki were challenged by a lethal dose (40 LD_{50}) of A/PR8 (H1N1) virus. All the mice immunized with poly(I:C) combined with A/PR8 (H1N1) and A/Yamagata (H1N1) vaccine survived, while the survival rate of mice immunized with poly(I:C) combined with A/Guizhou (H3N2) was 40% at 10 days after the challenge. No mouse survived in the groups of unimmunized or B/Ibaraki vaccine-immunized mice at 8 days after the challenge (Fig. 3B). Taken together, these observations indicate that intranasal vaccine with poly(I:C) results in the cross-protective immune responses against homologous or heterologous viruses infection in the upper RT and against lethal influenza virus challenge.

Induction of antigen-specific T-cell response by intranasal administration of vaccine with poly(I:C). To examine whether intranasally administered influenza virus vaccine induces T-cell response against homologous or heterologous influenza viruses, mice were immunized by A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), or B/Ibaraki vaccine with poly(I:C). T cells collected from the spleens of these mice 1 week after the booster immunization were enriched and cultured with irradiated antigen-presenting cells in the presence or absence of A/PR8 vaccine at 0.1, 1, or 10 μg/ml. The T cells from the mice immunized with A/PR8 and A/Yamagata viruses proliferated in an antigen dose-dependent manner; however, no proliferative effect was recognized in the mice vaccinated with A/Guizhou and B/Ibaraki virus (Fig. 4A). These results suggest that the intranasally administered influenza virus vaccine with poly(I:C) does induce T-cell activation with homologous antigen.

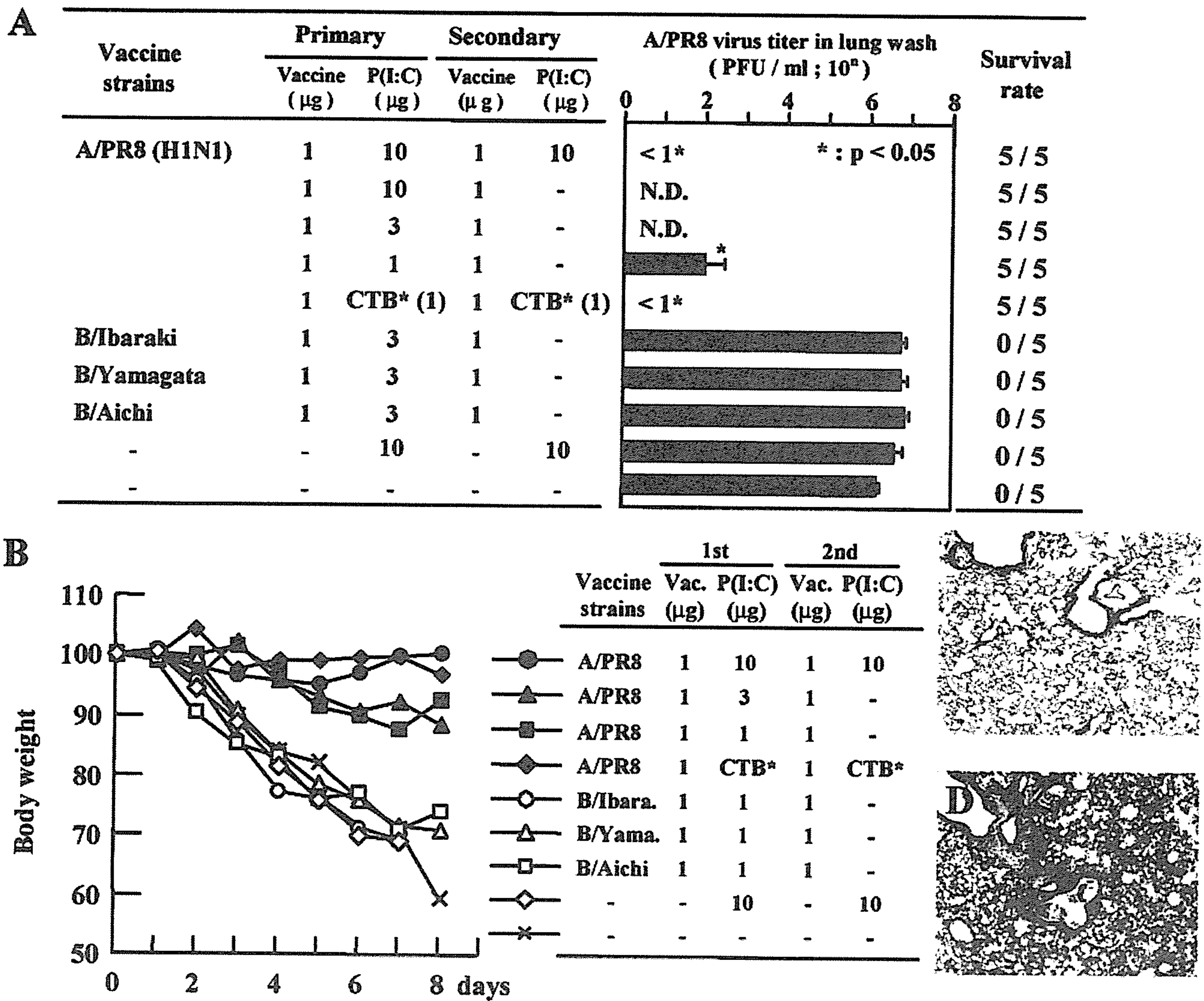

FIG. 2. (A) Virus titers in the lung wash fluids from the mice that received primary intranasal immunization with 1 to 10 μg of poly(I:C) as an adjuvant. Secondary immunization was performed 4 weeks after primary immunization with or without the adjuvant. The mice were infected intranasally with 1,000 PFU (40 LD_{50}) of PR8 influenza virus 2 weeks after the second immunization. The survival rate of each experimental group is shown on the right side of the bar graph. Each column represents the mean ± SD. N.D., not determined. (B) Body weight change after virus challenge. Each point represents the relative ratio for initial body weight (mean) of 5 mice for each day after the challenge. (C) Histopathological finding of a lung immunized intranasally with A/PR8 vaccine with poly(I:C) followed by 1,000 PFU (40 LD_{50}) of A/PR8 virus at 8 days after the challenge (×40; H&E). (D) Histopathological finding of a lung immunized intranasally with B/Aichi vaccine with poly(I:C) followed by 1,000 PFU (40 LD_{50}) of A/PR8 virus at 8 days after the challenge (×40; H&E).

We also examined the IFN-γ production in the supernatant of T cells derived from the spleens of the mice immunized with A/PR8 vaccine in vitro (Fig. 4B). We found that the A/PR8 (H1N1) vaccine induced IFN-γ production from the T cells derived from the mice immunized with A/PR8 (H1N1) and A/Yamagata (H1N1) virus in an antigen dose-dependent manner (Fig. 4B); however, the T cells from the mice immunized with A/Guizhou (H3N2) and B/Ibaraki viruses did not produce a significant amount of IFN-γ in response to A/PR8 (H1N1). These results suggest that T-cell responses against heterologous influenza viruses were weak and intranasal administration of influenza virus vaccine with poly(I:C) induces systemic antigen-specific T-cell responses.

Induction of TLR3 expression by intranasal administration of vaccine with poly(I:C). To define the mechanism by which intranasal administration of poly(I:C) with influenza virus HA vaccine functions as a mucosal adjuvant, we examined mRNA expression levels of Toll-like receptors 3 and 4, receptors for double-stranded RNA, and lipopolysaccharide, respectively. The expression of TLR3 in the NALT was up-regulated 30-fold in influenza virus-infected mice (Fig. 5A) and sixfold in mice vaccinated with poly(I:C) (Fig. 5B). The up-regulation of TLR3 in the vaccinated mice persisted for at least 72 h (Fig. 5B); meanwhile, TLR4 was not changed in these animals. The TLR responses were not detected in the mice treated with vaccine alone (Fig. 5C). These results suggested that up-regulation of TLR3 in the NALTs could enhance the adjuvant effect of poly(I:C).

Induction of IFN and Th1- and Th2-related cytokines by intranasal administration of vaccine with poly(I:C). We next

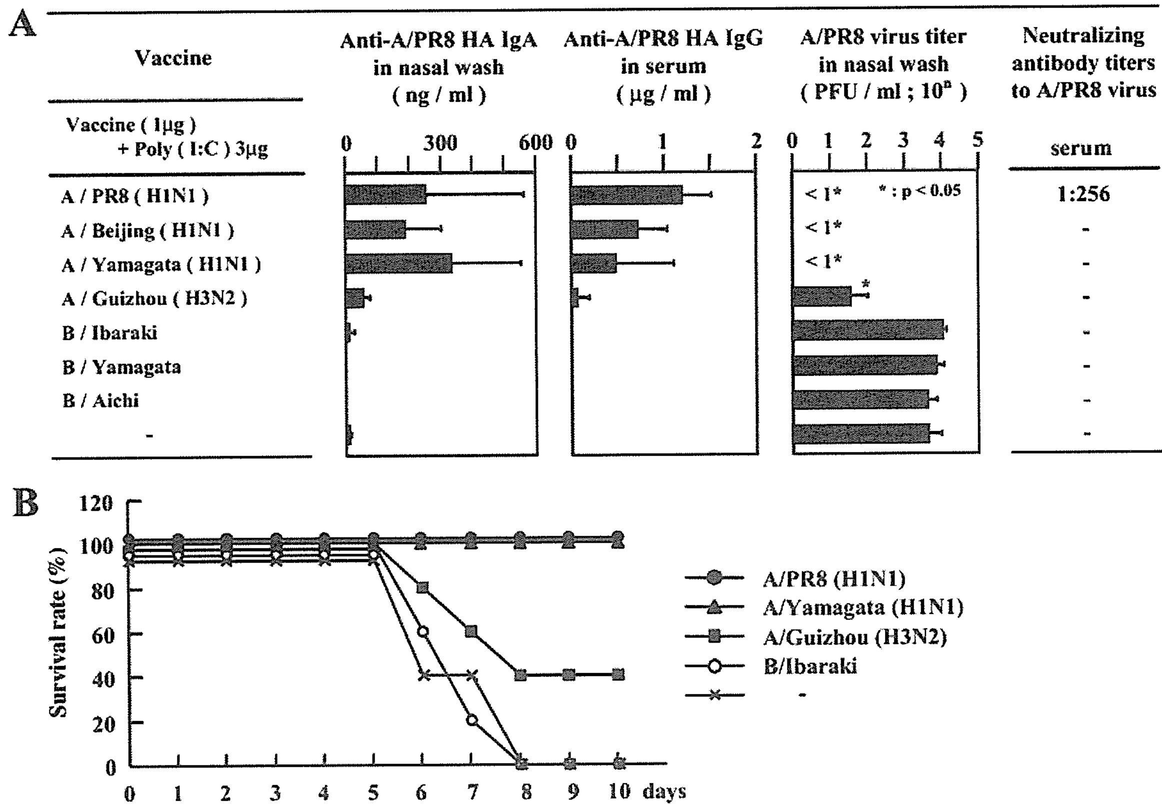

FIG. 3. (A) Cross-protective antibody responses against PR8 HA in the mice immunized intranasally with A/PR8 (H1N1), A/Beijing (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), B/Ibaraki, B/Yamagata, and B/Aichi vaccine with poly(I:C) as an adjuvant. Secondary immunization was performed 4 weeks after primary immunization without the adjuvant. The same groups of mice were infected with 1,000 PFU in 2 μl of PR8 influenza virus 2 weeks after the second immunization. The nasal wash fluid was collected 3 days after virus challenge. The virus titer was measured by plaque assay. Each column represents the mean ± SD. The serum collected at 2 weeks after the booster was analyzed for the presence of neutralizing antibodies against homologous or heterologous influenza virus. Inhibition of the virus was assessed by the additional reduction in infectivity beyond the background of naive mice. Samples were run in duplicate, and data are presented per group, where the ability to inhibit 50% of infection at the indicated dilution is shown. The dash indicates lack of reduction of infectivity. (B) The survival curve of the mice immunized with poly(I:C) and the various vaccines after the lethal A/PR8 (H1N1) challenge. Mice were immunized with 3 μg of A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki vaccine with 10 μg of poly(I:C) as an adjuvant. Secondary immunization was performed 4 weeks after primary immunization with the same amount of vaccine with the adjuvant. The survival rates of the mice until 10 days after the virus challenge are presented in a line graph.

investigated the expression of interferons and cytokines in the NALTs after administration of influenza virus vaccine with poly(I:C). Rapid induction of IFN-α, IFN-β, and IFN-γ (at 6 h after vaccination) and return to the basal levels at 24 h were observed. However, vaccine without poly(I:C) did not induce expression of these interferons (Fig. 6A to C). The mRNA expression levels of cytokines in the NALTs were also examined. IL-4 mRNA expression was increased at 72 h after vaccination with poly(I:C). In addition, IL-12 p40 was up-regulated from 6 to 24 h following vaccination with poly(I:C) (Fig. 6D to F). These observations suggested that up-regulation of IL-4 and IL-12 p40 results in production of anti-HA-specific immunoglobulins.

Safety of intranasal and intracerebral injection of poly(I:C). To examine the safety of poly(I:C) in intranasal administration, an excess amount of poly(I:C) and bacteria-derived adjuvant CTB* were intranasally given to mice daily for 9 days. The body weight of the mice administered 10 μg of poly(I:C) was not significantly changed, while that of the mice administered 10 μg of CTB* was relatively decreased (Fig. 7A). Histopathological examination revealed that the nasal areas of the mice administered poly(I:C) had no pathological changes, as well as those of the PBS-treated mice (Fig. 7B and D). Meanwhile, mucus exudation with inflammatory cells was recognized in the nasal areas of the mice treated with CTB* (Fig. 7C).

As the nasal cavity is anatomically connected to the brain via the olfactory nerves, we also examined the effects of intracerebral administration of poly(I:C) and CTB*. One mouse administered 10 μg of CTB* and 2 mice administered 25 μg of CTB* died with marked reduction of body weight (<15%) on day 4 after intracerebral injection. On the other hand, all the mice intracerebrally injected various doses (0.25 to 25 μg) of poly(I:C) survived for 7 days without body weight change (Fig. 8A). Histological examination of the brains demonstrated that cerebral hemorrhage was detected in the mice injected with 10 μg of CTB* (Fig. 8C); however, no significant change was recognized in the poly(I:C)-treated group (Fig. 8B) and PBS-treated group (Fig. 8D). This suggests that intranasal and intracerebral administrations of poly(I:C) are harmless to mice.

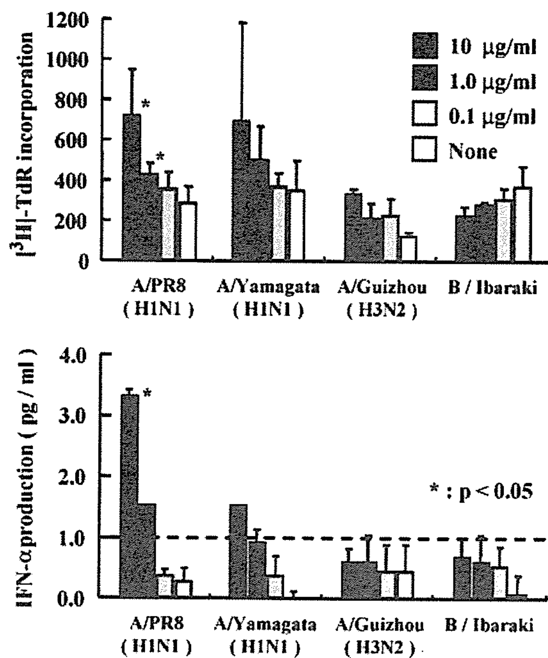

FIG. 4. In vitro responses of A/PR8 (H1N1) influenza virus-specific T cells derived from mice vaccinated with A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki viruses. The mice were intranasally administered 1 μg of each vaccine with 10 μg of poly(I:C) and then boosted with the same dose of the reagents at 3 weeks after priming. Spleens were isolated at 1 week after the boost and stimulated with T-cell-depleted splenocytes that had been pulsed with the indicated concentration of A/PR8 vaccine. These cells were cultured for 4 days and [^{3}H]thymidine was added 8 h prior to the harvest. (B) Production of IFN-γ in the culture supernatant of the cells prepared in the same manner as the cells shown in Fig. 4A. The results are represented as a means of two independent experiments.

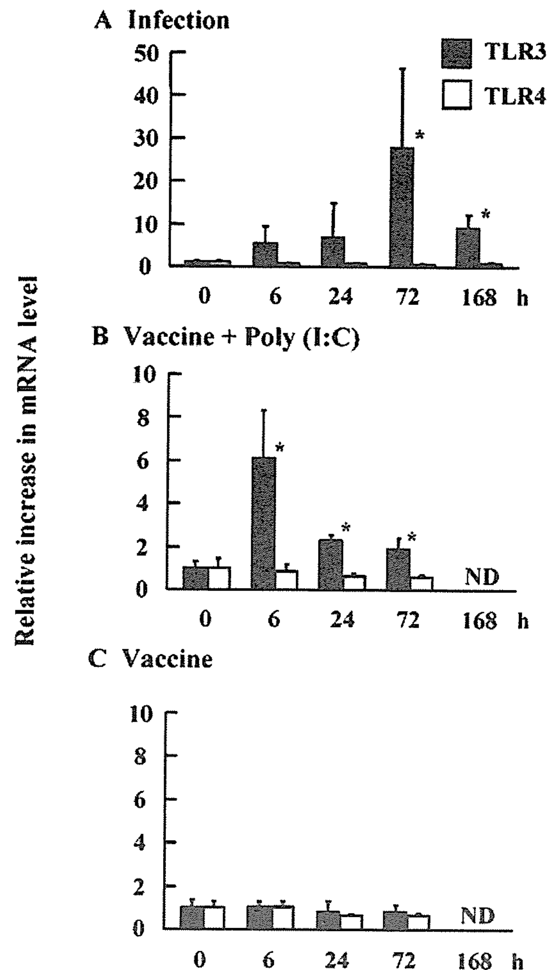

FIG. 5. Expression of TLR3 and TLR4 mRNAs in the NALTs. Total RNAs were extracted from the NALTs of mice infected with 1,000 PFU of A/PR8 (A) and intranasally immunized with the HA vaccine with poly(I:C) (B) and the HA vaccine alone (C). To determine the mRNA expression levels of TLR3 or TLR4 in the NALTs, real-time quantitative RT-PCR was performed (n = 3). *, $P < 0.05$ versus the pretreated group (0 h). ND, not determined.

DISCUSSION

In the present study, we clearly demonstrated that poly(I:C) is an effective mucosal adjuvant when administered intranasally with influenza virus vaccine. It has been reported that effective immunization strategies to protect against influenza virus infection involve induction of mucosal immune responses at the nasal mucosal epithelium, which is the initial target of virus infection (9, 28). For effective protection against influenza virus infection at the mucosa, bacterial toxin-derived adjuvants, such as CTB or *Escherichia coli* heat-labile enterotoxin (LT), have been administered in conjunction with immunization (28, 30, 33). To reduce the toxicity of bacterial toxin-derived adjuvants, mutant toxins (7, 8) or low doses of CTB with trace amounts (0.1%) of holotoxin (25) were applied, and the bacterial toxins became harmless for experimental animal models. However, there are still problems associated with application of bacterial toxin-derived adjuvants to human vaccines. An effective prophylactic method against influenza virus infection in humans must be both safe and effective. We have demonstrated that a synthetic dsRNA adjuvant could induce high titers of anti-HA antibodies comparable to those seen after inoculation of CTB*, when administered intranasally once with the vaccine at the first immunization followed by a booster inoculation of vaccine alone. The immune response was detected in both nasal wash fluid and serum, which resulted in complete protection against influenza viral challenge in both the upper RT-restricted influenza model and the lung infection pneumonia model. In the two-dose immunization regimen, PR8 HA vaccine combined with 1 μg of poly(I:C) at the first immunization conferred complete protection against lethal lung infection (40 LD_{50}) of PR8 influenza virus.

The advantage of nasal vaccination against influenza virus infection is the induction of secretory IgA at the mucosal epithelium, resulting in efficient production of cross-protective immunity compared with serum IgG (26). In fact, we observed the cross-protective effect of nasal vaccination of poly(I:C)-combined vaccination. In the present study, we used HA vaccines from various strains of influenza virus, including A/PR8 (H1N1), A/Beijing (H1N1), A/Yamagata (H1N1), and A/Guizhou (H3N2), with 3 μg of poly(I:C) at the first immunization in the two-dose regimen. Among these vaccines, anti-PR8 HA secretory IgA in the nasal wash fluid and anti-PR8

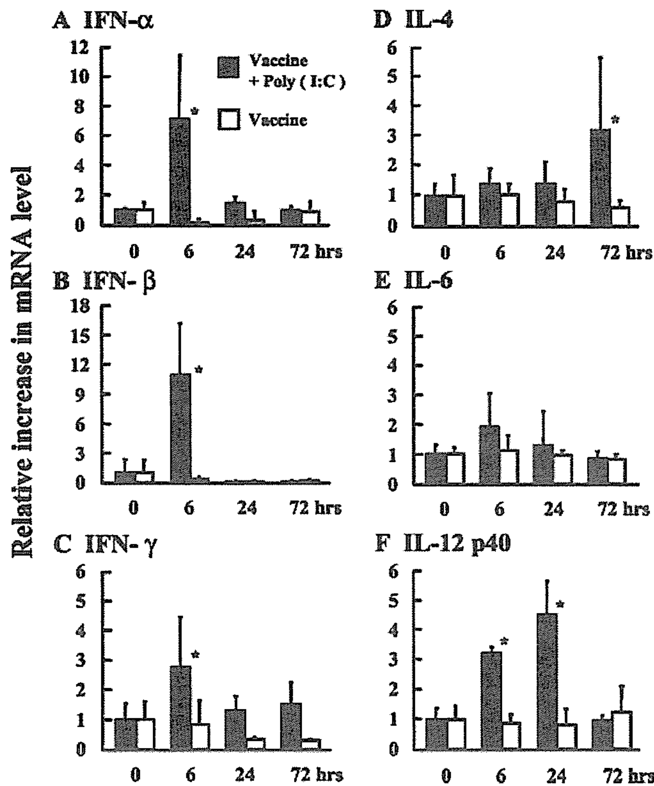

FIG. 6. Expression of cytokine mRNAs in the NALTs. Total RNA was extracted from the NALTs of mice intranasally treated with 1 μg of PR8 vaccine with or without poly(I:C). The mRNA levels of IFN-α (A), IFN-β (B), IFN-γ (C), IL-4 (D), IL-6 (E), and IL-12 p40 (F) in the NALTs were determined with real time RT-PCR (n = 3). *, $P < 0.05$ versus the pretreated group (0 h).

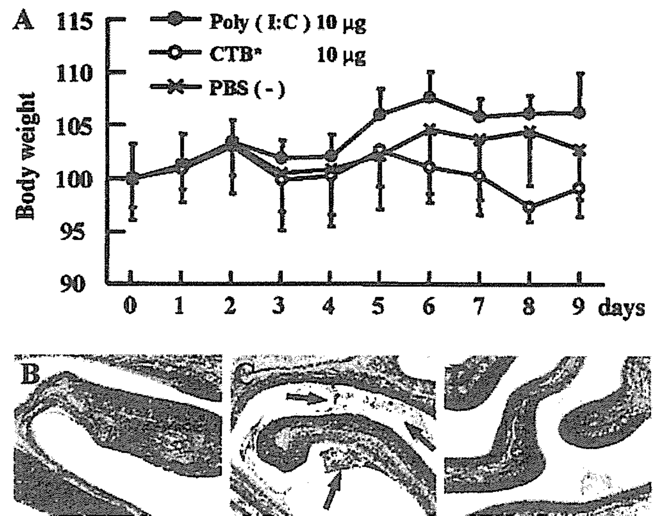

FIG. 7. (A) Body weight of mice with intranasal administration of 10 μg of poly(I:C) or 10 μg of CTB* daily for 9 days. Each point represents the mean relative ratio to initial body weight (mean ± SD [%]) of 5 mice in each day. (B to D) Histopathological findings of the nasal cavities of the mice intranasally administered 10 μg of poly(I:C) (B), 10 μg of CTB* (C), and PBS (D) daily for 9 days (×100; H&E). Black arrows indicate the mucus exudation with inflammatory cells.

HA IgG in the serum were detected in the mice immunized with the same H1N1 virus strains. However, the neutralizing activity of the serum against A/PR8 virus was exclusively recognized in the mice immunized with homologous virus (Fig. 3). Although we have examined the neutralizing activity in the nasal wash fluid, no neutralizing activity in the nasal wash fluid was detected in any group. The concentration of anti-A/PR8 HA IgA in the nasal wash fluid is much lower than that of anti-A/PR8 HA IgG in the serum, because the nasal wash fluid was diluted by PBS for collection from the nasal mucosa. It seems that the concentration of anti-A/PR8 HA IgA is much lower than the physiological concentration in the nasal mucosa; therefore, the neutralizing activity in the nasal wash fluid is not detected. However, mice that have the cross-reactive IgA to A/PR8 virus in the nasal wash fluid were completely protected against viral challenge from not only homologous but also heterologous viruses. Even mice immunized with influenza virus vaccine against the H3N2 strain produced low doses of secretory IgA and serum IgG, which represented a 10^2-fold reduction of viral titer after A/PR8 influenza virus challenge. We observed the antigen-specific T-cell responses and their weak cross-reactivity in the mice treated with heterologous viruses (Fig. 4). Thus, both homologous and heterologous protection against A/PR8 influenza virus challenge may be achieved by production of cross-reactive IgA.

The mechanism of the adjuvant effect of dsRNA is still unclear. As denatured poly(I:C) did not cause any adjuvant effect, the double-stranded structure of poly(I:C) seemed to play a pivotal role in production of IgA and IgG following intranasal immunization with influenza virus HA vaccine. It is known that dsRNA is recognized by TLR3 and RNA helicase, retinoic acid-inducible gene I, and induces activation of NF-κB and production of IFN-α/β (1, 39). Early administration of IFN-α/β during an immune response markedly increases primary antibody response against soluble antigens (14). We demonstrated up-regulation of TLR3 expression but not of TLR4 in the NALTs following influenza virus infection or intranasal administration of vaccine with poly(I:C) (Fig. 5). The peak of up-regulation of TLR3 at 72 h after infection corresponded to that of influenza virus replication in the nasal area at 72 h postinfection. Because the up-regulated TLR3 is the receptor for the poly(I:C) adjuvant, we presume that the up-regulation of TLR3 enhances the signals transduced from poly(I:C). As the influenza virus itself up-regulates the expression of TLR3, we expect the vaccine with poly(I:C) mimics the state of viral infection so that the protective immune response can be elicited. Moreover, administration of poly(I:C) induced expression of IFN-α/β and Th1- and Th2-related cytokines, such as IFN-γ, IL-12 p40, and IL-4. It has recently been reported that the exposure of naive B cells to the cytokine IL-4 and/or antigen leads to a state of "priming," in which subsequent aggregation of major histocompatibility complex class II molecules induces the mobilization of calcium ions and cell proliferation (12). A significant production of IL-4 in the NALTs immunized intranasally with vaccine with poly(I:C) may contribute to the priming of naive B cells together with the antigen. Thus, poly(I:C) might act to bridge the gap between innate and adaptive immunity.

Prophylactic agents, including vaccines, should be sufficiently safe. As the nasal cavity and the forebrain have direct

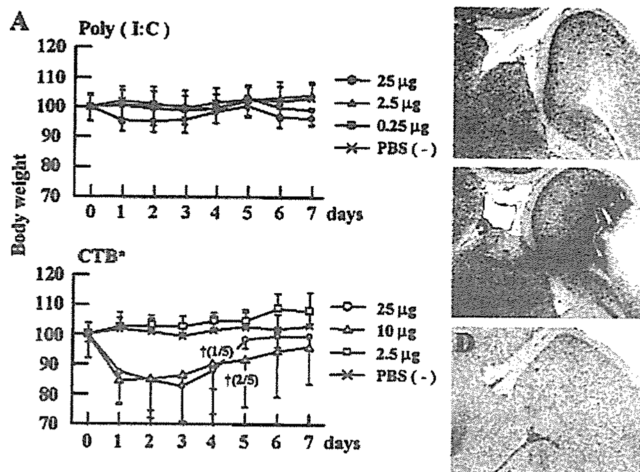

FIG. 8. (A) Body weight of mice after intracerebral injection of various doses [poly(I:C), 0.25, 2.5, and 25 μg; CTB*, 2.5, 10, and 25 μg] of poly(I:C) and CTB*. Each point represents the relative ratio to initial body weight (mean ± SD [%]) of 5 mice in each day. Histopathological findings of the brains injected with 10 μg of poly(I:C) (B), 10 μg of CTB* (C), and PBS (D) at day 4 after injection (×100; H&E).

communication via the olfactory nerve, the safety of nasal administration of the vaccine with adjuvant for the central nervous system should be confirmed. We demonstrated the safety of poly(I:C) to the central nervous system by direct intracerebral injection. Here, we propose that intranasal administration of influenza virus HA vaccine with poly(I:C) by the two-dose regimen is an effective and safe vaccination method.

ACKNOWLEDGMENTS

We are grateful to U. Suzuki (Kitasato Institute) and T. Tanaka (Toray Industries, Inc.) for providing us the materials. We are grateful to A. Harashima for technical assistance.

REFERENCES

1. **Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell.** 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **413:**732–738.
2. **Asahi, Y., T. Yoshikawa, I. Watanabe, T. Iwasaki, H. Hasegawa, Y. Sato, S. Shimada, M. Nanno, Y. Matsuoka, M. Ohwaki, Y. Iwakura, Y. Suzuki, C. Aizawa, T. Sata, T. Kurata, and S. Tamura.** 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J. Immunol. **168:**2930–2938.
3. **Bower, J. F., X. Yang, J. Sodroski, and T. M. Ross.** 2004. Elicitation of neutralizing antibodies with DNA vaccines expressing soluble stabilized human immunodeficiency virus type 1 envelope glycoprotein trimers conjugated to C3d. J. Virol. **78:**4710–4719.
4. **Clements, M. L., S. O'Donnell, M. M. Levine, R. M. Chanock, and B. R. Murphy.** 1983. Dose response of A/Alaska/6/77 (H3N2) cold-adapted reassortant vaccine virus in adult volunteers: role of local antibody in resistance to infection with vaccine virus. Infect. Immun. **40:**1044–1051.
5. **Couch, R. B., and J. A. Kasel.** 1983. Immunity to influenza in man. Annu. Rev. Microbiol. **37:**529–549.
6. **Davenport, F. M., A. V. Hennessy, F. M. Brandon, R. G. Webster, C. D. Barrett, Jr., and G. O. Lease.** 1964. Comparisons of serologic and febrile responses in humans to vaccination with influenza A viruses or their hemagglutinins. J. Lab. Clin. Med. **63:**5–13.
7. **Hagiwara, Y., T. Iwasaki, H. Asanuma, Y. Sato, T. Sata, C. Aizawa, T. Kurata, and S. Tamura.** 2001. Effects of intranasal administration of cholera toxin (or Escherichia coli heat-labile enterotoxin) B subunits supplemented with a trace amount of the holotoxin on the brain. Vaccine **19:**1652–1660.
8. **Hagiwara, Y., K. Komase, Z. Chen, K. Matsuo, Y. Suzuki, C. Aizawa, T. Kurata, and S. Tamura.** 1999. Mutants of cholera toxin as an effective and safe adjuvant for nasal influenza vaccine. Vaccine **17:**2918–2926.
9. **Hasegawa, H., S. Kadowaki, H. Takahashi, T. Iwasaki, S. Tamura, and T. Kurata.** 2000. Protection against influenza virus infection by nasal vaccination in advance of sublethal irradiation. Vaccine **18:**2560–2565.
10. **Jacobs, B. L., and J. O. Langland.** 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology **219:**339–349.
11. **Johnson, P. R., S. Feldman, J. M. Thompson, J. D. Mahoney, and P. F. Wright.** 1986. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J. Infect. Dis. **154:**121–127.
12. **Jordan, M. B., D. M. Mills, J. Kappler, P. Marrack, and J. C. Cambier.** 2004. Promotion of B cell immune responses via an alum-induced myeloid cell population. Science **304:**1808–1810.
13. **Kris, R. M., R. Asofsky, C. B. Evans, and P. A. Small, Jr.** 1985. Protection and recovery in influenza virus-infected mice immunosuppressed with anti-IgM. J. Immunol. **134:**1230–1235.
14. **Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough.** 2001. Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity **14:**461–470.
15. **Liew, F. Y., S. M. Russell, G. Appleyard, C. M. Brand, and J. Beale.** 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur. J. Immunol. **14:**350–356.
16. **Mestecky, J., and J. R. McGhee.** 1987. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv. Immunol. **40:**153–245.
17. **Murphy, B. R.** 1994. Mucosal immunity to viruses., p. 333. *In* P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober, and J. Biendnstock (ed.), Handbook of mucosal immunology. Academic Press, Inc., New York, N.Y.
18. **Murphy, B. R., and M. L. Clements.** 1989. The systemic and mucosal immune response of humans to influenza A virus. Curr. Top. Microbiol. Immunol. **146:**107–116.
19. **Murphy, B. R., and R. G. Webster.** 1996. Orthomyxoviruses, p. 1397–1445. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology. Lippincott-Raven, Philadelphia, Pa.
20. **Nedrud, J. G., X. P. Liang, N. Hague, and M. E. Lamm.** 1987. Combined oral/nasal immunization protects mice from Sendai virus infection. J. Immunol. **139:**3484–3492.
21. **Phelan, M. A., R. E. Mayner, D. J. Bucher, and F. A. Ennis.** 1980. Purification of influenza virus glycoproteins for the preparation and standardization of immunological potency testing reagents. J. Biol. Stand. **8:**233–242.
22. **Ramphal, R., R. C. Cogliano, J. W. Shands, Jr., and P. A. Small, Jr.** 1979. Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis. Infect. Immun. **25:**992–997.
23. **Renegar, K. B., and P. A. Small, Jr.** 1994. Passive immunization: systemic and mucosal, p. 347. *In* P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober, and J. Biendnstock (ed.), Handbook of mucosal immunology. Academic Press, Inc., New York, N.Y.
24. **Takasuka, N., H. Fujii, Y. Takahashi, M. Kasai, S. Morikawa, S. Itamura, K. Ishii, M. Sakaguchi, K. Ohnishi, M. Ohshima, S. Hashimoto, T. Odagiri, M. Tashiro, H. Yoshikura, T. Takemori, and Y. Tsunetsugu-Yokota.** 2004. A subcutaneously injected UV-inactivated SARS coronavirus vaccine elicits systemic humoral immunity in mice. Int. Immunol. **16:**1423–1430.
25. **Tamura, S., K. Ishihara, K. Miyata, C. Aizawa, and T. Kurata.** 1995. Mechanism of enhancement of the immune responses to influenza vaccine with cholera toxin B subunit and a trace amount of holotoxin. Vaccine **13:**339–341.
26. **Tamura, S., Y. Ito, H. Asanuma, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, and T. Kurata.** 1992. Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. J. Immunol. **149:**981–988.
27. **Tamura, S., T. Iwasaki, A. H. Thompson, H. Asanuma, Z. Chen, Y. Suzuki, C. Aizawa, and T. Kurata.** 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. J. Gen. Virol. **79**(Pt. 2):291–299.
28. **Tamura, S., H. Kurata, H. Funato, T. Nagamine, C. Aizawa, and T. Kurata.** 1989. Protection against influenza virus infection by a two-dose regimen of nasal vaccination using vaccines combined with cholera toxin B subunit. Vaccine **7:**314–320.
29. **Tamura, S., K. Miyata, K. Matsuo, H. Asanuma, H. Takahashi, K. Nakajima, Y. Suzuki, C. Aizawa, and T. Kurata.** 1996. Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. J. Immunol. **156:**3892–3900.
30. **Tamura, S., Y. Samegai, H. Kurata, T. Nagamine, C. Aizawa, and T. Kurata.** 1988. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. Vaccine **6:**409–413.
31. **Tamura, S., A. Yamanaka, M. Shimohara, T. Tomita, K. Komase, Y. Tsuda, Y. Suzuki, T. Nagamine, K. Kawahara, H. Danbara, et al.** 1994. Synergistic action of cholera toxin B subunit (and Escherichia coli heat-labile toxin B

subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. Vaccine **12**:419–426.
32. **Tamura, S. I., H. Asanuma, Y. Ito, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, T. Kurata, and A. Oya.** 1992. Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. Eur. J. Immunol. **22**:477–481.
33. **Tamura, S. I., Y. Samegai, H. Kurata, K. Kikuta, T. Nagamine, C. Aizawa, and T. Kurata.** 1989. Enhancement of protective antibody responses by cholera toxin B subunit inoculated intranasally with influenza vaccine. Vaccine **7**:257–262.
34. **Tobita, K.** 1975. Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. Med Microbiol. Immunol. (Berlin) **162**:23–27.
35. **Tobita, K., A. Sugiura, C. Enomote, and M. Furuyama.** 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med. Microbiol. Immunol. (Berlin) **162**:9–14.
36. **Underdown, B. J., and J. M. Schiff.** 1986. Immunoglobulin A: strategic defense initiative at the mucosal surface. Annu. Rev. Immunol. **4**:389–417.
37. **Watanabe, I., T. M. Ross, S. Tamura, T. Ichinohe, S. Ito, H. Takahashi, H. Sawa, J. Chiba, T. Kurata, T. Sata, and H. Hasegawa.** 2003. Protection against influenza virus infection by intranasal administration of C3d-fused hemagglutinin. Vaccine **21**:4532–4538.
38. **Yetter, R. A., S. Lehrer, R. Ramphal, and P. A. Small, Jr.** 1980. Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. Infect. Immun. **29**:654–662.
39. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. **5**:730–737.

JOURNAL OF VIROLOGY, Mar. 2005, p. 2847–2858 Vol. 79, No. 5
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.5.2847–2858.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Involvement of the Toll-Like Receptor 9 Signaling Pathway in the Induction of Innate Immunity by Baculovirus†

Takayuki Abe,[1] Hiroaki Hemmi,[2] Hironobu Miyamoto,[1] Kohji Moriishi,[1] Shinichi Tamura,[3] Hiroshi Takaku,[4] Shizuo Akira,[2] and Yoshiharu Matsuura[1]*

Research Center for Emerging Infectious Diseases,[1] Department of Host Defense,[2] and Laboratory of Prevention of Viral Diseases,[3] Research Institute for Microbial Diseases, Osaka University, Osaka, and Department of Industrial Chemistry and High Technology Research Center, Chiba Institute of Technology, Chiba,[4] Japan

Received 11 July 2004/Accepted 6 October 2004

We have previously shown that mice inoculated intranasally with a wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) are protected from a lethal challenge by influenza virus. However, the precise mechanism of induction of this protective immune response by the AcNPV treatment remained unclear. Here we show that AcNPV activates immune cells via the Toll-like receptor 9 (TLR9)/MyD88-dependent signaling pathway. The production of inflammatory cytokines was severely reduced in peritoneal macrophages (PECs) and splenic CD11c$^+$ dendritic cells (DCs) derived from mice deficient in MyD88 or TLR9 after cultivation with AcNPV. In contrast, a significant amount of alpha interferon (IFN-α) was still detectable in the PECs and DCs of these mice after stimulation with AcNPV, suggesting that a TLR9/MyD88-independent signaling pathway might also participate in the production of IFN-α by AcNPV. Since previous work showed that TLR9 ligands include bacterial DNA and certain oligonucleotides containing unmethylated CpG dinucleotides, we also examined the effect of baculoviral DNA on the induction of innate immunity. Transfection of the murine macrophage cell line RAW264.7 with baculoviral DNA resulted in the production of the inflammatory cytokine, while the removal of envelope glycoproteins from viral particles, UV irradiation of the virus, and pretreatment with purified baculovirus envelope proteins or endosomal maturation inhibitors diminished the induction of the immune response by AcNPV. Together, these results indicate that the internalization of viral DNA via membrane fusion mediated by the viral envelope glycoprotein, as well as endosomal maturation, which releases the viral genome into TLR9-expressing cellular compartments, is necessary for the induction of the innate immune response by AcNPV.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has long been used as a biopesticide and as an efficient tool for recombinant protein production in insect cells (39, 42). Subsequently, its efficacy for the delivery of high-level expression of foreign genes under the control of mammalian promoters in infected mammalian cells was also demonstrated (12, 26, 48). Since it causes no visible cytopathic effects, even at high titers, and does not replicate in mammalian cells (49), this baculovirus is now recognized as a useful viral vector, not only for the expression of foreign proteins in insect cells, but also for gene delivery to mammalian cells (4, 9, 12, 16, 26, 28, 37, 45, 48, 49, 53, 54).

AcNPV was also shown to be capable of stimulating interferon (IFN) production in mammalian cell lines and can confer protection from lethal encephalomyocarditis virus infections in mice (18). We demonstrated that intranasal inoculation with AcNPV induces a strong innate immune response and protects mice from a lethal challenge of influenza A and B viruses (1). Furthermore, inoculation with baculovirus induces the secretion of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-12, in RAW264.7, a murine macrophage cell line. However, the precise mechanism of induction of the protective immune response by a pretreatment with AcNPV remained unclear.

Members of the IL-1 receptor/Toll-like receptor (TLR) superfamily are key mediators of innate and adaptive immunity (5). Toll, the first member of this superfamily to be identified, was initially discovered as a factor involved in dorsoventral axis formation in fly embryos and was later shown to participate in host defense mechanisms (38). A family of TLRs exists in mammals and has been shown to play an important role not only in the recognition of a wide variety of infectious pathogens and their products, but also in protection of the host from infections with pathogens. So far, 11 TLR family members and their corresponding ligands have been identified, with TLR1 being the only orphan receptor among them. Different TLRs have been shown to mediate immune responses to a variety of different pathogen-derived elements. For example, TLR4, TLR5, and TLR9 are essential for the recognition of lipopolysaccharides (LPS), bacterial flagellin, and bacterial DNA containing unmethylated CpG motifs, respectively (21, 24, 27, 46). TLR2 is implicated in the recognition of peptidoglycan (PGN) and lipopeptides (7, 13, 50, 57), while TLR6 can associate with TLR2 and recognize PGN and lipopeptides derived from mycoplasma (44). On the other hand, TLR3 has been shown to activate immune cells in response to virus-derived double-stranded RNA (6). Although synthetic imidazoquinoline com-

* Corresponding author. Mailing address: Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

† This study is dedicated to the memory of Ikuko Yanase.

pounds and guanosine analogs with antiviral activities have been shown to activate TLR7 and TLR8 (25, 36), it was recently demonstrated that single-stranded RNAs from RNA viruses are the natural ligands of these receptors (17, 23). The most recently identified TLR, termed TLR11, senses bacteria that cause infections of the bladder and kidney (60). In summary, TLRs recognize specific components derived from pathogens and activate a signaling cascade that causes proinflammatory cytokine production and subsequent immune responses.

TLRs share a common cytoplasmic Toll–IL-1 receptor (TIR) domain. MyD88, also a TIR domain-containing protein, associates with TLRs and acts as an adapter that recruits IL-1 receptor-associated kinase and TNF receptor-associated factor 6 (TRAF6) to TLRs. Macrophages isolated from MyD88-deficient mice fail to activate NF-κB and Jun N-terminal protein kinase or to produce inflammatory cytokines in response to microbial components such as lipopeptides, LPS, and CpG-rich bacterial DNA (20, 52), indicating that MyD88 is a critical component in the signaling pathway that leads to the production of inflammatory cytokines.

Viruses are obligate intracellular parasites; accordingly, viral proteins synthesized in host cells bear modifications that reflect the identity and characteristics of the host. Therefore, viral particles do not display exclusively pathogen-associated molecular patterns. Although the mechanisms by which the innate immune response is induced by viral infection are poorly understood, there is increasing evidence suggesting that TLRs function to detect viruses and trigger inflammatory responses. For instance, respiratory syncytial virus and mouse mammary tumor virus activate innate immunity through TLR4 (22, 34, 47), which is a signaling receptor for LPS. Similarly, hemagglutinin from wild-type measles virus was reported to activate TLR2 (10), which also recognizes certain elements of gram-positive bacteria and fungi. Herpes simplex virus type 1 (HSV-1) and human cytomegalovirus have also been shown to recognize TLR2 (15, 35), while vaccinia virus encodes proteins containing amino acid sequences similar to the Toll/IL-1 receptor domain and inhibits IL-1-, IL-18-, and TLR4-mediated signal transduction (11).

It was recently shown that HSV-1 and -2, whose genomes contain abundant CpG motifs, can induce angiogenesis and a variety of diseases, including herpes stromal keratitis, that produce chronic inflammatory responses via a TLR9/MyD88-dependent signaling pathway (33, 40, 61). HSV-1 and -2 are also able to trigger alpha interferon (IFN-α) secretion from plasmacytoid dendritic cells through TLR9/MyD88-dependent signaling (33, 40). The TLR9-mediated recognition of HSV by immunocompetent cells suggests that this recognition pathway may be important for the recognition of other DNA viruses.

For this study, we characterized the innate immune response induced by AcNPV. Peritoneal macrophages and splenic $CD11c^+$ dendritic cells obtained from TLR9 or MyD88 knockout mice exhibited severe reductions in proinflammatory cytokine production following stimulation with AcNPV, whereas a significant amount of IFN-α was still detectable in these cells. In addition, the frequency of CpG motifs in the AcNPV genome was similar to that of bacterial DNA and significantly higher than that of mammalian DNA. Furthermore, stimulation by AcNPV was eliminated by a treatment with inhibitors of endosomal acidification. These results indicate that the internalization of viral AcNPV DNA via membrane fusion by envelope glycoproteins found in the endosome is required for the induction of a TLR9/MyD88-dependent innate immune response.

MATERIALS AND METHODS

Mice and cell culture. C57BL/6 mice were purchased from Clea Japan, Inc., Tokyo, Japan. MyD88-deficient ($MyD88^{-/-}$) mice were established as previously described (2) and backcrossed more than eight times with C57BL/6 mice. $TLR9^{-/-}$ mice were generated as previously described (24). The mice were injected intraperitoneally with 2 ml of 4% thioglycolate (Sigma-Aldrich Co., St. Louis, Mo.), and cells were harvested 3 days later by peritoneal lavage. The mouse macrophage cell line RAW264.7 was purchased from Riken Cell Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml at 37°C in a 5% CO_2 humidified incubator.

Viruses and reagents. AcNPV was propagated in *Spodoptera frugiperda* (Sf-9) cells in Sf-900II insect medium supplemented with 10% (vol/vol) heat-inactivated FCS. A mutant baculovirus, AcNPVΔ64, which lacks the gp64 envelope protein and possesses the green fluorescent protein gene under the control of the polyhedrin promoter in the gp64 gene locus, was generated (Y. Kitagawa et al., unpublished data). AcNPV and AcNPVΔ64 were purified as previously described (1). The inactivation of AcNPV was performed with a Stratalinker 2400 (Stratagene, La Jolla, Calif.) using short-wavelength UV radiation (UVC, 254 nm) at a distance of 5 cm for 30 min on ice (1.6×10^4 mJ/cm^2). The inactivation of infectivity was verified by a plaque assay with Sf-9 cells.

AcNPV DNA was isolated from the purified virions by a treatment with 10 mg of proteinase K (Sigma-Aldrich)/ml and 10% sodium dodecyl sulfate (SDS) in sterile phosphate-buffered saline (PBS) for 2 h at 55°C. The viral DNA was purified by phenol-chloroform-isoamyl alcohol extraction, precipitated at 12,000 × *g*, and resuspended in sterile endotoxin-free Tris-buffered saline. RNAs were removed by incubation with RNase A (10 mg/ml) (Wako Pure Chemical Industries, Osaka, Japan) for 1 h at 37°C, and the viral DNA was extracted as described above. The resultant DNA exhibited a single band by electrophoresis, and neither protein nor chromosomal DNA of insect cells was detected.

Phosphorothioate-stabilized mouse CpG (mCpG) oligodeoxynucleotides (ODN1668) (TCC-ATG-ACG-TTC-CTG-ATG-CT) and human CpG (hCpG) oligodeoxynucleotides (ODN2006) (TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT) were purchased from Invitrogen (Tokyo, Japan). Guanosine, 2′-deoxy-G, 8-bromo-G, 7-methyl-G, 7-allyl-8-oxo-G (loxoribine) was purchased from Invivogen (San Diego, Calif.). LPS derived from *Salmonella enterica* serovar Minnesota (Re-595), PGN derived from *Staphylococcus aureus*, monodansylcadaverine (MDC), and chloroquine were purchased from Sigma-Aldrich. Bafilomycin A1 and ammonium chloride were purchased from Wako Pure Chemical Industries. An anti-p39 mouse monoclonal antibody was kindly provided by G. F. Rohrmann. The virus stocks and the other TLR ligands were free of endotoxin (<0.01 endotoxin units/ml), as determined by use of a Pyrodick endotoxin measure kit (Seikagaku Co., Tokyo, Japan).

Production of authentic and truncated forms of gp64 proteins. cDNAs encoding a deletion mutant of gp64 lacking the transmembrane region (gp64ΔTM) as well as a wild-type version of gp64 were obtained by PCRs with AcNPV DNA as a template. The same 5′ primer (5′-CATAAGCTTATGGTAAGCGCTATT GTTTTATAT-3′) was used to amplify the gp64 and gp64ΔTM cDNAs, and the 3′ primers were 5′-GATTCTAGAATATATTGTCTATTACGGTTTCT-3′ and 5′ GATTCTAGAATCGAAGTCAATTTAGCGGCCAA-3′, respectively. cDNAs were subcloned into HindIII and XbaI sites in pIB/V5-His (Invitrogen). The sequences of the recombinant plasmids, pIBgp64/V5-His and pIBgp64ΔTM/V5-His, were confirmed by DNA sequencing. These plasmids were transfected into Sf-9 cells by the use of Unifector (B-Bridge International, Inc., San Jose, Calif.). After 3 days of incubation, the recombinant gp64 proteins were purified from cell lysates or supernatants by use of a column of nickel-nitrilotriacetic acid beads (QIAGEN, Valencia, Calif.). The protein concentrations were determined by use of a Micro BCA protein assay kit (Pierce, Rockford, Ill.). The recombinant proteins were analyzed by SDS–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) under reducing conditions, stained with GelCord Blue staining reagent (Pierce), and detected by immunoblotting analysis with an antihexahistidine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Isolation of peritoneal cells and cytokine production. To evaluate cytokine production from macrophages in vitro, we seeded thioglycolate-elicited perito-

neal cells (PECs) into 96-well plates at a concentration of 2×10^5 cells/well and stimulated them with various doses of AcNPV and loxoribine. After 24 h of incubation, the culture supernatants were collected and analyzed for cytokine production. The concentrations of IL-12 p40 and IFN-α in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISAs). ELISA kits for OptEIA mouse IL-12 p40 Set and mouse IFN-α were purchased from BD PharMingen (San Diego, Calif.) and PBL Biomedical Laboratories (New Brunswick, N.J.), respectively. Total RNAs were isolated by the use of Sepazol-RNA I (Nacalai Tesque, Kyoto, Japan), electrophoresed, and transferred to nylon membranes. Hybridization was performed with the indicated cDNA probes as previously described (2). cDNA probes specific for IL-12 p40 were established as previously described (31). To determine the effects of infection with AcNPV on cytokine production, we seeded the mouse macrophage cell line RAW264.7 into six-well plates at a concentration of 10^6 cells/well and stimulated them with various TLR ligands, with or without endosomal inhibitors such as chloroquine, bafilomycin A1, MDC, and ammonium chloride. For cell stimulation, AcNPV (5 μg/ml), LPS (10 ng/ml), PGN (2.5 μg/ml), and mCpG (200 ng/ml) were used.

Preparation of splenic dendritic cells and cytokine secretion. To prepare splenocytes containing dendritic cells (DCs), we cut spleen tissues into small fragments and incubated them with RPMI 1640 containing 400 U of collagenase (Wako)/ml and 15 μg of DNase (Sigma-Aldrich)/ml at 37°C for 20 min. For the last 5 min, 5 mM EDTA was added, and single-cell suspensions were prepared after red blood cell lysis. $CD11c^+$ cells were purified by magnetic cell sorting with anti-CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions and were used as splenic DCs. Enriched cells containing >90% $CD11c^+$ cells were seeded into 96-well plates at a concentration of 10^5 cells/well and stimulated with various doses of AcNPV or loxoribine. Culture supernatants were collected, and the production of IL-12 p40 and IFN-α was determined by ELISAs.

Indirect immunofluorescence assay and flow cytometric analysis. 293T cells transfected with a plasmid encoding human TLR9 were dislodged with PBS containing 5 mM EDTA 48 h after transfection. The cells were incubated with PBS containing 2% FCS and an anti-Flag (M2) monoclonal antibody (1:1,000) (Santa Cruz Biotechnology) for 1 h at 4°C, washed twice with PBS containing 2% FCS, and further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma-Aldrich) in PBS containing 2% FCS for 1 h at 4°C. The cells were then fixed with 4% paraformaldehyde for 20 min, and the surface expression of human TLR9 was observed by fluorescence microscopy (UFX-II microscope; Nikon, Tokyo, Japan). Intracellular staining was examined after permeabilization with 0.5% Triton X-100. Stained cells were also analyzed by flow cytometry with a FACSCalibur instrument (Becton Dickinson, San Jose, Calif.), and the data were analyzed with CellQuest software (Becton Dickinson).

NF-κB–luciferase reporter gene assays with 293T cells. 293T cells were transfected with an NF-κB-dependent luciferase reporter plasmid (pELAM-Luc) together with human TLR9 expression vectors by the use of Lipofectamine 2000 (Life Technologies, Grand Island, N.Y.). pELAM-Luc (kindly provided by D. T. Golenbock) contains a human E-selectin promoter introduced into the pGL3 reporter plasmid (Promega, Inc., Madison, Wis.). The human TLR9 expression vector (kindly provided by T. H. Chuang) consists of a preprotrypsin signal peptide and a Flag epitope tag followed by an in-frame human TLR9 cDNA sequence (14). At 24 h posttransfection, the cells were stimulated with hCpG DNA (10 μg/ml) or AcNPV DNA (10 μg/ml) for 24 h. The luciferase activity was determined as previously described (49) and calculated as the degree of induction compared with an untreated control.

Detection of AcNPV capsid protein in murine macrophage cells by Western blot analysis. RAW264.7 murine macrophage cells (10^6 cells/well) infected with AcNPV at a dose of 40 μg/ml were washed extensively after 1 h of adsorption and harvested after 4 or 6 h of incubation. The cells were lysed in buffer containing 1% Triton X-100, 135 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% glycerol, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). The lysed sample was separated by SDS–12.5% PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). An anti-p39 mouse monoclonal antibody was used to detect the AcNPV capsid protein, which was visualized with the SuperSignal West Femto chemiluminescent substrate (Pierce).

RESULTS

Immune system activation by AcNPV is not mediated by viral envelope glycoprotein. It was previously reported that an IFN-stimulating preparation purified from Sf-9 cells infected with AcNPV exhibited IFN production both in vitro and in vivo and that induction was inhibited by monoclonal antibodies against the AcNPV envelope glycoprotein gp64 (18). To verify these observations, we constructed a mutant baculovirus lacking gp64, which we called AcNPVΔ64, and examined its ability to stimulate an immune response in RAW264.7 cells, which are highly sensitive to TLR stimulation and respond by producing inflammatory cytokines at a level comparable to that observed in primary macrophages (1). The absence of gp64 in purified particles of AcNPVΔ64 was confirmed by immunoblotting (Fig. 1A). The mutant virus lost the ability to induce TNF-α production in inoculated RAW264.7 cells (Fig. 1B), a result that is consistent with the previous observation that gp64 appears to play an important role in the induction of the immune response by AcNPV (18). Because some microbial products are known to induce cytokine production in macrophages, it was important to eliminate the possibility that contamination with microbial products contributed to the immune system

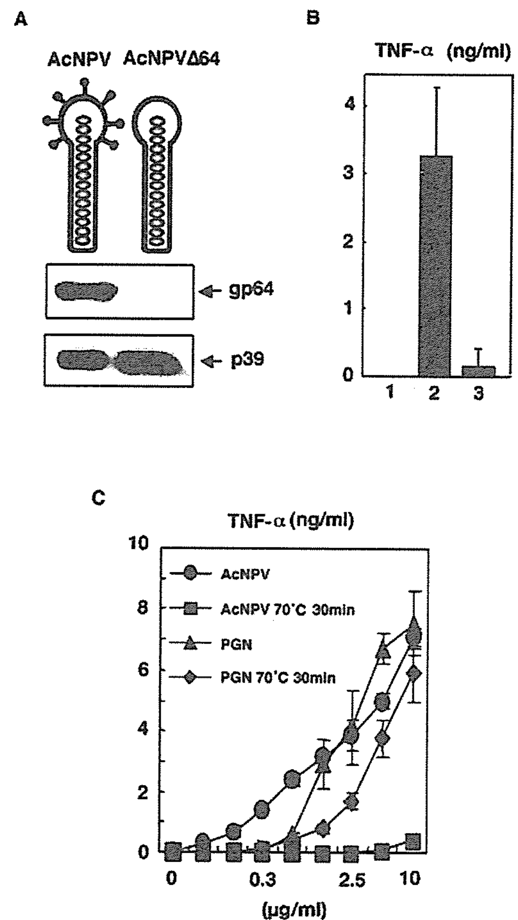

FIG. 1. Immune system activation of macrophages by heat-denatured or gp64-deficient AcNPV. (A) Purified particles of the mutant virus, AcNPVΔ64, lack gp64, as assayed by immunoblotting. (B) The production of TNF-α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (5 μg/ml) (bar 2) or AcNPVΔ64 (5 μg/ml) (bar 3) was determined 24 h after inoculation by a sandwich ELISA. 1 is an uninfected control. Data are shown as means ± SD. (C) AcNPV and PGN were incubated at 70°C for 30 min. Treated and untreated samples were inoculated into RAW264.7 cells (10^6 cells/well) and incubated for 24 h. The production of TNF-α was determined by a sandwich ELISA. Data are shown as means ± SD.

activation by AcNPV. Although the stimulation of macrophages by AcNPV was completely eliminated by incubation at 70°C for 30 min (Fig. 1C), stimulation by the bacterial components PGN and LPS was resistant to heat treatment (Fig. 1C and data not shown). These data indicate that the activation of macrophages by AcNPV is mediated by heat-labile viral components rather than by LPS and PGN.

To further verify the involvement of gp64 in immune system stimulation by baculovirus, we prepared expression plasmids encoding both wild-type gp64 and a C-terminally truncated gp64 protein (gp64ΔTM) with a C-terminal His_6 tag to allow for purification. Upon transfection of Sf9 cells, both recombinant proteins were detected, while gp64ΔTM was efficiently secreted into the culture supernatant (Fig. 2A). The protein from cells expressing gp64ΔTM was purified by column chromatography, producing a single band corresponding to gp64ΔTM and comparable to viral gp64 (Fig. 2B). We also tried to obtain the wild-type gp64 protein from the cell lysates but could not purify it to a homogeneous band (data not shown).

The activities of AcNPV, gp64ΔTM, and PGN on RAW264.7 cells were then examined. A dose-dependent induction of TNF-α and IL-6 was observed for RAW264.7 cells treated with AcNPV and PGN, whereas cytokine production was not observed for cells treated with gp64ΔTM (Fig. 2C). In addition, gp64ΔTM was not able to induce IFN-α production in RAW264.7 cells (Fig. 2D). Furthermore, the pretreatment of macrophage cells with gp64ΔTM inhibited immune system activation by AcNPV but had no effect on the activation by PGN (Fig. 2E), suggesting that the gp64ΔTM protein still retained some of the biological functions of the wild-type gp64 protein, at least in terms of its interaction with host cells. These results indicated that gp64 is an essential element of AcNPV-induced immune system activation in RAW264.7 cells but that it does not directly participate in the reaction. Viral components other than gp64 may be more directly involved in this process.

AcNPV induces inflammatory cytokine production through a MyD88/TLR9-dependent pathway. Immune cells from MyD88- or TLR-deficient mice are unresponsive to TLR ligands, as assayed by their levels of cytokine production (5). Therefore, we used PECs and splenic $CD11c^+$ DCs obtained from MyD88- and TLR-deficient mice to determine whether or not the TLR signaling pathway is responsible for the activation by AcNPV. Thioglycolate-elicited PECs were isolated from wild-type, $MyD88^{-/-}$, $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR9^{-/-}$ mice and examined by ELISA and Northern blot analysis for the induction of IL-12 following exposure to AcNPV. Wild-type macrophages inoculated with AcNPV produced large amounts of IL-12 in a dose-dependent manner, whereas MyD88- or TLR9-deficient macrophages had severely reduced IL-12 production (Fig. 3A). PECs from $TLR2^{-/-}$ and $TLR4^{-/-}$ mice produced IL-12 at wild-type levels in response to AcNPV (Fig. 3A).

Loxoribine is a potent inducer of cytokine production in macrophages and functions through a TLR7-dependent pathway (36). PECs from wild-type, $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR9^{-/-}$ mice all produced IL-12 in response to loxoribine, whereas no IL-12 production was observed in PECs from $MyD88^{-/-}$ mice (Fig. 3A). The transcription of IL-12 p40 mRNA was also impaired in MyD88- and TLR9-deficient macrophages stimulated with AcNPV (Fig. 3B). We further examined the response of splenic $CD11c^+$ DCs to AcNPV and loxoribine. Wild-type and $TLR4^{-/-}$ splenic $CD11c^+$ DCs produced IL-12 in response to AcNPV in a dose-dependent manner, whereas the production of IL-12 was severely impaired in $MyD88^{-/-}$ and $TLR9^{-/-}$ mice (Fig. 3C). In response to loxoribine, splenic $CD11c^+$ DCs from $TLR4^{-/-}$ and $TLR9^{-/-}$ mice exhibited higher IL-12 production levels than wild-type cells, whereas the production of IL-12 was completely inhibited in $MyD88^{-/-}$ mice (Fig. 3C). These results indicate that AcNPV induces the production of inflammatory cytokines in immunocompetent cells through a MyD88/TLR9-dependent pathway.

AcNPV produces IFN-α through a MyD88/TLR9-independent pathway. IFNs are important mediators of the early host defense against various viral infections. Since AcNPV has also been shown to be a potent inducer of IFN-α (Fig. 2D) (18), we investigated whether IFN-α production induced by AcNPV is dependent on the MyD88 and TLR9 signaling pathways. Although IFN-α induction by the TLR9 ligand, CpG oligonucleotides, was completely abolished in PECs and splenic $CD11c^+$ DCs derived from $MyD88^{-/-}$ or $TLR9^{-/-}$ mice (data not shown), IFN-α production in response to AcNPV was less impaired (Fig. 4A). This contrasted sharply with the complete loss of IL-12 production observed for these cells (Fig. 3). Macrophages from $MyD88^{-/-}$ and $TLR9^{-/-}$ mice exhibited a slight reduction in IFN-α and IFN-β mRNA transcription in response to AcNPV (Fig. 4B). These results indicate that AcNPV induces the production of inflammatory cytokines in immunocompetent cells through a MyD88/TLR9-dependent pathway, while other MyD88/TLR9-independent pathways are also involved in the production of IFNs.

AcNPV DNA stimulates immune system activation in macrophage cell lines. CpG motifs present in the genomes of many bacteria are unmethylated, whereas eukaryotic genomes are much more likely to undergo methylation. Previous work demonstrated that bacterial DNAs and certain oligonucleotides containing unmethylated CpG dinucleotides can stimulate PECs and DCs (19, 32). In addition, TLR9 is essential for the immune response to CpG-rich DNA, since TLR9-deficient mice are refractory to such stimulation (24). The frequency of bioactive CpG motifs in the AcNPV genome was similar to that observed for *Escherichia coli* and HSV DNAs (61) and significantly higher than that in murine and entomopoxvirus DNAs (Table 1).

To determine the methylation status of the AcNPV genome, we digested DNAs isolated from AcNPV, Sf-9 cells, *E. coli*, and 293T cells with the restriction enzyme HpaII, which cannot cleave when the cytosine adjacent to the cleavage site (CC ↓ GG) is methylated. While DNA isolated from 293T cells was refractory to HpaII digestion, DNAs from AcNPV, Sf-9 cells, and *E. coli* were sensitive to HpaII digestion, indicating that most of the CpG dinucleotides in AcNPV were unmethylated (Fig. 5A).

To determine the ability of AcNPV DNA to stimulate an immune response in vitro, we purified the viral DNA from virions. RAW264.7 cells were then treated with purified viral DNA or PGN with or without liposomes (Fig. 5B). The transfection of viral DNA with liposomes resulted in the production

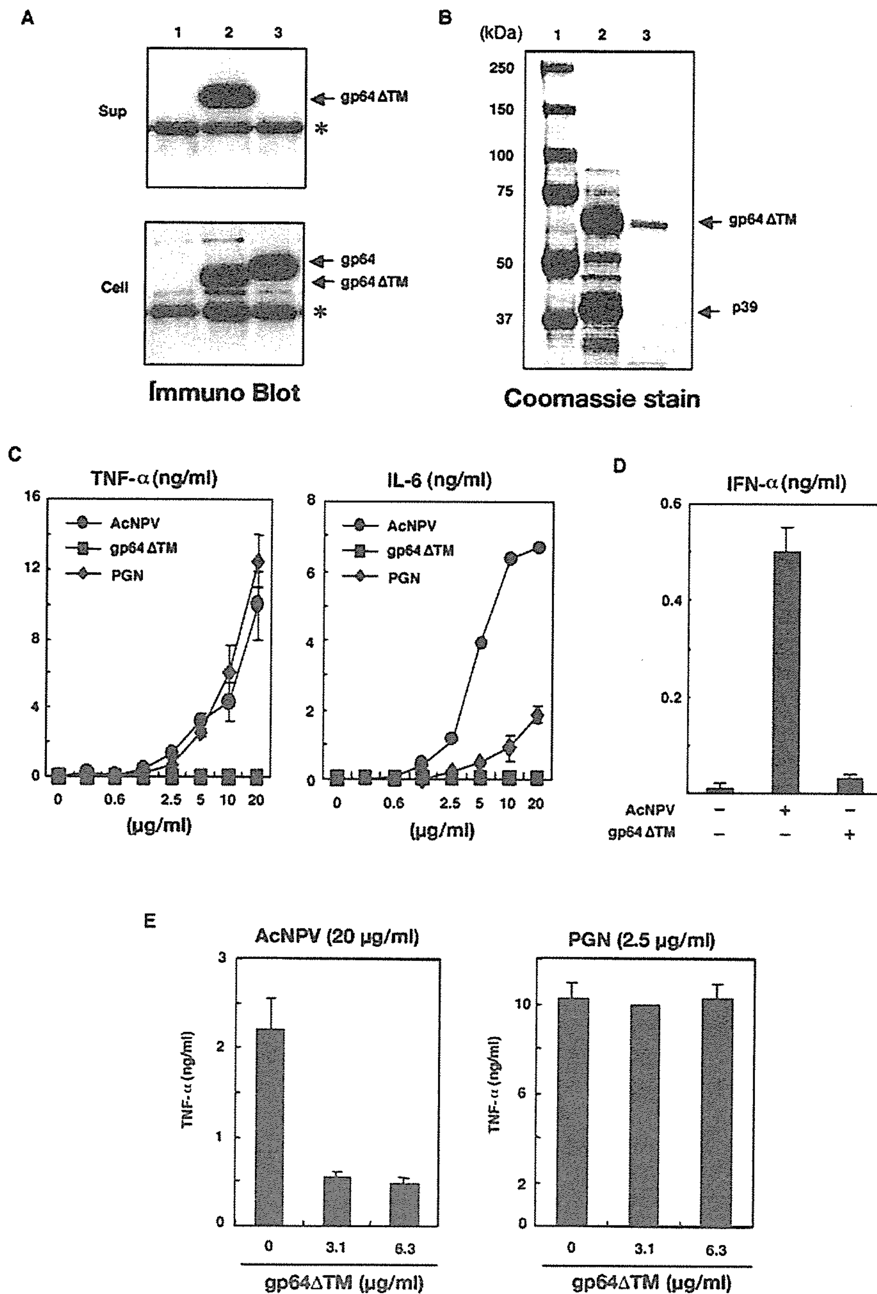

FIG. 2. Immune system activation by AcNPV in macrophages is not mediated by gp64. (A) Wild-type gp64 and a deletion mutant lacking the transmembrane region of the gp64 envelope protein (gp64ΔTM) were expressed in Sf-9 cells. Whole-cell lysates and culture supernatants were subjected to SDS-PAGE under reducing conditions and visualized by immunoblotting with an antihexahistidine monoclonal antibody. Lane 1, cells transfected with pIB/V5-His; lanes 2 and 3, cells transfected with pIBgp64ΔTM/V5-His and pIBgp64/V5-His, respectively. The heavy chains of the antibody are indicated by asterisks. (B) Purified AcNPV virions (lane 2) and gp64ΔTM (lane 3) were analyzed by SDS-PAGE and Coomassie blue staining. Lane 1, molecular mass markers. (C) Activation of mouse macrophage RAW264.7 cells (10^6 cells/well) treated with the indicated amounts of AcNPV or gp64ΔTM. The production of TNF-α and IL-6 in culture supernatants after 24 h of incubation was determined by sandwich ELISAs. PGN was used as a positive control. Data are shown as means ± SD. (D) Production of IFN-α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (5 μg/ml) or gp64ΔTM (5 μg/ml), as determined by a sandwich ELISA after 24 h of incubation. Data are shown as means ± SD. (E) Production of TNF-α in RAW264.7 cells (10^6 cells/well) inoculated with AcNPV (20 μg/ml) or PGN (2.5 μg/ml), with or without a pretreatment with the indicated amounts of gp64ΔTM for 2 h at 37°C. After 24 h of incubation, the production of TNF-α in culture supernatants was determined by a sandwich ELISA. Data are shown as means ± SD.

of TNF-α, but this effect was not observed in the absence of liposomes. The enhancement of TNF-α production by liposomes was not observed in cells treated with PGN, and the addition of liposomes alone did not elicit TNF-α production (Fig. 5C). These results indicate that the internalization of viral DNA is necessary for the activation of the AcNPV-mediated TLR9 signaling pathway. Thus, the impaired immune system activation by AcNPVΔ64 in macrophages may result from a

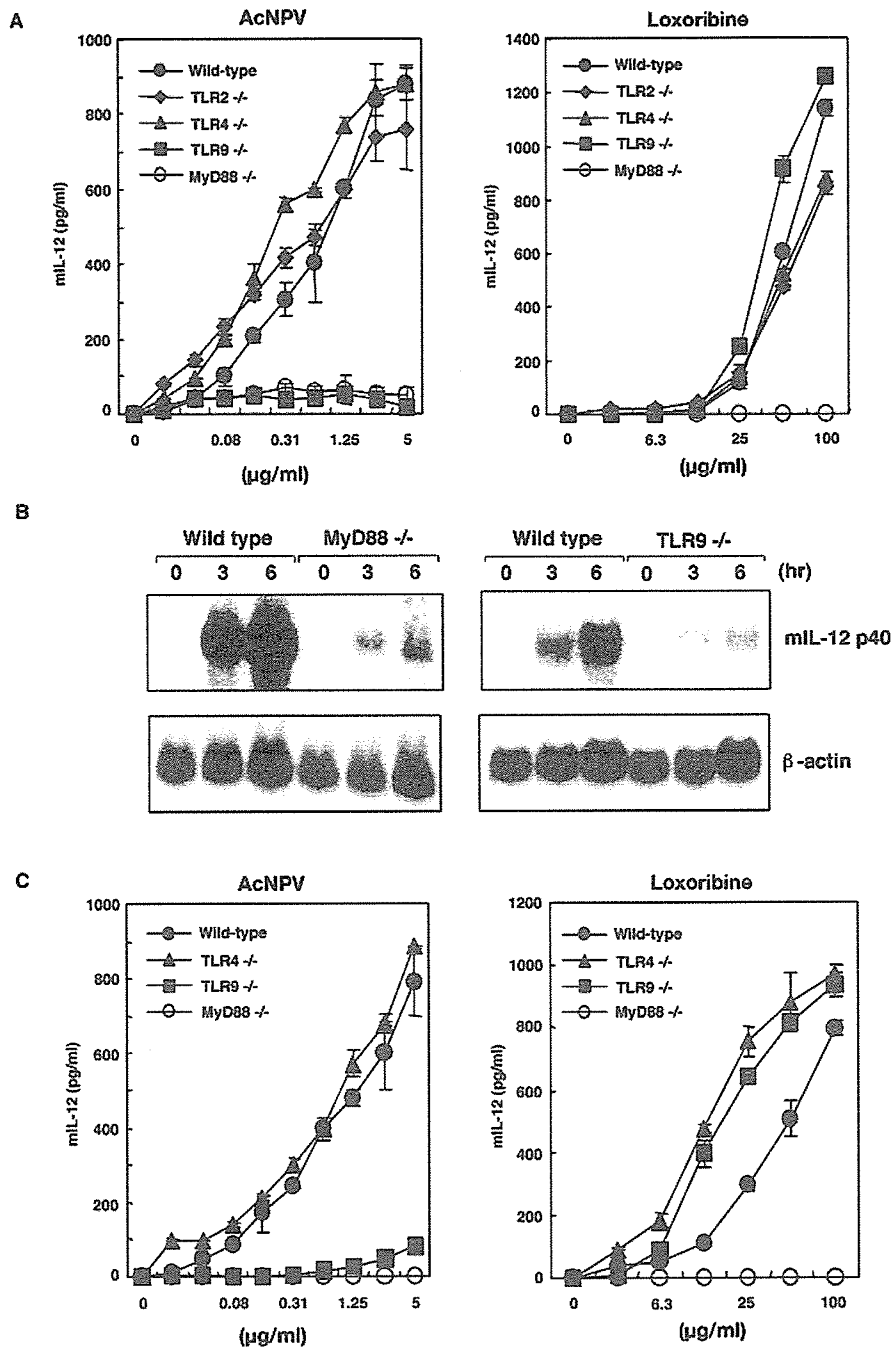

FIG. 3. AcNPV activates PECs and DCs in a MyD88/TLR9-dependent manner. (A) PECs (2×10^5 cells/well) from wild-type (C57BL/6) or MyD88-, TLR2-, TLR4-, or TLR9-deficient mice were stimulated with the indicated amounts of AcNPV or loxoribine. The production of IL-12 p40 in culture supernatants was measured by a sandwich ELISA. Data are shown as means ± SD. (B) Northern blot analysis of murine macrophage cells stimulated with AcNPV. PECs (6×10^6 cells/well) from wild-type or MyD88- or TLR9-deficient mice were stimulated with AcNPV (10 μg/ml) for the indicated times. Total RNAs were extracted and subjected to Northern blot analysis. (C) Splenic $CD11c^+$ DCs were prepared from wild-type or MyD88-, TLR4-, or TLR9-deficient mice and enriched by magnetic cell sorting. Splenic DCs (10^5 cells/well) were stimulated with the indicated amounts of AcNPV or loxoribine for 24 h. The production of IL-12 p40 in supernatants was measured by a sandwich ELISA. Data are shown as means ± SD.

failure to internalize viral DNA via gp64-mediated membrane fusion.

To further confirm that viral DNA activates the signaling pathway following internalization via gp64, we inactivated AcNPV by UV irradiation and examined the production of TNF-α in RAW264.7 cells. UV irradiation diminished the AcNPV-mediated induction of TNF-α, but the addition of liposomes restored the activation (Fig. 5C). These results

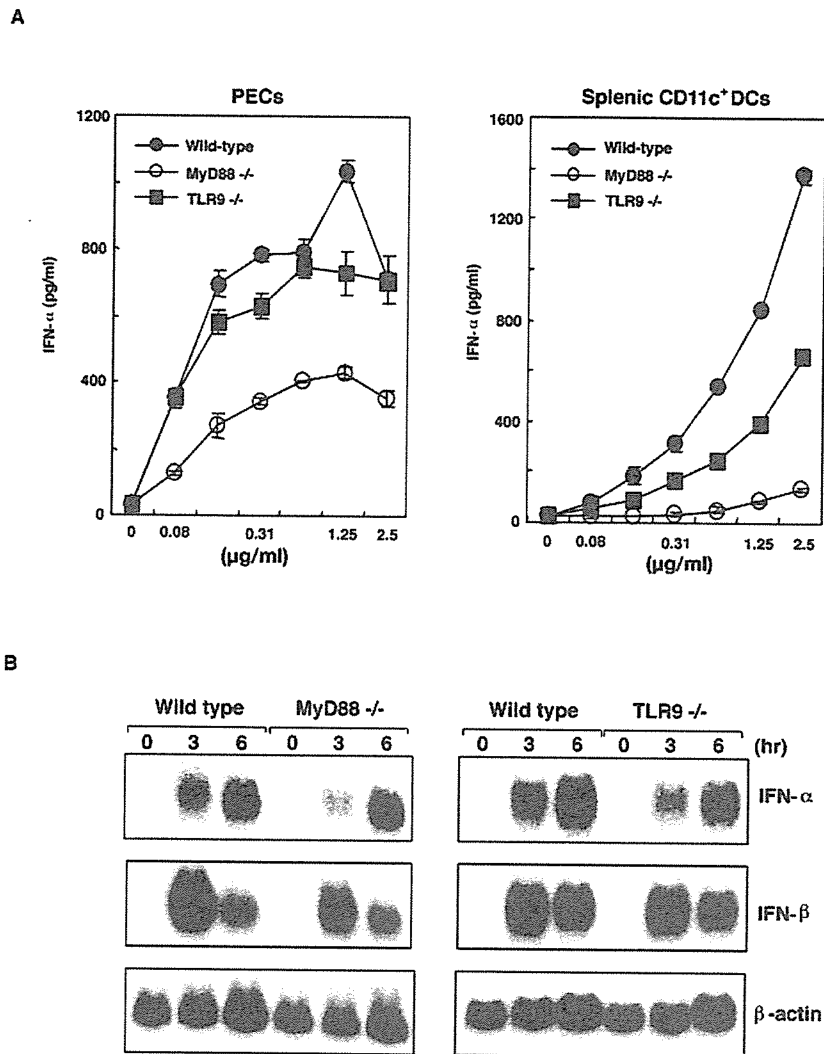

FIG. 4. IFN production by AcNPV is mediated by a MyD88/TLR9-independent process. (A) PECs (2×10^5 cells/well) and splenic CD11c$^+$ DCs (1×10^5 cells/well) were prepared from wild-type or MyD88- or TLR9-deficient mice and stimulated with the indicated amounts of AcNPV or loxoribine for 24 h. The production of IFN-α in culture supernatants was measured by a sandwich ELISA. Data are shown as means ± SD. (B) Northern blot analysis of murine macrophage cells stimulated with AcNPV. PECs (6×10^6 cells/well) from wild-type or MyD88- or TLR9-deficient mice were stimulated with AcNPV (10 μg/ml) for the indicated times. Total RNAs were then extracted and subjected to Northern blot analysis.

suggest that the denaturation of gp64 by UV irradiation impaired the fusion capability of the envelope protein, thus inhibiting the internalization of viral DNA into the cell via membrane fusion.

AcNPV DNA induces NF-κB activation through human TLR9. Signaling via TLRs occurs through the sequential recruitment of the adapter molecule MyD88 and the serine-threonine kinase IL-1 receptor-associated kinase, which leads to the activation of mitogen-activated protein kinases and the nuclear factor NF-κB (51). To assess whether or not the expression of human TLR9 confers cellular responsiveness to AcNPV DNA, we transfected 293T cells with a human TLR9 expression plasmid and a pELAM luciferase reporter plasmid together with AcNPV or hCpG, which was used as a positive control (Fig. 6A). Although NF-κB activation was not observed for cells transfected with undigested AcNPV DNA,

TABLE 1. CpG motif frequencies in AcNPV and other genomes[a]

Motif	Frequency of appearance				
	E. coli	Mouse	HSV-1	AcNPV	AmEPV
CACGTT	1.30	0.11	0.76	0.90	0.17
AGCGTT	1.70	0.17	0.42	1.12	0.15
AACGTC	0.60	0.11	0.73	0.98	0.17
AGCGTC	1.30	0.15	0.85	0.85	0.15
GGCGTC	1.40	0.15	4.0	1.10	0.02
GGCGTT	2.50	0.15	1.51	1.37	0.10
Average	1.53	0.14	1.38	1.05	0.13

[a] The frequency at which each CpG hexamer appeared in the *E. coli*, mouse, HSV-1, AcNPV, and *Amsacta moorei* entomopoxvirus genomes was determined by using published sequence data. The GenBank accession numbers for the complete genomes of AcNPV and AmEPV are NC 001623 and NC 002520, respectively. The complete genomes of *E. coli* K-12 and HSV-1 and mouse chromosome sequences were described previously (61).