Journal of Medical Virology 79:811–819 (2007)

Prophylactic Effects of Chitin Microparticles on Highly Pathogenic H5N1 Influenza Virus

Takeshi Ichinohe,[1,2†] Noriyo Nagata,[1] Peter Strong,[3] Shin-ichi Tamura,[1] Hidehiro Takahashi,[1] Ai Ninomiya,[4] Masaki Imai,[4] Takato Odagiri,[4] Masato Tashiro,[4] Hirofumi Sawa,[5] Joe Chiba,[2] Takeshi Kurata,[1] Tetsutaro Sata,[1] and Hideki Hasegawa[1]*

[1]Department of Pathology, National Institute of Infectious Diseases, Gakuen, Musashimurayama-shi, Tokyo, Japan
[2]Department of Biological Science and Technology, Tokyo University of Science, Yamazaki, Noda, Chiba, Japan
[3]CMP Therapeutics Ltd., Oxford, UK
[4]Department of Virology III, National Institute of Infectious Diseases, Gakuen, Musashimurayama-shi, Tokyo, Japan
[5]Department of Molecular Pathobiology, 21st Century COE Program for Zoonosis Control, Hokkaido University Research Center for Zoonosis Control, Kita-ku, Sapporo, Japan

Highly pathogenic avian influenza virus (H5N1) is an emerging pathogen with the potential to cause great harm to humans, and there is concern about the potential for a new influenza pandemic. This virus is resistant to the antiviral effects of interferons and tumor necrosis factor-α. However, the mechanism of interferon-independent protective innate immunity is not well understood. The prophylactic effects of chitin microparticles as a stimulator of innate mucosal immunity against a recently obtained strain of H5N1 influenza virus infection were examined in mice. Clinical parameters and the survival rate of mice treated by intranasal application of chitin microparticles were significantly improved compared to non-treated mice after a lethal influenza virus challenge. Flow cytometric analysis revealed that the number of natural killer cells that expressed tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and that had migrated into the cervical lymph node was markedly increased (26-fold) after intranasal treatment with chitin microparticles. In addition, the level of IL-6 and interferon-gamma-inducible protein-10 (IP-10) in the nasal mucosa after H5N1 influenza virus challenge was decreased by prophylactic treatment with chitin microparticles. These results suggest that prophylactic intranasal administration of chitin microparticles enhanced the local accumulation of natural killer cells and suppressed hyper-induction of cytokines, resulting in an innate immune response to prevent pathogenesis of H5N1 influenza virus. ***J. Med. Virol. 79:811–819, 2007.***
© 2007 Wiley-Liss, Inc.

KEY WORDS: influenza virus H5N1; innate immunity; chitin

INTRODUCTION

Avian influenza A subtype H5N1 outbreaks involving fatal human respiratory disease were reported in Hong Kong in 1997 (H5N1/97) [Claas et al., 1998; Subbarao et al., 1998]. The subsequent re-emergence of human H5N1 disease with high fatality rates has been reported in southern China [Peiris et al., 2004], Vietnam [Tran et al., 2004], Thailand [Grose and Chokephaibulkit, 2004], Cambodia, Indonesia, Turkey, and Iraq. At least 278 laboratory-confirmed cases of human infection with a fatality rate of greater than 50% were reported to the World Health Organization [2007] from January 2003 to March 2007. It has been reported that an oseltamivir-resistant H5N1 influenza virus (A/Hanoi/30408/2005) was isolated from a Vietnamese girl [Le et al., 2005], and H5N1 influenza viruses isolated from Hong Kong (A/Hong Kong/156/97, A/Hong Kong/483/97, and A/Hong Kong/486/97) were resistant to the antiviral effects of interferons and tumor necrosis factor-α [Seo et al., 2002].

Natural killer cells eliminate tumor cells and cells infected by viruses, including influenza virus, via their cytotoxic activity and production of cytokines [Biron and Brossay, 2001; Cooper et al., 2001; Gazit et al., 2006]. Natural killer cells are rapidly recruited to sites of infection, and can inhibit viral replication and dissemination through the respiratory tract

†Research Fellow of the Japanese Society for the Promotion of Science.

*Correspondence to: Hideki Hasegawa, Department of Pathology, National Institute of Infectious Diseases 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan.
E-mail: hasegawa@nih.go.jp

Accepted 5 February 2007
DOI 10.1002/jmv.20837
Published online in Wiley InterScience (www.interscience.wiley.com)

© 2007 WILEY-LISS, INC.

WILEY InterScience® DISCOVER SOMETHING GREAT

[Stein-Streilein et al., 1983; Stein-Streilein and Guffee, 1986, and unpublished data by P. Strong]. Thus, natural killer cells play a role in the early stage of host defense against viral infection, and also bridge the subsequent adaptive anti-viral immune responses [Kos and Engleman, 1996; Biron, 1997; Biron et al., 1999; Andoniou et al., 2005; O'Leary et al., 2006]. Although the precise mechanism of the innate immune response to highly pathogenic H5N1 influenza virus is still unknown, the role of natural killer cells in innate immunity against viral infection seems to be important.

Chitin (a natural polysaccharide of *N*-acetyl-D-glucosamine), one of the most abundant polysaccharides in nature, is an essential component of fungal walls and the exoskeletons of crabs, shrimp, and insects. Chitin is non-allergenic, non-toxic, bio-degradable and biocompatible. Chitin-derived products are now widely used in the medical, veterinary, cosmetic, health supplement, and environmental industries [Okamoto et al., 1993]. Chitosan, a highly deacetylated form of chitin, has been used as a vaccine adjuvant due to its muco-adhesive properties, and has been shown to enhance antibody responses to mucosally delivered vaccine antigens [Bacon et al., 2000]. Chitin microparticles (1–20 μm in diameter), in contrast to chitosan, have strong immunomodulatory properties. Previous studies showed that chitin microparticles had effective adjuvant activity with an inactivated influenza vaccine [Hasegawa et al., 2005] or with an HIV-DNA vaccine [Hamajima et al., 2003]. Chitin microparticles, when administered intranasally, have also been found to reduce symptoms of respiratory allergy and allergic asthma [Strong et al., 2002; Ozdemir et al., 2006]. Other studies using chitin microparticles have demonstrated their Th1-inducing properties and shown that phagocytosis of chitin microparticles by macrophages through involves the mannose receptor and results in the production of IL-12, IL-18, and tumor necrosis factor-α, which in turn stimulated natural killer cells to produce IFN-γ [Shibata et al., 1997a,b, 1998].

In the present study, prophylactic use of intranasally applied chitin microparticles to stimulate innate mucosal immunity to lethal H5N1 influenza virus challenge is investigated. It is shown that intranasal pretreatment with chitin microparticles induces expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a large proportion of natural killer cells in the cervical lymph node, and suppresses viral load and hyper-induction of cytokines that may play a role in the pathogenesis of H5N1 [Chan et al., 2005; de Jong et al., 2006].

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from Japan SLC. Mice were kept under specific-pathogen-free conditions approved by the Institution Animal Care and Use Committee of National Institute of Infectious Diseases.

Viruses

The mouse-adapted strains of A/Puerto Rico/8/34 (A/PR8, H1N1) and wild-type A/Vietnam/1194/04 (VN1194, H5N1) viruses were used in this study. The A/PR8 virus was passaged 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day fertile chicken eggs. The VN1194 virus isolated from patients with H5N1 disease in Vietnam in 2004 was prepared in Mardin-Darby canine kidney (MDCK) cells without any additional special steps for mouse adaptation. These viruses were stored at −80°C and viral titers were quantified by plaque assay.

Preparation of Chitin Microparticles, Poly(I:C) and LPS

Chitin microparticles prepared from shrimp derived chitin was kindly provided by P. Strong (CMP Therapeutics Ltd., Oxford, UK). Particle size was determined by Christison Particle Technologies (Gateshead, UK) using a Model 780 Accusizer and the average particle size was 10 μm. The sterility of the chitin microparticles was confirmed by plating on agar plates. The concentration of endotoxin in the chitin microparticles preparations was examined by Limulus Amebocyte Lysate Assay (BioWhittaker, Wokingham, UK) and shown to be less than 1 EU/ml. Synthetic dsRNA [poly(I:C)] was kindly provided by Toray Industries, Inc. (Kamakura, Kanagawa, Japan). Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO).

Pretreatment with Chitin Microparticles, Poly(I:C) and LPS and Virus Infection in Mice

To assess the efficacy of intranasal pretreatment with innate immune stimulators as prophylactic agents against influenza (A/PR8, H1N1) and highly pathogenic avian influenza (VN1194, H5N1) strains, chitin microparticles (100 μg), poly(I:C) (10 μg), LPS (1 μg) or PBS were administered intranasally to mice. CMP treatments were performed once a day for 2 or 3 days and other treatments were performed once (6 hr) before viral challenge. Previous experiments established the optimum dosing schedule for CMP, poly(I:C), and LPS. These amounts of each of the innate stimulators were sufficient to generate adjuvant activity against influenza virus infection when they were administered intranasally with vaccine [Ichinohe et al., 2005, 2006]. Five mice for each experimental group were anesthetized with diethyl ether and received an intranasal application of 10 μl of PBS containing chitin microparticles, poly(I:C) or LPS (5 μl/each nostril) prior to influenza virus infection. Mice were anesthetized 6 hr after final administration, and 2 μl of a suspension of influenza virus (A/PR8 or VN1194) was dropped into each nostril (4 μl per mouse). Virus titers of nasal washes were measured 3 days after inoculation of the influenza virus. H5N1 virus infection experiments were carried out in biosafety level 3 containment facilities approved

by the Guides for Animal Experiments Performed at the National Institute of Infectious Diseases.

Titration of Virus

Mice were given 100 μg of chitin microparticles or PBS twice intranasally at 30 and 6 hr before infection, then infected with 1,000 PFU of H5N1 influenza virus. Mice (n = 3 mice per time point) were sacrificed and tissues were collected 3, 5, 8, or 10 days post-infection. Viral titration in the frontal lobe, trigeminal nerve ganglia, brain stem, cervical lymph node, spleen, liver, kidney, large intestine, muscle, serum, nasal wash, and lung wash of infected mice was determined by plaque assay using MDCK cells (Fig. 4). Lung washes and nasal washes were prepared in PBS containing 0.1% bovine serum albumin, as described previously [Asahi et al., 2002] and used for viral titration after removing cellular debris by centrifugation. Tissue homogenates (1–10%, w/v) were prepared in PBS containing 0.1% bovine serum albumin, centrifuged at 9,170*g* for 10 min, and supernatants were inoculated into cells in the presence of 10 μg/ml acetylated trypsin (Sigma).

Flow Cytometry

Mice were given 100 μg of chitin microparticles intranasally once a day for 3 days, and sacrificed 6 hr after the final administration to collect the cervical lymph node. The number of natural killer cells in the local lymphoid tissue was analyzed by three-color flow cytometry. Single cell suspensions were prepared from the cervical lymph node and red blood cells were depleted by incubation in 0.83% NH4Cl. Cells were incubated with 5 μg/ml of anti-mouse CD16/32 antibody (eBioscience, San Diego, CA) to block nonspecific binding, then incubated with FITC-labeled anti-mouse pan-natural killer cell antibody (Dx5, eBioscience) and phycoerythrin (PE)-labeled anti-mouse tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) antibody (N2B2, eBioscience). To quantitate the total number of live cells an aliquot of cells were incubated with propidium iodide (PI, final concentration; 5 μg/ml). Samples were analyzed with a flow cytometer (FACS-Calibur, BD Biosciences, San Jose, CA), and the data were analyzed with CELLQuest software.

Multiplex Cytokine Assays

Mice were given 100 μg of chitin microparticles or PBS intranasally once a day for 2 days, then infected with 1,000 PFU of H5N1 influenza virus. After the challenge, mice were sacrificed to collect nasal washes at 3, 5, 8, or 10 days post-infection. Samples of nasal washes were analyzed for 20 cytokines (FGF basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF) by Luminex 200^{TM} (Luminex Co., Austin, TX) using mouse cytokine twenty-plex antibody bead kit (BioSource International, Inc. Camarillo, CA), according to the manufacturer's instructions. Briefly, Multiplex beads were vortexed and sonicated for 30 s and 25 μl of beads suspension was added to each well of a 96 well filter plate and washed twice with wash buffer. The nasal wash were diluted 1:1 in assay diluent and loaded onto a Millipore Multiscreen BV 96-well filter plate to which 50 μl of incubation buffer had been added to each well. Serial dilutions of cytokines standards were prepared in parallel and added to the plate. Samples were incubated on a plate shaker in the dark at room temperature for 2 hr. The plate was applied to a Millipore Multiscreen Vacuum Manifold, washed twice with 200 μl wash buffer, and 100 μl of biotinylated Anti-Mouse Multi-Cytokine Detector Antibody was added to each well. The plate was shaken again as above for 1 hr applied to a Millipore Multiscreen Vacuum Manifold, and washed twice with 200 μl wash buffer. One hundred microliters of Streptavidin R-phycoerythrin was added directly to each well, plate was shaken again as above for 30 min, applied to the vacuum manifold, and washed twice. One hundred microliters of wash buffer was added to each well and the plate was shaken for 3 min. The assay plate was analyzed using the Bio-Plex Luminex 100 XYP instrument. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a five parameter curve-fitting algorithm applied for standard curve calculation.

Statistics

Comparisons between experimental groups were made using the Student's *t*-test for paired observations; $P < 0.05$ was considered statistically significant.

RESULTS

Protective Effect of Chitin Microparticles on A/PR8 (H1N1) or A/Vietnam (H5N1) Influenza virus Infection

To assess the efficacy of intranasal pretreatment with chitin microparticles as a prophylactic treatment against avirulent (A/PuertoRico/8/34/, H1N1) or highly pathogenic avian influenza virus infection (A/Vietnam/1194/2004, H5N1), a chitin microparticle suspension was given intranasally to mice. After H1N1 viral challenge, mice pretreated intranasally with either poly(I:C) or LPS given 6 hr prior to infection showed a marked reduction in virus titer in their nasal wash (Fig. 1A). Mice treated with three daily doses of CMP showed a partial but significant reduction of nasal-wash virus titers (Fig. 1A). The protective effect of chitin microparticles against H5N1 influenza virus infection was also examined. Mice treated intranasally with three daily doses of chitin microparticles showed a marked reduction in virus titer in their nasal washes, although mice pretreated with either poly(I:C) or LPS showed only a partial reduction in virus titers in their nasal

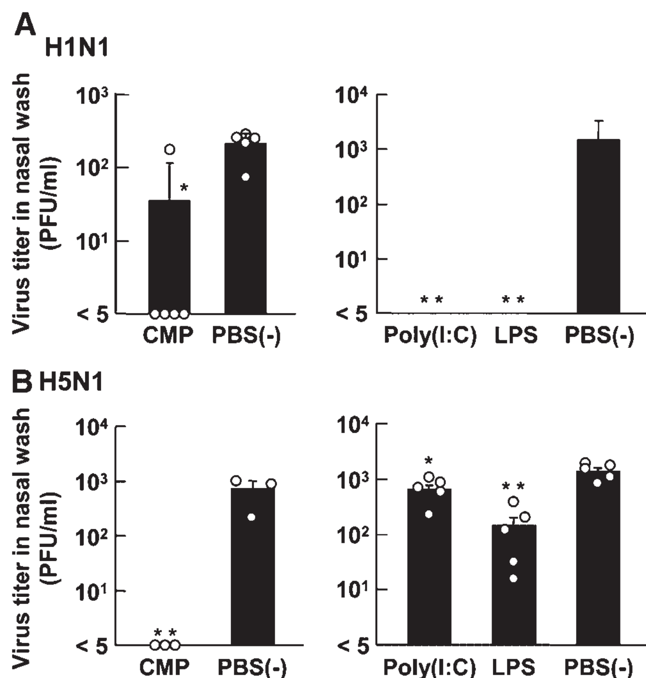

Fig. 1. Protective effect of innate immune stimulators against H1N1 and H5N1 influenza virus infection in BALB/c mice. **A**: Mice (n = 5 mice per group) received 100 μg of chitin microparticles (CMP) once a day for 3 days, or 10 μg of poly(I:C), 1 μg of LPS, or PBS once 6 hr before viral challenge, then were infected intranasally with 100 PFU of A/PR8 (H1N1) influenza viruses in 2 μl suspension for each nostril. Virus titers in nasal washes 3 days post-inoculation are shown. **B**: Mice were pretreated as above and infected intranasally with 1,000 PFU of VN1194 (H5N1) influenza viruses in 2 μl suspension for each nostril. Virus titers in nasal washes 3 days post-inoculation are shown. Data represents the means ± standard error (SE). Open circles indicate values for individual mice. Asterisks indicate significant differences compared with infected controls: *$P < 0.05$; **$P < 0.01$.

washes (Fig. 1B). These results suggest that chitin microparticles can stimulate an immunological reaction that leads to reduction of viral replication in vivo.

Pretreatment With Chitin Microparticles Protects Mice From Lethal Infection of H1N1 and H5N1

The mortality of mice after inoculation with a lethal dose of H1N1 or H5N1 influenza virus was monitored. Chitin microparticles (100 μg) was given intranasally once a day for 3 days before infection with 100 PFU (4 LD50) of H1N1 virus in a 20 μl suspension (Fig. 2A,B). All the control mice pre-treated with PBS were dead 9 days after infection, with marked clinical symptoms of disease and marked reduction of body weight. Mice pretreated with chitin microparticles also lost weight and developed clinical symptoms, but 15 days after infection, 60% of mice recovered their body weight, and survived for the duration of the experiment (at least 40 days after infection).

The prophylactic effect of chitin microparticles against lethal H5N1 influenza virus infection was also examined. Mice were given 100 μg of chitin microparticles intranasally once a day for 2 days, or PBS 6 hr before intranasal infection with 1,000 PFU of H5N1 influenza viruses. Interestingly, intranasal administration of chitin microparticles led to a significant improvement in survival and fewer clinical symptoms compared with PBS-treated control mice (Fig. 2C). These results suggested that intranasal pretreatment with chitin microparticles protects mice against both H1N1 and H5N1 lethal infections.

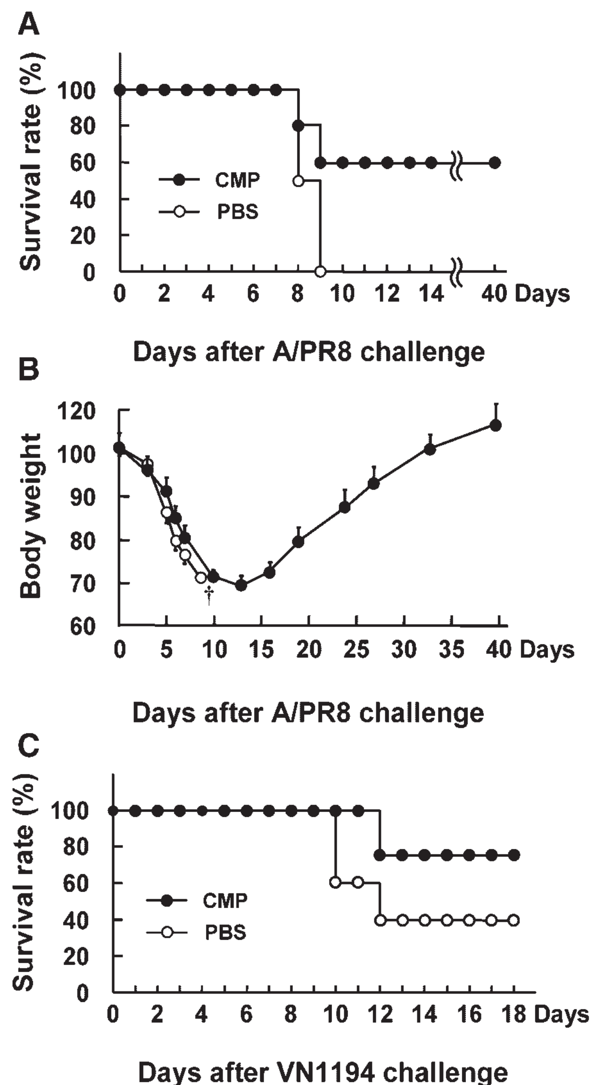

Fig. 2. Prophylactic effect of chitin microparticles (CMP) against H1N1-induced pneumonia or H5N1 influenza virus infection. Mice (n = 4–5 mice per group) were administered 100 μg of chitin microparticles to the lung intranasally in a volume of 20 μl PBS once a day for 3 days (closed circles) or PBS (open circles), then challenged with a lethal dose (4 LD50) of A/PR8/H1N1 influenza virus in 20 μl PBS to the lung. Survival curves **(A)** and body weight changes over time **(B)** after virus challenge are shown. Body weights are plotted as a percentage of the average initial weight. The open cross indicates the time-point at which all mice in a group succumbed to disease. Infected mice were monitored for 40 days. **C**: Mice (n = 5 mice per group) received 100 μg of chitin microparticles once a day for 2 days (closed circles), or PBS (open circles), then infected intranasally with 1,000 PFU of VN1194 (H5N1) influenza virus in 2 μl suspension for each nostril. Survival curves after virus challenge are shown. The survival rates were monitored for 18 days.

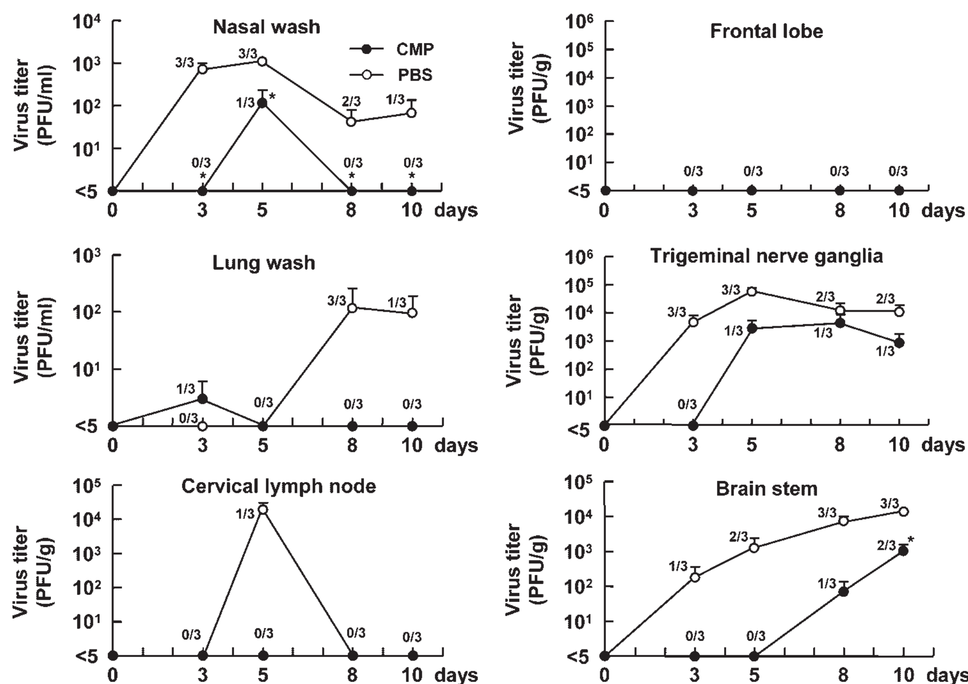

Fig. 3. Virus titers in various organs after challenge with 1,000 PFU of VN1194 (H5N1). Mice were administered 100 μg of chitin microparticles (CMP) intranasally once a day for 2 days (closed circle) or PBS (open circle), and infected with 1,000 PFU of VN1194 influenza virus. Mice were sacrificed to collect organs 3, 5, 8, or 10 days post-inoculation. Virus titers in the indicated organs after viral challenge are shown. Data represents the means ± SE, and represents the numbers of mice that failed to clear viruses/total number of mice ($n = 3$). Asterisks indicate significant differences compared to the PBS control group; $P < 0.05$.

Reduction of H5N1 Influenza Virus Titer in Multiple Organs by Pretreatment with Chitin Microparticles

To examine the efficiency of viral spread of A/Vietnam/1194/04 (H5N1) after nasal infection in mice, viral titers in multiple tissues from mice intranasally infected with 1,000 PFU of H5N1 were measured 3, 5, 8, and 10 days after infection with or without chitin microparticles pretreatment ($n = 3$ mice per time point). Mice were given 100 μg of chitin microparticles or PBS intranasally twice at 30 and 6 hr before infection, then infected with 1,000 PFU of H5N1 influenza viruses. When the mice were infected with a small volume (2 μl in each nostil) of virus suspension without chitin microparticles pretreatment, virus titers were detected in nasal washes and in the trigerminal nerve ganglia of all mice 3 days post-inoculation, and were highest at 5 days post-inoculation (open circles in Fig. 3). Thereafter, virus was detected in the lung and brain stem 8 days post-inoculation (open circles in Fig. 3). Interestingly, live virus was not detected in the frontal lobe of the cerebrum, which is directly connected to the nasal cavity via the olfactory nerve. No live virus was detected in the spleen, liver, kidney, large intestine, muscle, or serum of mice (data not shown).

In the nasal wash and brain stem of chitin microparticles-treated mice, the virus titer was significantly reduced compared to PBS-treated mice (closed circles in Fig. 3). In addition, virus titers tended to be much lower in lung washes, cervical lymph node, and terminal nerve ganglia in the chitin microparticles-treated group compared to the control group (closed circles in Fig. 3). Live virus titers were not detected in the frontal lobe of the cerebrum.

Migration of Natural Killer Cells Expressing Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) in the Cervical Lymph Node After Intranasal Administration of Chitin Microparticles

Previous work suggested that chitin microparticles given intranasally induced an accumulation of natural killer cells in local lymphoid tissue (unpublished data). To define the mechanism of the protective effect of chitin microparticles against highly pathogenic H5N1 influenza virus infection, the migration of natural killer cells into the cervical lymph node was examined. The proportion of natural killer cells in the cervical lymph node increased 6-fold in chitin microparticles-treated mice compared with PBS-treated mice 6 hr after chitin microparticles administration (Fig. 4A). As tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays an important role in the immune response of natural killer cells to influenza virus infection [Ishikawa et al., 2005], the number of natural killer cells expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on their cell surface was counted. The number of tumor necrosis factor-

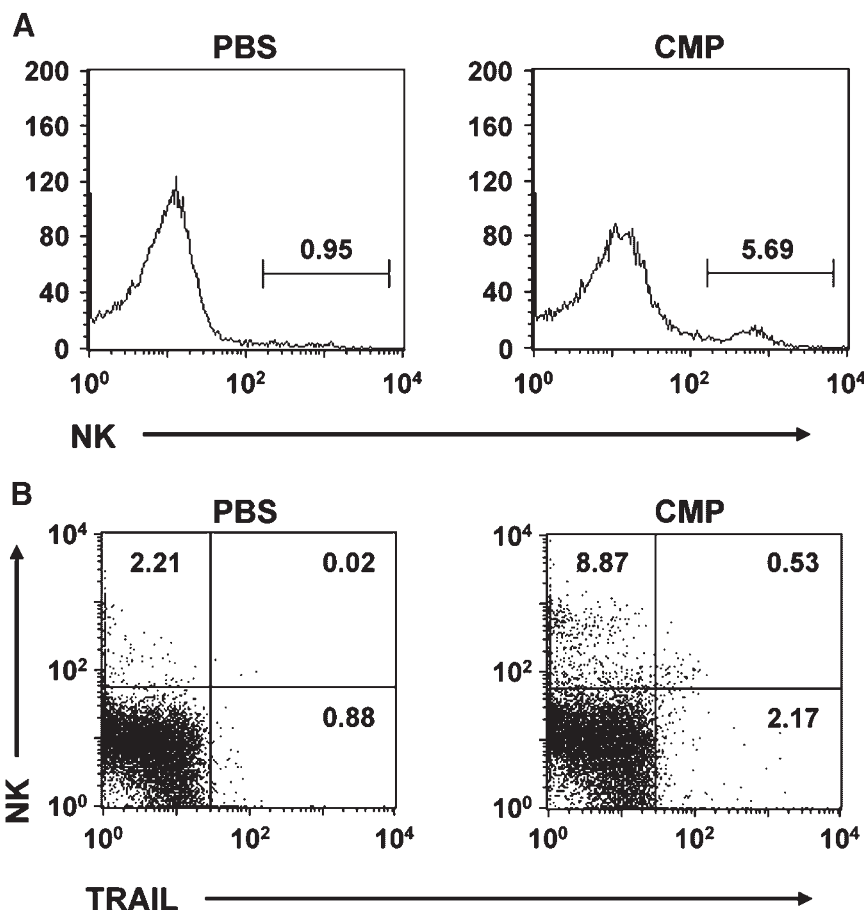

Fig. 4. Increased natural killer (NK) cell migration into the cervical lymph node after intranasal chitin microparticles (CMP) treatment. Mice were administered 100 μg of chitin microparticles intranasally once a day for 3 days or PBS, and sacrificed to collect the cervical lymph node 6 hr after the final administration. cervical lymph node cells of 5 mice were pooled and stained with PI and anti-mouse CD49bFITC and/or anti-mouse tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) PE antibodies. Viable cells (PI−) were selected with forward and side scatter gated for lymphocytes. The expression of CD49b and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was analyzed in this population. **A**: Numbers in each histogram above the marker indicate the percentage of 'live'-gated cells deemed panCD49b+. **B**: CD49b and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) profile of cells recovered from the cervical lymph node of mice pretreated with chitin microparticles or PBS. Figure is representative of three experiments.

related apoptosis-inducing ligand (TRAIL)-expressing natural killer cells increased 26-fold in the cervical lymph node of chitin microparticles treated mice compared with PBS-treated mice (Fig. 4B). These results suggest that migration of natural killer cells expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) into local lymph nodes might play a role in the reduction of virus titer and improvement of clinical symptoms in virus infected mice.

Reduction of IL-6 and Interferon-Gamma-Inducible Protein-10 (IP-10) Secretion From Nasal Mucosa by Pretreatment with Chitin Microparticles in H5N1 Influenza Virus Infection

Chitin microparticles might also play a role in the reduction of inflammatory cytokines and chemokines that accompany H5N1 influenza virus infection. To examine this possibility, the level of secreted cytokines described in the Materials and Methods in nasal mucosa was examined in mice infected with H5N1 virus with or without chitin microparticles pretreatment. Among them the secretion of IL-6 in nasal mucosa was up-regulated by H5N1 influenza virus infection 10 days post-inoculation, while in the chitin microparticles-treated group, there was a marked reduction of IL-6 secretion (Fig. 5A). Likewise, the secretion of interferon-gamma-inducible protein-10 (IP-10) was inhibited in mice pretreated with chitin microparticles compared to the PBS-treated group 8 days post-inoculation (Fig. 5B). On the other hand tumor necrosis factor-α was not detected in nasal mucosa of mice after H5N1 influenza virus infection (data not shown). These results suggested that chitin microparticles pretreatment may inhibit the hyper-induction induction of cytokines and chemokines that are relevant to the pathogenesis of H5N1 influenza virus in infected mice.

DISCUSSION

The present study demonstrated that intranasal administration of a suspension of chitin microparticles

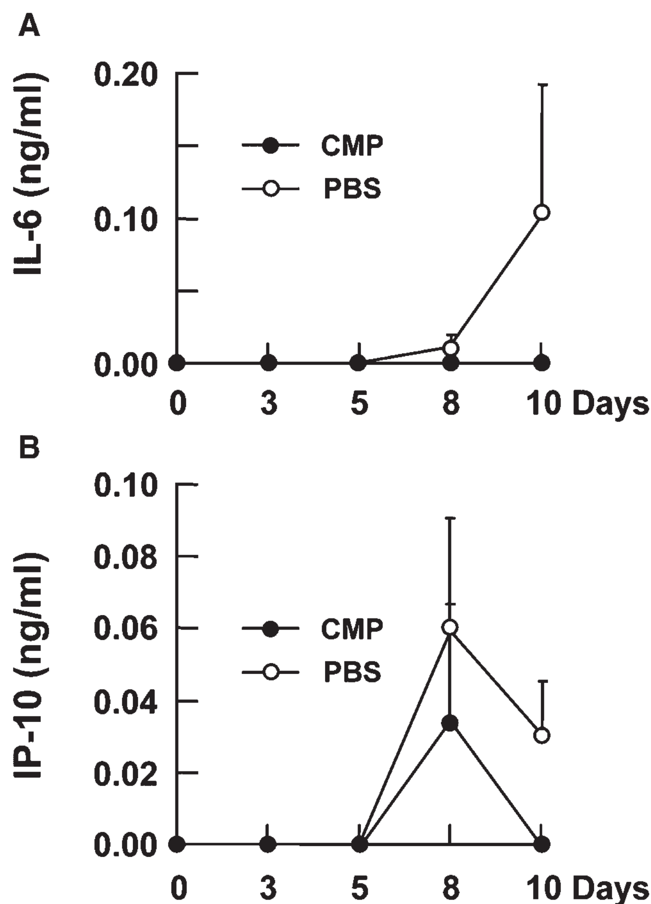

Fig. 5. Kinetics of IL-6 (**A**) and interferon-gamma-inducible protein-10 (IP-10) (**B**) secretion in the nasal mucosa after H5N1 influenza virus infection. Mice (n = 3) were administered 100 μg of chitin microparticles (CMP) intranasally once a day for 2 days (closed circles) or PBS (open circles), then infected with 1,000 PFU of VN1194 (H5N1) influenza virus. After viral challenge, mice were sacrificed to collect nasal washes 3, 5, 8, or 10 days post-inoculation. The levels of each cytokine in nasal washes after the viral challenge are shown. Data represents the means ± SE.

has protective effects against lethal H5N1 influenza virus infection of the upper respiratory tract in mice. These findings were consistent with results showing the protective effects against avirulent influenza virus (A/PR8, H1N1) infection in mice by intranasal administration of chitin microparticles into the lung (unpublished data by P. Strong). It is previously reported that chitin microparticles had a mucosal adjuvant effect when co-administered with an influenza hemagglutinin vaccine and increased both the mucosal and systemic humoral responses that provided complete protection against challenge with the homologous influenza virus H1N1 or H5N1 in mice [Hasegawa et al., 2005; Asahi-Ozaki et al., 2006; Ichinohe et al., 2006]. The current study demonstrates hitherto unrecognized effects of chitin microparticles in enhancing innate protection against infection with a highly pathogenic avian influenza virus strain.

It has been reported that the highly pathogenic H5N1 influenza virus induces high levels of pro-inflammatory cytokines and chemokines that may play a role in the pathogenesis of [Chan et al., 2005; de Jong et al., 2006]. It is demonstrated that intranasal administration of chitin microparticles induced the accumulation of natural killer cells expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the cervical lymph node and suppressed viral load and hyper-induction of cytokines. These results suggested that recruitment of natural killer cells expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) into local sites of infection (Fig. 4) and suppression of pro-inflammatory cytokines and chemokines (e.g. IL-6 and interferon-gamma-inducible protein-10 in Fig. 5) may contribute to a reduction of clinical symptoms and enhance protection against lethal H5N1 influenza virus infection.

The importance of natural killer cells in viral defense has been demonstrated most convincingly in a patient who lacked natural killer cells and was therefore susceptible to virus infections [Biron et al., 1989]. Mice in which natural killer cells have been depleted demonstrated increased mortality after infection with influenza viruses [Stein-Streilein and Guffee, 1986]. Therefore, it might be expected that the enhancement of natural killer cells activity by chitin microparticles and the accumulation of natural killer cells locally into the site of infection and an enhancement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on their surface (Fig. 4) might increase resistance to viral infection. In natural killer cells or T-cells, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays an important role in the immune response to influenza virus infection [Ishikawa et al., 2005]. Presumably due to this recruitment of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-expressing natural killer cells, H5N1 virus titers were suppressed in nasal washes, lung washes, cervical lymph node, trigeminal nerve ganglia and in the brain stem of mice pretreated with chitin microparticles compared to mice in the control group (Fig. 3). Consistent with these findings, pretreatment of mice with chitin microparticles led to a significant improvement in survival rate and reduction in clinical symptoms following H5N1 virus infection (Fig. 2A,B) and H1N1 virus infection (Fig. 2C).

Finally, the present studies suggest that intranasal administration of chitin microparticles boosts innate immunity and protects mice from infection by the highly pathogenic H5N1 influenza virus infection in the upper respiratory tracts. It is proposed that if mice are given chitin microparticles as a daily prophylactic they would have enhanced protection against infection with H5N1. This prophylactic effect is elicited by activation of natural killer cells and regulation of inflammatory cytokines such as IL-6 and interferon-gamma-inducible protein-10 (IP-10). The adjuvant effects of chitin microparticles are also expected [Hasegawa et al., 2005; Asahi-Ozaki et al., 2006; Ichinohe et al., 2006] in inducing adaptive immunity following infection. Identification of therapeutic innate immunity enhancing agents such as chitin microparticles may lead to

antiviral strategies against the highly pathogenic H5N1 influenza virus and may have relevance as part of a first line defense against H5N1 outbreaks.

ACKNOWLEDGMENTS

We are grateful to Dr. Wilina Lim (Department of Health, The government of Hong Kong) for providing us A/Vietnam/1194/04 (H5N1) influenza virus strain, and Dr. U. Suzuki and Dr. K. Komase (Kitasato Institute, Saitama) and Dr. T. Tanaka (Toray Industries, Inc.) for providing the materials and Dr. M. Moriyama for helpful discussion. This work was supported in part by grants from the Ministry of Health, Labor, and Welfare, and Research on Health Sciences focusing on Drug Innovation.

REFERENCES

Andoniou CE, van Dommelen SL, Voigt V, Andrews DM, Brizard G, Asselin-Paturel C, Delale T, Stacey KJ, Trinchieri G, Degli-Esposti MA. 2005. Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. Nat Immunol 6:1011–1019.

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.

Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, Sawa H, Moriyama M, Tashiro M, Sata T, Kurata T, Hasegawa H. 2006. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect 8:2706–2714.

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.

Biron CA. 1997. Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol 9:24–34.

Biron CA, Brossay L. 2001. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 13:458–464.

Biron CA, Byron KS, Sullivan JL. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 320: 1731–1735.

Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 17:189–220.

Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS. 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res 6:135.

Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351:472–477.

Cooper MA, Fehniger TA, Caligiuri MA. 2001. The biology of human natural killer-cell subsets. Trends Immunol 22:633–640.

de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Van Vinh Chau N, Khanh TH, Dong VC, Qui PT, Van Cam B, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 12:1203–1207.

Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, Achdout H, Hanna J, Qimron U, Landau G, Greenbaum E, Zakay-Rones Z, Porgador A, Mandelboim O. 2006. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat Immunol 7:517–523.

Grose C, Chokephaibulkit K. 2004. Avian influenza virus infection of children in Vietnam and Thailand. Pediatr Infect Dis J 23: 793–794.

Hamajima K, Kojima Y, Matsui K, Toda Y, Jounai N, Ozaki T, Xin KQ, Strong P, Okuda K. 2003. Chitin micro-particles (CMP): a useful adjuvant for inducing viral specific immunity when delivered intranasally with an HIV-DNA vaccine. Viral Immunol 16:541–547.

Hasegawa H, Ichinohe T, Strong P, Watanabe I, Ito S, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T. 2005. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. J Med Virol 75:130–136.

Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H. 2005. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 79:2910–2919.

Ichinohe T, Watanabe I, Tao E, Ito S, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Moriyama M, Chiba J, Komase K, Suzuki Y, Kurata T, Sata T, Hasegawa H. 2006. Protection against influenza virus infection by intranasal vaccine with surf clam microparticles (SMP) as an adjuvant. J Med Virol 78:954–963.

Ishikawa E, Nakazawa M, Yoshinari M, Minami M. 2005. Role of tumor necrosis factor-related apoptosis-inducing ligand in immune response to influenza virus infection in mice. J Virol 79:7658–7663.

Kos FJ, Engleman EG. 1996. Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. Cell Immunol 173:1–6.

Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437:1108.

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.

O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol 7:507–516.

Ozdemir C, Yazi D, Aydogan M, Akkoc T, Bahceciler NN, Strong P, Barlan IB. 2006. Treatment with chitin microparticles is protective against lung histopathology in a murine asthma model. Clin Exp Allergy 36:960–968.

Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 363:617–619.

Seo SH, Hoffmann E, Webster RG. 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8:950–954.

Shibata Y, Foster LA, Kurimoto M, Okamura H, Nakamura RM, Kawajiri K, Justice JP, Van Scott MR, Myrvik QN, Metzger WJ. 1998. Immunoregulatory roles of IL-10 in innate immunity: IL-10 inhibits macrophage production of IFN-gamma-inducing factors but enhances NK cell production of IFN-gamma. J Immunol 161:4283–4288.

Shibata Y, Foster LA, Metzger WJ, Myrvik QN. 1997a. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of *N*-acetyl-D-glucosamine, in mice. Infect Immun 65:1734–1741.

Shibata Y, Metzger WJ, Myrvik QN. 1997b. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. J Immunol 159:2462–2467.

Stein-Streilein J, Bennett M, Mann D, Kumar V. 1983. Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection. J Immunol 131:2699–2704.

Stein-Streilein J, Guffee J. 1986. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. J Immunol 136:1435–1441.

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.

Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N. 1998. Characterization of an avian influenza A

(H5N1) virus isolated from a child with a fatal respiratory illness. Science 279:393–396.

Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, Pham TS, Vo CD, Le TQ, Ngo TT, Dao BK, Le PP, Nguyen TT, Hoang TL, Cao VT, Le TG, Nguyen DT, Le HN, Nguyen KT, Le HS, Le VT, Christiane D, Tran TT, Menno de J, Schultsz C, Cheng P, Lim W, Horby P, Farrar J. 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 350:1179–1188.

World Health Organization. 2007. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2007_03_12/en/index.html