vaccines conferred a partial but significant reduction in lung wash virus titers (Fig. 2A), while the virus titer in the lung washings of mice immunized with *G. frondosa*-adjuvanted vaccine was not altered compared to that of the control mice (Fig. 2A). The hemagglutination inhibition titer from each group corresponded with the IgG titer in lung washings and serum (Fig. 2A).

To examine the protective effects of these mycelia extract-adjuvanted vaccines against lethal influenza virus challenge, mice were inoculated with a lethal dose (40 LD_{50}) of influenza A/PR8 virus (Fig. 2B). Mice that had been immunized previously with *P. linteus*- or poly(I:C)-adjuvanted vaccine exhibited no remarkable change in body weight 14 days after virus challenge. In mice that had been immunized with vaccine adjuvanted with mycelia extracts from *M. gracilenta*, *G. frondosa*, or *L. edode*, body weights decreased gradually until day 6 after virus challenge, and then recovered from days 7 to 14 (Fig. 2B). Control mice that had been immunized with non-adjuvanted vaccine suffered from marked loss of body weight. The survival rate of mice immunized with *P. linteus*-adjuvanted vaccine was 100% at 14 days after virus challenge, suggesting that *P. linteus*-adjuvanted vaccine protected the mice against lethal lung infection as effectively as vaccine containing the poly(I:C) adjuvant (Fig. 2B). Meanwhile, the survival rates of mice immunized with vaccines adjuvanted with mycelial extracts from *M. gracilenta*, *G. frondosa*, or *L. edode* ranged from 40% to 60% at day 14 after lethal lung infection (Fig. 2B). All mice immunized with non-adjuvanted vaccine were deceased by 7 days after challenge.

Intranasal Administration of *Phellinus linteus*-Adjuvanted H5N1 Vaccine Protects Mice From Highly Pathogenic H5N1 Influenza Virus Variant Challenge

Next, the efficacy of *P. linteus*-adjuvanted vaccine against homologous (A/Vietnam/1194/2004) and heterologous (A/Indonesia/6/2005) H5N1 influenza virus challenge was examined in BALB/c mice (Fig. 3). The mice were immunized twice intranasally with 1 μg of formalin-inactivated whole H5N1 virus vaccine (NIBRG14) alone, or in combination with 500 μg of *P. linteus* extract. At 2 weeks after the final immunization, the mice were challenged by intranasal administration of 1,000 PFU of H5N1 influenza viruses.

The concentrations of anti-NIBRG14 IgA and IgG antibodies in nasal washings and serum, respectively, were much higher in animals immunized intranasally with *P. linteus*-adjuvanted NIBRG14 vaccine than in mice immunized with the vaccine alone or in non-immunized mice (Fig. 3A). In response to homologous viral challenge (A/Vietnam/1194/2004), the mice immunized with *P. linteus*-adjuvanted vaccine showed a significant reduction in virus titer compared with control mice (Fig. 3A). The mice vaccinated with *P. linteus*-adjuvanted vaccine survived longer than 14 days post-infection, while the mice immunized with

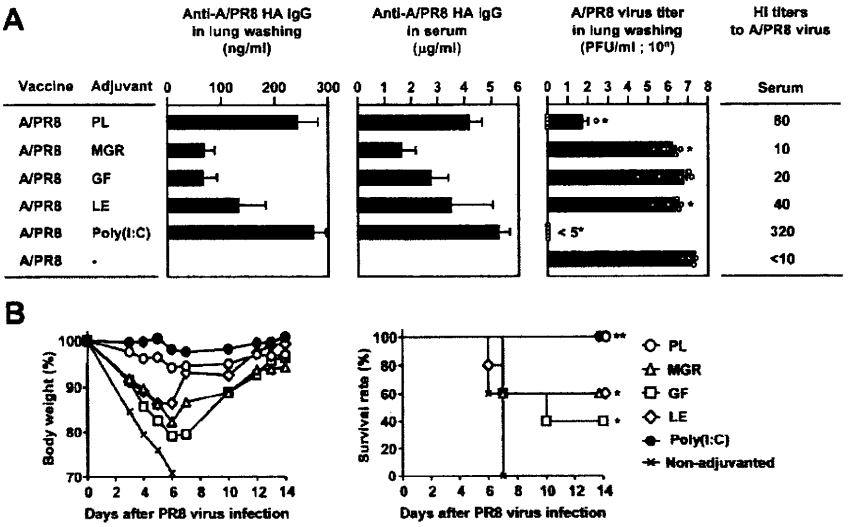

Fig. 2. **A**: Anti-A/PR8 hemagglutinin-specific IgG antibodies in lung washings and serum, and A/PR8 virus titer in lung washings. The mice were immunized twice intranasally with 1 μg of hemagglutinin vaccine alone, or in combination with extracts of mycelia from *P. linteus* (PL), *Macrolepiota gracilenta* (MGR), *Grifola frondosa* (GF), *L. edodes* (LE), or poly(I:C). Two weeks after the final immunization, the immunized mice were challenged by administration of 1,000 PFU (40 LD_{50}) of A/PR8 influenza viruses into the lung, and samples were collected 3 days after the challenge. The concentrations of IgG antibodies and virus titers from five mice from each group were measured by ELISA and plaque assay using MDCK cells, respectively. Each column represents the mean ± SE of five mice per group and open circles indicate individual animals. The virus titers were statistically compared to those of non-adjuvanted mice ($^*P < 0.05$). The hemagglutination inhibition (HI) titers against homologous A/PR8 influenza virus in the serum were measured at 2 weeks after the final immunization. The data are presented per group, and expressed as reciprocals of the highest dilution that completely inhibits hemagglutination of four HA units of the virus. **B**: Body weight and survival curves of the immunized mice after lethal A/PR8 virus challenge. Each point represents the ratio relative to the initial body weight (average) of five mice for each day after challenge (left panel). The survival rates were monitored for 14 days (right panel). $^{**}P < 0.01$ versus control mice, log-rank test.

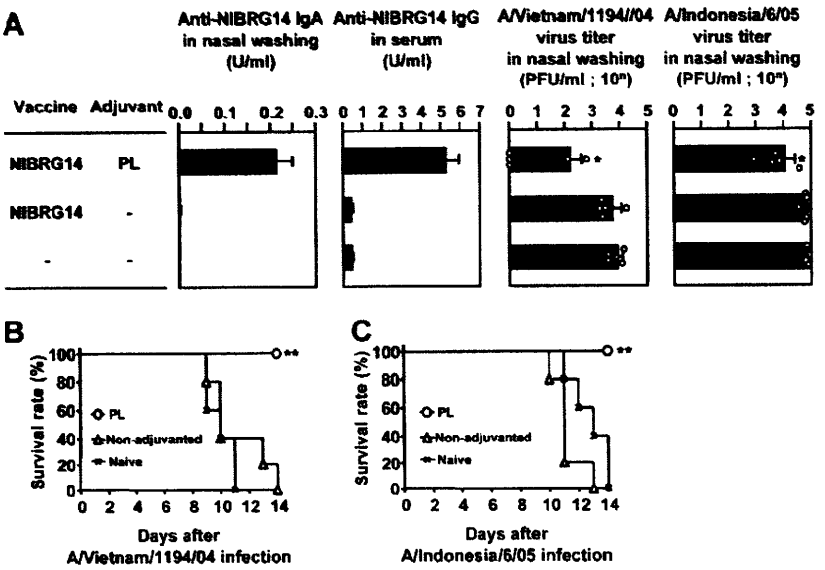

Fig. 3. Anti-NIBRG14-specific IgA and IgG responses, and H5N1 virus titers in nasal washings and survival rates after lethal challenge with homologous influenza A/Vietnam/1194/04 and heterologous influenza A/Indonesia/6/05 viruses. A: Anti-NIBRG14-specific IgA and IgG responses and H5N1 virus titer in the nasal washings. The mice were immunized twice intranasally with vaccine alone, or in combination with mycelia extracts of *Phellinus linteus* (PL), then challenged by intranasal administration of 1,000 PFU of influenza A/Vietnam/1194/04 or influenza A/Indonesia/6/05 virus 14 days after the final immunization. Nasal washings and serum samples were collected 3 days after the challenge. The concentrations of IgA and IgG antibodies and virus titers from five mice from each group were measured by ELISA and plaque assay using MDCK cells, respectively. Each column represents the mean values ± SE of five mice per group, and open circles indicate individual animals. The virus titers were statistically compared to those of non-immunized mice ($^{*}P < 0.05$). (**B,C**) The survival curves of mice immunized according to the same schedule as in Figure 2A after lethal influenza A/Vietnam/1194/04 (B) or influenza A/Indonesia/6/05 (C) virus challenge is depicted. The survival rates were monitored for 14 days. $^{**}P < 0.01$ versus control mice, log-rank test.

non-adjuvanted vaccine or non-immunized mice succumbed to disease by days 14 and 11, respectively (Fig. 3B). In the group challenged with heterologous A/Indonesia/6/2005 virus, mice immunized with *P. linteus*-adjuvanted vaccine showed a significant reduction in virus titer compared to the control mice (Fig. 3A) and survived longer than 14 days post-infection (Fig. 3C), while none of the mice immunized with non-adjuvanted vaccine or non-immunized mice survived more than 14 days post-infection (Fig. 3C). None of the surviving mice exhibited any clinical signs of infection, such as ruffled hair or emaciation, following the virus challenge. These results clearly indicate that intranasal administration of H5N1 vaccine in combination with *P. linteus* extract protects mice against highly pathogenic homologous and heterologous influenza A virus H5N1 infection.

Intranasal Immunization With the Hemagglutinin Vaccine With Mycelia Extract Induces a Weak Systemic T-Cell Response

To examine whether intranasal administration of influenza vaccine induces a T-cell response, levels of interferon-γ were measured in supernatants of T-cells from spleen and cervical lymph nodes of immunized mice in co-culture with antigen-presenting cells and PR8 vaccine (Fig. 4). Briefly, T-cells isolated from the spleen or cervical lymph nodes of mice 7 days after the final immunization were cultured with irradiated antigen-presenting cells in the presence or absence of A/PR8 vaccine at 0.1, 1.0 or 10 μg/ml. Low but significant levels of interferon-γ were detected in the splenic T-cells of mice immunized with *P. linteus*-adjuvanted vaccine (Fig. 4A). However, there was no significant effect on interferon-γ production in splenic T-cells of mice immunized by A/PR8 vaccine adjuvanted with *M. gracilenta*, *G. frondosa*, or *L. edode* mycelial extracts. Similarly, *P. linteus*-adjuvanted vaccine induced little T-cell response in cervical lymph nodes (Fig. 4B). These results suggest that immunization with a combination of vaccine and mycelia extract induces a relatively weak T-cell response.

MyD88 Participates in Mycelia Extract Stimulated Production of Proinflammatory Cytokines in Bone Marrow-Derived Dendritic Cells

Crude mycelia extract contains proteoglycans, hemicellulase, and β-glucans [Ukawa et al., 2000] and activates innate immune responses via CD14/TLR4 or dectin-1 (a β-glucan-specific C-type lectin receptor)-dependent pathways [Saijo et al., 2007; Taylor et al., 2007]. Because MyD88 is a general adaptor/regulator molecule for the Toll/IL-1R family of receptors [Medzhitov et al., 1998], cytokine production was measured in wild-type and MyD88-deficient bone marrow-derived dendritic cells following stimulation with mycelia extract. Bone marrow-derived dendritic

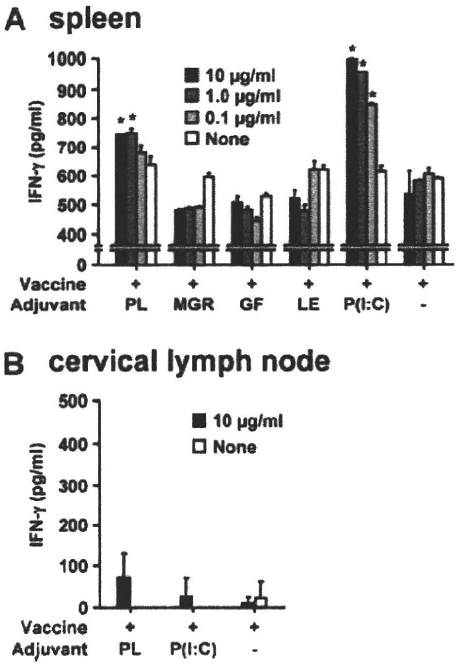

Fig. 4. In vitro responses of influenza A/PR8 virus-specific T-cells derived from mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelial extracts or poly(I:C). Spleens (**A**) and cervical lymph nodes (**B**) were isolated 1 week after the final immunization and re-stimulated with T-cell-depleted splenocytes that had been pulsed with the indicated concentration of A/PR8 hemagglutinin vaccine. Production of interferon-γ in the culture supernatant was measured by ELISA at 4 days after the antigen re-stimulation. These results are presented as the means of two independent experiments. $^*P < 0.05$ versus non-stimulated sample.

cells from wild-type or MyD88-deficient mice were stimulated with lipopolysaccharide, Zymosan, or mycelia extracts in vitro for 24 hr, and their ability to secrete TNF-α was examined. After stimulation with Zymosan, *P. linteus*, *M. gracilenta*, *G. frondosa*, or *L. edode* extracts, TNF-α production was partially but significantly reduced in MyD88-deficient dendritic cells in comparison to wild-type dendritic cells, and was drastically reduced after lipopolysaccharide stimulation in MyD88-deficient dendritic cells as compared to wild-type (Fig. 5). All mycelia extracts strongly induced TNF-α and IL-6 as much as stimulation with lipopolysaccharide or Zymosan in bone marrow-derived dendritic cells, but not IL-12 or p70. In addition, while lipopolysaccharide and Zymosan strongly enhanced CD40 expression in dendritic cells, treatment with mycelial extracts from *P. linteus*, *M. gracilenta*, *G. frondosa*, or *L. edode* only modestly enhanced CD40 expression in dendritic cells. These results indicate that proinflammatory cytokine production in bone marrow-derived dendritic cells in response to stimulation by mycelial extracts is partly dependent on MyD88 and that the adjuvant activity of mycelia extracts may be achieved through activation of dendritic cells.

DISCUSSION

The results of the present study clearly demonstrate that extracts of mycelia from edible mushrooms, especially *P. linteus*, are an effective mucosal adjuvant when administered intranasally with influenza vaccine. Nasal immunization induced not only an increase in mucosal secretory IgA, but also a high titer of anti-HA IgG in the serum. This immune reaction resulted

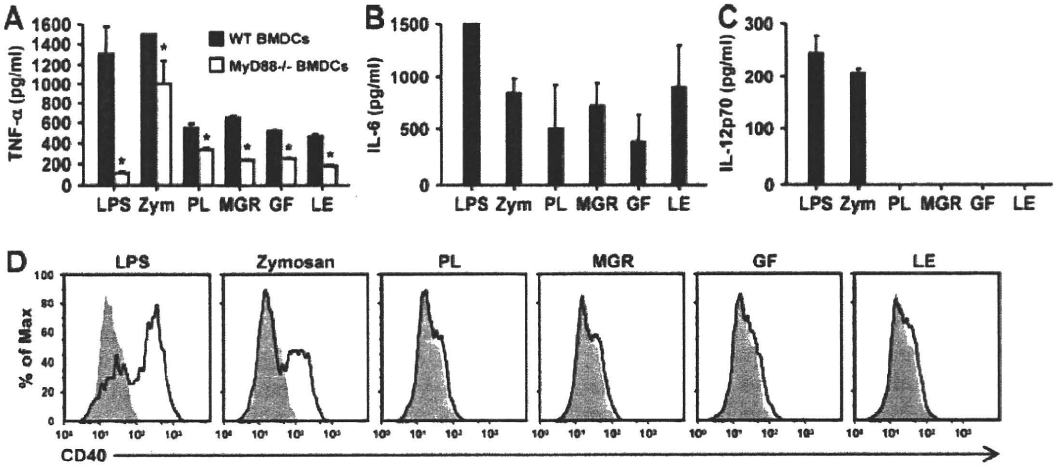

Fig. 5. Tumor necrosis factor (TNF)-α (**A**), IL-6 (**B**), and IL-12p70 production from bone marrow-derived dendritic cells (BMDCs). BMDCs (5×10^5 cells/well) from wild-type (filled columns) or MyD88-deficient mice (open columns) were stimulated with lipopolysaccharide (LPS), Zymosan (Zym.), *Phellinus linteus* (PL), *Macrolepiota gracilenta* (MGR), *Grifola frondosa* (GF), or *Lentinula edodes* (LE) for 24 hr as described in Materials and Methods Section. The culture supernatants were collected and the concentration of TNF-α (A), IL-6 (B) and IL-12p70 (**C**) were measured by ELISA. Data represent the means ± SD of duplicate samples. $^*P < 0.05$, wild-type versus MyD88-deficient dendritic cells. The expression of the co-stimulatory molecule CD40 was measured by flow cytometry after 24 h of stimulation (**D**). The gray histograms show the expressions on the unstimulated cells, and the bold lines show the expression of CD40.

in cross-protective immune responses against both homologous and heterologous influenza variants, including highly pathogenic H5N1 influenza virus isolates. Administration of the formalin-inactivated whole H5N1 vaccine (NIBRG14) or the PR8 HA vaccine combined with *P. linteus* extract following a two-dose immunization protocol was able to confer protection against infection with lethal influenza A/Vietnam/1194/2004 (H5N1) virus, influenza A/Indonesia/6/2005 (H5N1) virus, and lethal lung infection (40 LD_{50}) by PR8 influenza virus, respectively. These results indicate that *P. linteus* extract is an effective mucosal adjuvant when administered intranasally with influenza vaccine. The *P. linteus*-adjuvanted vaccines induced poor T-cell responses, indicating that cross-protection may be mediated primarily by the mucosal immune response, probably via the activity of secretory IgA antibodies against viral proteins. It has been shown that polymeric immunoglobulin receptor-knockout mice do not secrete IgA and exhibit less cross-protective efficacy against variant influenza virus infection [Asahi et al., 2002]. Although neutralizing activity against heterologous A/Vietnam/1194/2004 (H5N1) virus was not detected in the nasal washings, this was likely due to dilution with PBS when the nasal washings were collected. The concentration of vaccine-specific IgA in the nasal wash samples was much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal washings may not have been detectable.

Intranasal vaccination is advantageous for protection against influenza virus infection due to the induction of secretory IgA at the mucosal surface, which elicits a more effective cross-protective immunity compared to serum IgG. In fact, the cross-protective effects of *P. linteus*-adjuvanted vaccine were observed even against virulent heterologous H5N1 variants. Antigen-specific T-cell responses were weak in mice that had been immunized intranasally with vaccine and mycelia extracts from *P. linteus*, indicating that homologous and heterologous protection is primarily accomplished by secretory IgA at the mucosal surface.

Although mycelia extracts did not activate dendritic cells to the same extent as lipopolysaccharide or Zymosan, they induced high levels of cytokines such as TNF-α and IL-6 as much as stimulation with lipopolysaccharide or Zymosan. It has been shown that dectin-1 mediates the phagocytosis of β-glucan-bearing ligands, including yeast-derived particles such as Zymosan [Herre et al., 2004]. The phagocytosis of pathogens is a critical host defence mechanism, not only for clearance of the invading microorganism, but also for the generation of antigenic fragments for presentation to $CD4^+$ T-cells to induce a subsequent adaptive immune response [Dzionek et al., 2001]. Toll-like receptors 3, 7, 8, and 9 are localized to intracellular compartments and specialize in recognition of viral nucleic acids in the endosome [Iwasaki and Medzhitov, 2004]. In this regard, concomitant administration of mycelial extracts and a toll-like receptor agonist, such as synthetic double-stranded RNA poly(I:C), synthetic imidazoquinoline compounds, or oligodeoxynucleotides containing cytosine–guanine motifs may be more effective than either mycelial extracts or toll-like receptor agonists alone, by reason of accumulation of vaccine with a toll-like receptor agonist into endosomal compartments that express toll-like receptors 3, 7, 8, and 9. In fact, complexing toll-like receptor 3 or 9 agonists to cationic liposomes markedly potentiated their ability to activate immune responses [Zaks et al., 2006]. These synergistic effects may contribute to the enhancement of mucosal adjuvant effects, leading to complete protection against viral challenge.

A major objective of intranasal influenza vaccine development is the design of an adjuvant that can provide effective mucosal immune activity and at the same time is stable and safe for clinical application in humans. Although poly(I:C) is a potent mucosal adjuvant, it has been associated with some adverse events during clinical trials of intravenous administration. Poly(I:C) induced a number of side effects in humans, including renal failure and hypersensitivity, in a previous clinical trial using dosages as high as 75 mg of poly(I:C)/m^2 at day 0 followed by daily administration from day 7 to a maximum of 35 days [Robinson et al., 1976]. Although the crude mycelia extracts used in the present study are not as effective as poly(I:C) as an adjuvant, identification of the active ingredients has the potential to produce an adjuvant as effective as poly(I:C). The mycelial extracts are derived from edible mushrooms and are separated by boiling, indicating that the active ingredients in the adjuvant are thermotolerant, whereas poly(I:C) loses adjuvant activity after boiling at 95 C for 5 min [Ichinohe et al., 2005]. It has also been shown that oral administration of mushroom extracts decreases IgE levels by modulation of Th1/Th2 balance. Inagaki et al. [2005] reported that oral administration of *P. linteus* significantly inhibited the IgE-dependent mouse triphasic cutaneous reaction, and Lim et al. [2005] demonstrated that *P. linteus* given orally decreased IgE concentration in serum and murine mesenteric lymph node lymphocytes, and increased concanavalin A-induced interferon-γ secretion in mesenteric lymphocytes. These characteristics offer great advantages for clinical application.

For the clinical application, we need to evaluate the effective dose of vaccine in human trials. The effectiveness of intranasal vaccine with injection dose (15 μg) in humans could be roughly assessed by the ability of ~0.1 μg vaccine to induce an immune response to provide protection against infection in BALB/c mice immunized intranasally according to a two-dose regimen [Tamura et al., 2005]. These data suggest that the vaccine dose in BALB/c mice in the current study (1 μg of vaccine per mouse) corresponded to ~10 times the dose in subcutaneous injection in human. Identification of the active ingredients in the crude mycelial extracts could reduce the doses. Further investigation is necessary to identify the active ingredients to produce more potent mucosal adjuvant.

In summary, intranasal immunization with influenza vaccine and extract of mycelia from *P. linteus*-induced cross-protective mucosal immunity against heterologous H5N1 influenza viruses in mice. Intranasal immunization with influenza vaccine containing *P. linteus* extract may thus represent a strategy to generate protective mucosal immunity in humans against newly emerging and highly pathogenic avian influenza viruses. Because prophylactic agents, including vaccines, must ensure sufficient safety for clinical use, further studies are required to determine whether such a nasal vaccine would be effective for use in humans.

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Short Communication

The First Autopsy Case of Pandemic Influenza (A/H1N1pdm) Virus Infection in Japan: Detection of a High Copy Number of the Virus in Type II Alveolar Epithelial Cells by Pathological and Virological Examination

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SUMMARY: We report the pathological and virological findings of the first autopsy case of the 2009 pandemic influenza (A/H1N1pdm) virus infection in Japan. A man aged 33 years with chronic heart failure due to dilated cardiomyopathy, mild diabetes mellitus, atopic dermatitis, bronchial asthma, and obesity died of respiratory failure and multiple organ dysfunction syndrome. Macroscopic examination showed severe plumonary edema and microscopically the lung sections showed very early exudative-stage diffuse alveolar damage (DAD). Immunohistochemistry revealed proliferation of the influenza (A/H1N1pdm) virus in alveolar epithelial cells, some of which expressed SAα2-3Gal on the cell surface. Influenza (A/H1N1pdm) virus genomic RNA and mRNA were also detected in alveolar epithelial cells. Real-time PCR revealed 723 copies/cell in the left lower lung section from which the influenza (A/H1N1pdm) virus was isolated. Electron microscopic analysis revealed filamentous viral particles in the lung tissue. The concentrations of various cytokines/chemokines in the serum and the autopsied lung tissue were measured. IL-2R, IL-6, IL-8, IL-10, IFN-α, MCP-1, and MIG levels were elevated in both. These findings indicated a case of viral pneumonia caused by influenza (A/H1N1pdm) virus infection, showing characteristic pathological findings of the early stage of DAD.

The 2009 pandemic influenza (A/H1N1pdm) virus causes severe respiratory disease, neurologic complications, and myocardial symptom in some patients (1–3). From August 15 through December 15, 2009, a total of 116 patients with confirmed infection with influenza (A/H1N1pdm) virus died in Japan (4). Autopsy verification of the cause of death is indispensable to elucidate the pathogenesis of influenza (A/H1N1pdm) virus infection. Pathological findings of fatal influenza (A/H1N1pdm) virus infection have recently been reported (5,6). Here, we report in detail the pathological and virological findings of the first autopsy case in Japan.

On August 20, 2009 (day 1), a man aged 33 years with chronic heart failure due to dilated cardiomyopathy, mild diabetes mellitus, atopic dermatitis, asthma, and obesity (BMI, 38) complained of cough and watery diarrhea. On day 6, he was admitted with progressive dyspnea, high fever (39°C) and diarrhea. On admission, a chest radiogragh showed nodular infiltrates in the lower lungs (Fig. 1a) and a chest computed tomography (CT) scan revealed severe bilateral consolidations (Fig. 1b). The leukocyte count was 5,210/mm^3 and CRP was 1.1 mg/dl. The nasopharyngeal swab specimens were negative for influenza virus type A antigen by the rapid test (ESPLINE® Influenza A&B Kit; Fujirebio, Tokyo, Japan), and therefore oseltamivir was not administered. On the morning of day 7, the patient required intubation and was placed on mechanical ventilation. At intubation, foamy, white and partly bloody fluid spouted out from the tube. Methylprednisolone pulse therapy and administration of a neutrophil elastase inhibitor (sivelestat sodium hydrate) were initiated for the treatment of acute respiratory distress syndrome (ARDS). The diagnosis of influenza (A/H1N1pdm) virus infection was confirmed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) testing on day 7. In spite of intensive care, the patient died of respiratory failure and multiple organ dysfunction syndrome on day 8. The chest radiograph showed progressive and confluent consolidations (Fig. 1c).

Autopsy revealed macroscopically severe pulmonary edema, hemorrhage, exudation and focal nodular lesions in the lungs (left, 730 g; right, 800 g) (Fig. 2a). The bilateral main bronchi were filled with foamy liquid, which was positive for influenza virus A antigen. The nodular lesions were palpable in the lower lungs, and corresponded to the nodular shadows of the chest radiographic findings (Fig. 1c). The heart was enlarged and all chambers were dilated (670 g), and the left ventricular wall and septal wall had irregular whitish patches, compatible with dilated cardiomyopathy (Figs. 2b and c). The brain was edematous and swollen (1,350 g). Hyperplastic solitary lymph follicles of the terminal ileum and rectal erosion were observed.

Tissue samples from all major organs were fixed in 20% buffered formalin and embedded in paraffin by an automated

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processor (SAKURA ETV-150CV; Sakura Finetek Japan, Tokyo, Japan). Each section was cut into 3 μm in thickness, mounted on silane-coated slides (Matsunami, Tokyo, Japan) and was examined histologically. Hematoxylin and eosin-stained lung sections demonstrated intra-alveolar edema with fibrin, erythrocytes and desquamated epithelial cells in alveolar spaces, hyperplasia of type II pneumocytes, and hemosiderin-laden macrophages (heart failure cells) (Figs. 3a, b, c, d, and e). No viral inclusion bodies or cytopathic changes were observed in pneumocytes. Neutrophil infiltration was not prominent. Regenerative hyperplasia and desquamation of the pseudostratified columnar epithelium of the bronchi were observed (Fig. 3a). Some sections demonstrated the feature of early exudative-stage diffuse alveolar damage (DAD) with hyaline membrane formation (Fig. 3c). The findings of more progressed proliferative-stage DAD were also observed in a few sections (Fig. 3e).

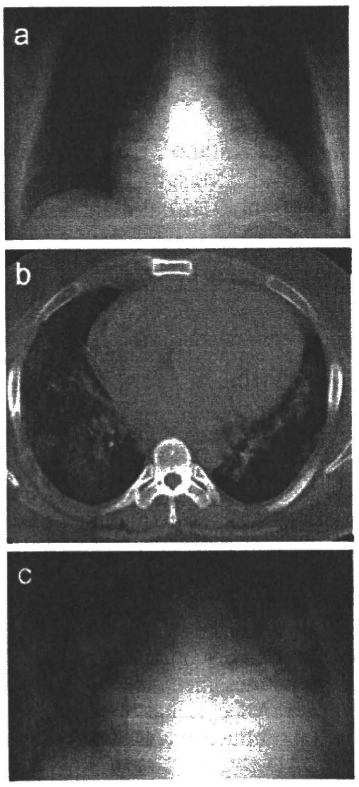

Fig. 1. Radiograph of the lung and chest computed tomography (CT). (a) The radiograph showed nodular infiltrates in lower lungs on day 6. (b) The chest CT scan revealed severe bilateral consolidations on day 6. (c) The radiograph showed progressive and confluent consolidations on day 8.

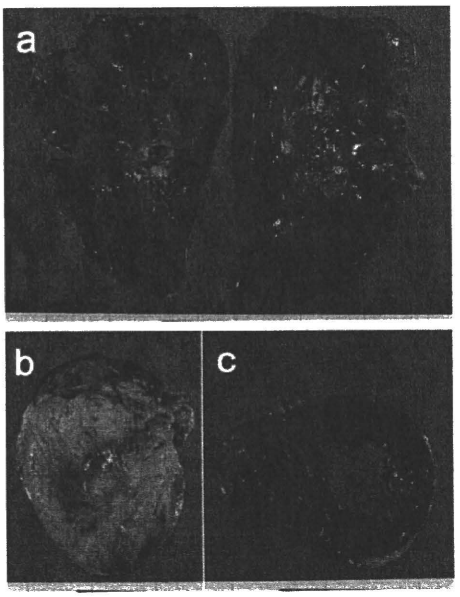

Fig. 2. Macroscopical findings. (a) Plumonary edema (left, 730 g; right, 800 g). (b, c) Enlarged heart (670 g) and dilated chambers compatible with dilated cardiomyopathy.

To evaluate the distribution of influenza (A/H1N1pdm) virus antigen, all sections were immunostained by an avidin-biotin complex immunoperoxidase method (LSAB2 kit/HRP/DAB; Dako Cytomation, Copenhagen, Denmark) using a mouse monoclonal antibody against influenza A nucleoprotein (InfA-NP) (7). Positive signals for InfA-NP antigen were detected primarily in the lung field sections, and only sparsely in a bronchial section (Figs. 3f, g, h, and i). No signals were detected in a trachea section or the other extrapulmonary tissue sections. Many viral antigen-positive cells were detected in the earlier-stage DAD lesions before hyaline membrane formation (Figs. 3f, g, h, and i) rather than in the progressed lesions (Fig. 3j). The signals were found in alveolar epithelial cells, both type I and type II pneumocytes (Figs. 3b, d, g, and i). The influenza (A/H1N1dm) virus genomic RNA (minus-strand RNA) and mRNA (plus-strand RNA) were detected in the same lesion by the in situ hybridization AT-tailing CSA (ISH-AT-CSA) method using strand-specific oligonucleotide probes for the NP region of the influenza (A/H1N1pdm) virus (sense probe: 5′-gcaaggttcaacacttcccagaaggtctggtgccgcaggt-ATATATATATATATATATAT-3′, anti-sense probe: 5′-acctgcgg caccagaccttctgggaagtgttgaaccttgc-ATATATATATATATATATAT AT-3′) (Figs. 4a and b) (8,9). This revealed that the virus had replicated in the alveolar epithelial cells. No signals were detected using an irrelevant probe as a negative control (Fig. 4c).

To characterize the virus-infected cells, confocal laser scanning microscopy was used to visualize double immunofluorescence staining for InfA-NP and for the cell type-specific marker proteins EMA (epithelial cells), SP-D (type II pneumocytes), cytokeratin AE1/AE3 (epithelial cells), CD68 (macrophages) and CD34 (endothelial cells) as previously described (10). Alexa Fluor 568-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, Oreg., USA) and Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes) were used as secondary antibodies. Almost all InfA-NP signals were detected in epithelial (EMA-positive) cells (Fig. 4d). They were also detected in SP-D-positive cells, suggesting type II pneumocytese (Fig. 4e). A few were detected in AE1/AE3-positive bronchial epithelial cells. None were detected in CD68-positive macrophages or CD34-positive endothelial cells (data not shown).

The influenza A virus binds to receptors containing terminal sialic acids linked to galactose on cell surface glycoproteins by a 2-3 linkage (SAα2-3Gal) and/or by a 2-6 linkage (SAα2-6Gal) (11). Swine influenza HA binds to both SAα2-6Gal and SAα2-3Gal (12). In the human respiratory organs, putative SAα2-6Gal receptors were mainly expressed in the epithelium of the upper respiratory tract and SAα2-3Gal receptors were expressed in the epithelium of the lower respiratory tract (data not shown). The sections were incubated

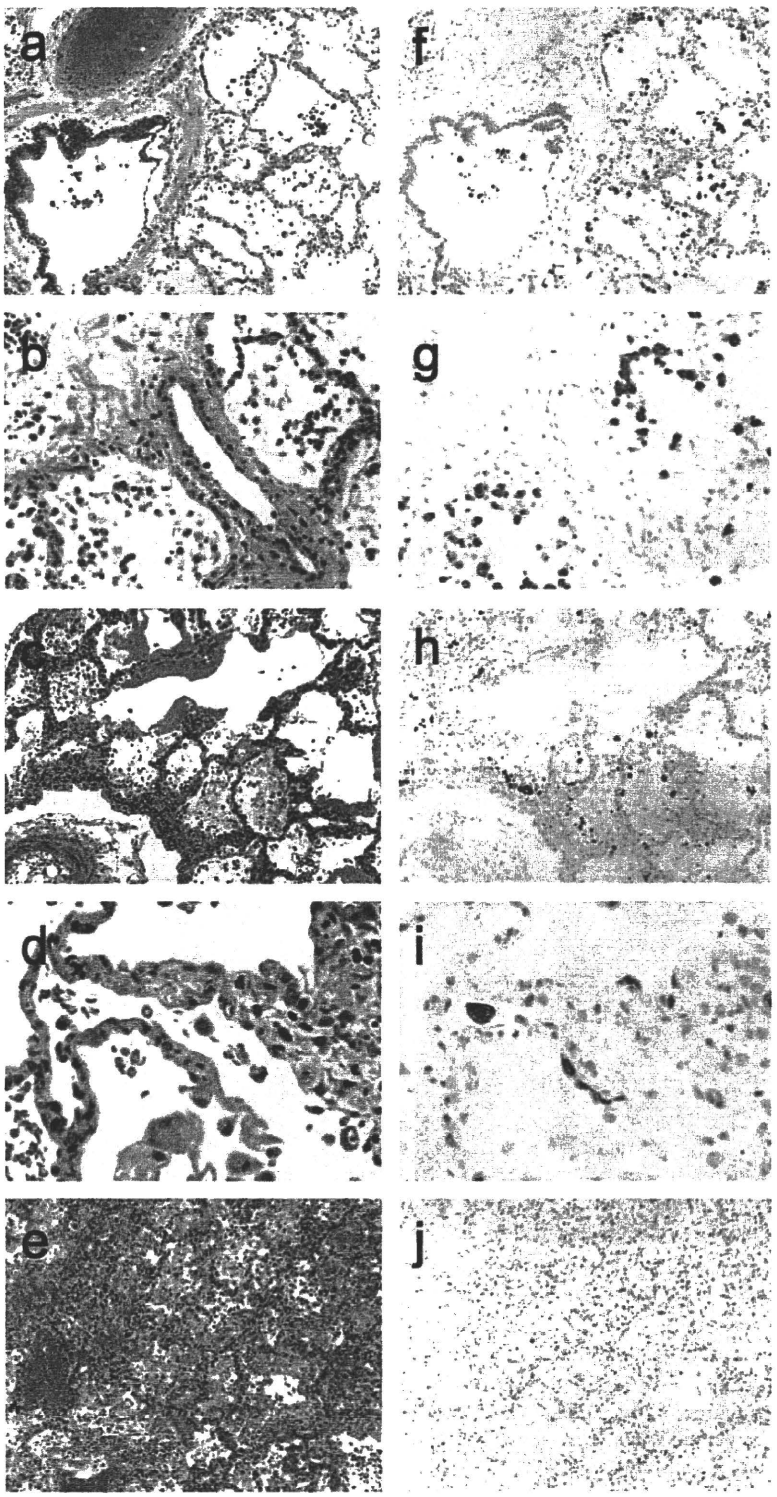

Fig. 3. Hematoxylin and eosin staining (a, b, c, d, and e) and immunohistochemistry for influenza A nucleoprotein (InfA-NP) (f, g, h, i, and j). Intra-alveolar edema with liquid, fibrin, erythrocytes and desquamated epithelial cells in alveolar spaces, hyperplasia of type II pneumocytes, and hemosiderin-laden macrophages (heart failure cells) were generally observed. (a) Regenerative hyperplasia and desquamation of the pseudostratified columnar epithelium of the bronchi were observed. (b, c, d) Early exudative-stage diffuse alveolar damage (DAD) with hyaline membrane formation. (e) More progressed proliferative-stage DAD were shown. (f, g, h, and i) A lot of viral antigen-positive cells were detected in the earlier-exudative stage DAD lesions before hyaline membranes formation. (i) The signals were also found in alveolar type I pneumocytes. (j) Few viral antigen-positive cells were detected in the progressed lesion. Original magnification, ×100 (a, c, e, f, h, and j), ×200 (b, g), ×400 (d, i).

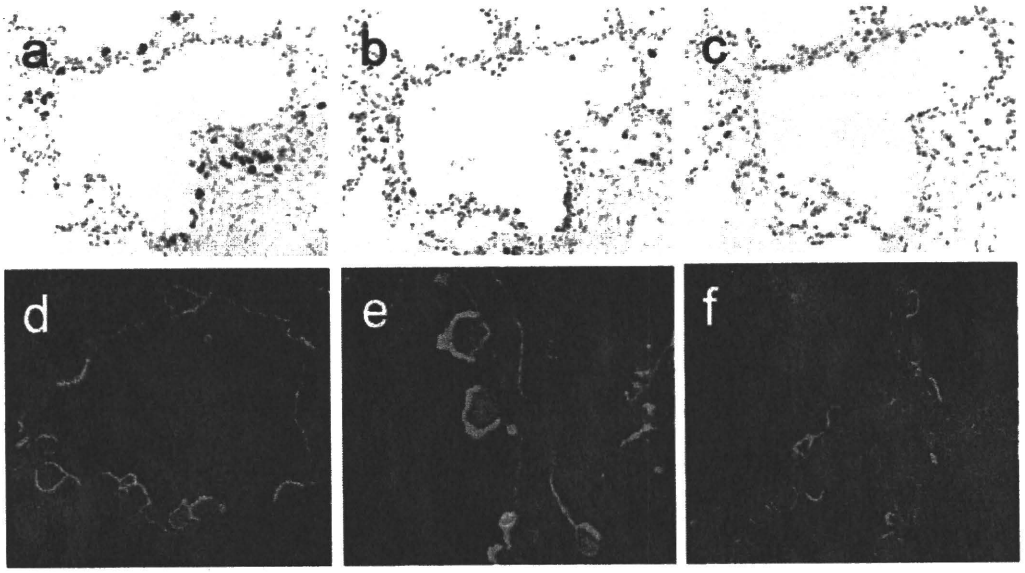

Fig. 4. The detections of influenza (A/H1N1pdm) virus-RNA using in situ hybridization AT-tailing-CSA method. (a) The influenza (A/H1N1pdm) virus genomic-RNA detected using a sense-probe. (b) The influenza (A/H1N1pdm) virus-mRNA detected using an anti-sense probe. (c) No signals were detected using an irrelevant probe. Original magnification, ×200. Double immunofluorescence staining. (d) Influenza virus A nucleoprotein (InfA-NP) (red) and EMA for epithelial cells (green) were co-localized. (e) InfA-NP (red) and SP-D for type II pneumocytes (green) were co-localized. (f) InfA-NP (red) were detected in the cells expressing sialic acids linked to galactose by a 2-3 linkage (SAα2-3Gal) (green). Original magnification, ×400.

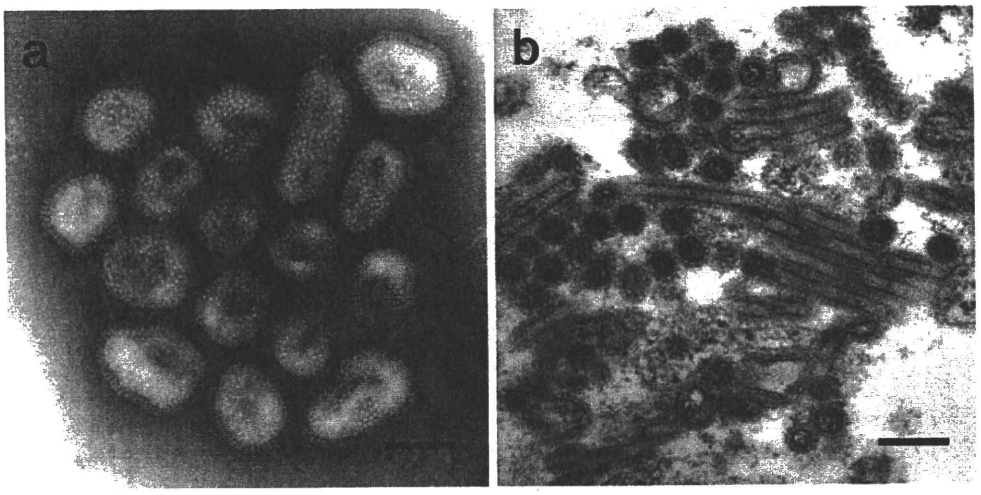

Fig. 5. Electron microscopy. (a) The negative staining feature of isolated influenza (A/H1N1pdm) virus particles in the culture supernatant. Scale bar, 100 nm. (b) The influenza (A/H1N1pdm) viral particles in filamentous forms in the lung sections. Scale bar, 200 nm.

with biotinylated-*Maackia amurensis* lectin II (MAA-II) (Vector Laboratories, Burlingame, Calif., USA) to detect SAα2-3Gal and with biotinylated-*Sambucus nigra* lectin I (SNA-I) (EY Laboratories, San Mateo, Calif., USA) to detect SAα2-6Gal. They were then incubated with Fluor 488-conjugated streptavidin (Molecular Probes). Double fluorescence staining and visualization with a confocal laser scanning microscope revealed that some infA-NP-positive cells expressed SAα2-3Gal on the cell surface (Fig. 4f), suggesting that A/H1N1pdm HA is able to bind to SAα2-3Gal receptors and to infect type II pneumocytes. Because few infA-NP are detected in bronchial epithelial cells on day 8 in this case, it was difficult to confirm that A/H1N1pdm HA was able to bind to SAα2-6Gal receptors, which are abundant on the surface of bronchial epithelial cells.

To examine the copy numbers of the influenza (A/H1N1pdm) virus, RNAs extracted from 4 paraffin-embedded sections (5 μm × 3) (left upper lung, left lower lung, left bronchus, middle trachea) were identified by quantitative real-time RT-PCR using an Mx3005P system (Stratagene, La Jolla, Calif., USA), which amplified a segment within the HA region of the A/H1N1pdm virus-RNA (13). The amount of human beta-actin mRNA in the DNase-treated RNA extracted from each section was also determined as an internal reference gene to provide a normalization factor for the amount of RNA isolated from a specimen (14). To amplify A/H1N1pdm-HA, forward (swH1N1-HA-F: 5′-CCCCATTGCA TTTGGGTAAA-3′) and reverse (swH1N1-HA-R: 5′-TGGA GAGTGATTCACACTGGAT-3′) primers were used with a labeled probe 5′-(FAM)AACATTGCTGGCTGGATCCTG

GGA(TAMRA)-3′. Real-time RT-PCR revealed 2.37×10^5 copies of A/H1N1pdm-RNA and 4.92×10^5 copies of beta-actin mRNA in the left lower lung section. The beta-actin mRNA copy number was 1,500 copies/cell (unpublished data). Therefore the influenza (A/H1N1pdm) virus-RNA copy number was calculated as 723 copies/cell in the left lower lung section. Similarly, it was calculated as 10 copies/cell in the left upper lung section, 2 copies/cell in the left bronchus and less than 1 copy/cell in the middle trachea. The differences in the copy numbers of influenza (A/H1N1pdm) virus-RNA among the sections were consistent with the differences in the numbers of InfA-NP-antigen-positive cells detected by immunohistochemistry.

A/H1N1pdm virus was isolated by inoculating 20% (w/v) homogenates of the autopsy lung tissue to Madin-Darby canine kidney (MDCK) cells in the presence of trypsin. The culture supernatants were harvested on the 3rd day post-inoculation as a stock virus. The virus titer, which was expressed as 50% of the tissue culture infectious dose ($TCID_{50}$)/ml on MDCK cells, was 7.5×10^5 $TCID_{50}$/ml. The whole sequence of the influenza virus proliferated in the lung was determined directly using an Illumina Genome Analyzer II (Illumina, San Diego, Calif., USA) and indirectly using the isolated strain. Both sequences mostly coincided with that of A/H1N1pdm. The former sequence was named A/Nagano/RC1-L/2009(H1N1) and the latter one was named A/Nagano/RC1/2009(H1N1). The negative staining feature of isolated A/H1N1pdm virus particles in the culture supernatant by electron microscopy (EM) is shown in Fig. 5a. In the lung tissue processed routinely for EM (15), outside of type II alveolar epithelial cells, influenza virus particles in filamentous rather than spherical forms were found (Fig. 5b).

The concentration of various cytokines/chemokines in the serum on day 5 and the autopsied lung tissue were measured by using a Human Cytokine 25-plex (BioSource International, Inc., Carmarillo, Calif., USA) and Luminex100TM (Luminex Co., Austin, Texas, USA) as described previously (16). IL-2R, IL-6, IL-8, IL-10, IFN-α, MCP-1, and MIG levels were elevated both in the serum on day 5 before corticosteroid treatment and the autopsy lung tissue on day 8. In addition to these data, an increase of IP-10 was observed in the serum and an increase of IFN-γ was detected in the lung tissue.

The patient had multiple risk factors for severe complications of influenza (A/H1N1pdm) virus infection, suggesting that he died of respiratory failure due to severe pulmonary edema caused by both chronic heart failure and influenza pneumonia. The lung sections showed very early exudative-stage DAD before hyaline membrane formation. Unexpectedly, a high level of proliferation of influenza (A/H1N1pdm) virus was detected in alveolar epithelial cells by immunohistochemistry, as shown in Fig. 3. We were able to detect this early stage of the disease even in the autopsied lung sections. This case provide clues to the pathogenesis of influenza pneumonia and suggests what occurs in the lungs after influenza (A/H1N1pdm) virus infection.

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Oseltamivir-Resistant Influenza Viruses A (H1N1) during 2007–2009 Influenza Seasons, Japan

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To monitor oseltamivir-resistant influenza viruses A (H1N1) (ORVs) with H275Y in neuraminidase (NA) in Japan during 2 influenza seasons, we analyzed 3,216 clinical samples by NA sequencing and/or NA inhibition assay. The total frequency of ORVs was 2.6% (45/1,734) during the 2007–08 season and 99.7% (1,477/1,482) during the 2008–09 season, indicating a marked increase in ORVs in Japan during 1 influenza season. The NA gene of ORVs in the 2007–08 season fell into 2 distinct lineages by D354G substitution, whereas that of ORVs in the 2008–09 season fell into 1 lineage. NA inhibition assay and M2 sequencing showed that almost all the ORVs were sensitive to zanamivir and amantadine. The hemagglutination inhibition test showed that ORVs were antigenetically similar to the 2008–09 vaccine strain A/Brisbane/59/2007. Our data indicate that the current vaccine or zanamivir and amantadine are effective against recent ORVs, but continuous surveillance remains necessary.

Influenza A and B viruses are major pathogens that represent a threat to public health with subsequent economic losses worldwide (*1*). Vaccination is the primary method for prevention; antiviral drugs are used mainly for prophylaxis and therapy. Currently, 2 classes of drugs, matrix 2 (M2) blockers and neuraminidase inhibitors (NAIs) are available, but M2 blockers such as amantadine and rimantadine are not commonly used because of the rapid generation of resistance and lack of efficacy against influenza B virus (*2–4*). The NAIs zanamivir and oseltamivir are widely used because of effects against influenza A and B viruses and a low frequency of resistance. NAI virus surveillance studies by several groups have demonstrated that <1% of viruses tested show naturally occurring resistance to oseltamivir as of 2007 (*5–10*), indicating limited human-to-human transmission of these viruses.

At the beginning of the 2007–08 influenza season, however, detection of a substantially increased number of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) was reported, mainly in countries in Europe where the prevalence varies, with the highest levels in Norway (67%) and France (47%) (*11–14*). These viruses showed a specific NA mutation with a histidine-to-tyrosine substitution at the aa 275 position (N1 numbering, H275Y), conferring high-level resistance to oseltamivir. Most of these ORVs were isolated from NAI-untreated patients and retained similar ability of human-to-human transmission to oseltamivir-sensitive influenza viruses A (H1N1) (OSVs) (*10,15*). In response to public health concerns about ORVs, the World Health Organization (WHO) directed Global Influenza Surveillance Network laboratories to intensify NAI surveillance and announced regularly updated summaries of ORV data collected from each laboratory on its website (*16*). This site reported that the global frequency increased from 16% (October 2007–March 2008) to 44% (April 2008–September 2008) to 95% (October 2008–January 2009), indicating that ORVs have spread rapidly around the world.

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Japan has the highest annual level of oseltamivir usage per capita in the world, comprising >70% of world consumption (*10*). Such high use of oseltamivir has raised concerns about emergence of OSVs with increased resistance to this drug. Moreover, in Japan, 2 recent influenza seasons were dominated by influenza viruses A (H1N1) (Figure 1). If a high prevalence of ORVs is observed, primary selection of oseltamivir treatment for influenza patients should be reconsidered. Thus, monitoring ORVs is a serious public health issue.

To estimate the frequency of ORVs and characterize these viruses, we analyzed 1,734 clinical samples isolated from the 2007–08 season and 1,482 isolates from the 2008–09 season by NA sequencing and/or NAI inhibition assay. The total frequencies were 2.6% in the 2007–08 season and 99.7% in the 2008–09 season, indicating that ORVs increased dramatically in Japan.

Materials and Methods

Virus Testing

Influenza sentinel clinics send clinical specimens to local public health laboratories for virus isolation. Several culture tissues, including MDCK, Caco-2, and LLC-MK2, are used for virus isolation. Without successful viral isolation, clinical specimens are analyzed directly. Influenza viruses were collected from all 47 prefectures in Japan for this study: 1,734 samples of influenza A (H1N1) were isolated during the 2007–08 season (September 2007–August 2008) and 1,482 samples of influenza A (H1N1) were isolated in the 2008–09 season (September 2008–April 2009). During the 2007–08 season, viruses were isolated primarily after December 2007. All influenza viruses A (H1N1) were subjected to full or partial (nt 615–1076) NA sequencing to detect H275Y substitution on the N1 NA protein. Representative influenza viruses A (H1N1), including ORVs and OSVs, were subjected to NA inhibition assay (number of tested viruses isolated during the 2007–08 and 2008–09 seasons was 306 and 58, respectively), full NA sequencing (891 and 83), hemagglutinin (HA) 1 sequencing (299 and 83), M2 sequencing (288 and 79), and hemagglutinin inhibition (HI) test (187 and 59).

Sequence Analysis

The phylogenetic tree of NA and HA1 genes was constructed by neighbor-joining methods. The phylogenetic tree was described by representative ORVs and OSVs isolated from several prefectures in Japan. Sequence information for isolates from other countries was obtained from the Global Initiative on Sharing Avian Influenza Data and the Los Alamos National Laboratory database. All amino acid positions in the phylogenetic tree were described by N1 numbering.

NA Inhibition Assay

The chemiluminescent NA inhibition assay was per-

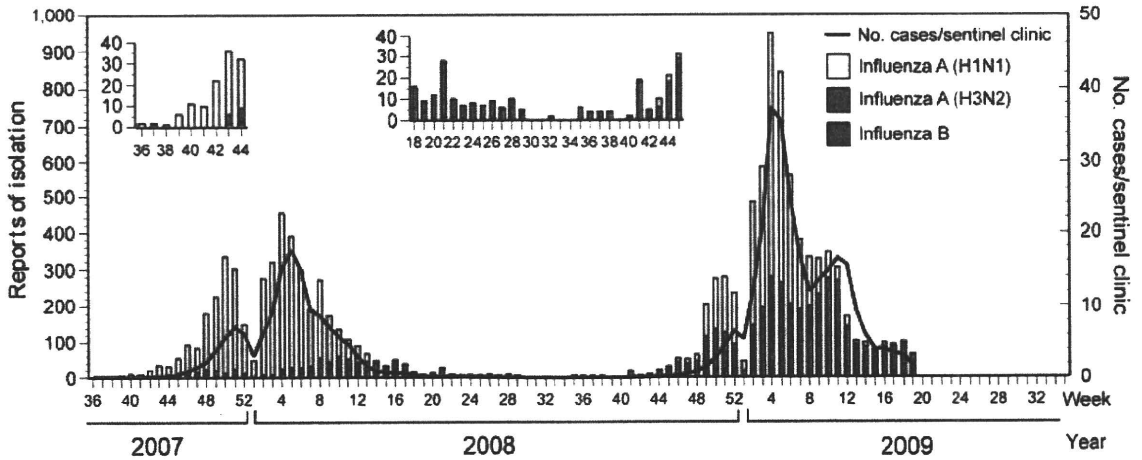

Figure 1. Weekly cases of influenza and isolation of influenza viruses in the 2007–08 and 2008–09 seasons in Japan (as of July 2, 2009). The National Epidemiologic Surveillance of Infectious Diseases (NESID) Network comprises the Ministry of Health, Labor and Welfare; the National Institute of Infectious Diseases; 76 local public health laboratories; ≈3,000 pediatric clinics; and 2,000 internal medical clinics. The NESID Network monitored influenza activity during the 2007–08 season (week 36, September 2007–week 35, August 2008) and 2008–09 season (week 36, September 2008–week 22, May 2009). Clinically diagnosed influenza-like cases were reported weekly by influenza sentinel clinics. **Boldface** line indicates weekly cases of influenza-like illness per influenza sentinel clinic (values shown in right bar). Bars indicate numbers of influenza A (H1N1) (yellow), A (H3N2) (blue), and B (red) isolates (values shown in left bar). Influenza activity started week 47 of 2007 and finished in week 14 of 2008 in the 2007–08 season and started week 49 of 2008 and finished in week 22 of 2009 in the 2008–09 season. Among all influenza isolates, influenza A (H1N1) consisted of 81% during 2007–08 and 49% during 2008–09. Seasonal influenza surveillance showed that influenza viruses A (H1N1) dominated the 2 recent influenza seasons in Japan.

formed by using the NA Star Kit (Applied Biosystems, Tokyo, Japan) with slight modifications of the instructions provided by the manufacturer. The final drug concentration ranged from 0.03 nmol/L to 6,500 nmol/L for oseltamivir and from 0.03 nmol/L to 12,500 nmol/L for zanamivir. Chemiluminescent light emission was measured by using an LB940 plate reader (Berthhold Technologies, Bad Wildbad, Germany). Drug concentrations required to inhibit NA activity by 50% (IC_{50}) were calculated by a 4-parameter method using MikroWin 2000 version 4 software (Mikrotek Laborsysteme GmbH, Overath, Germany).

Hemagglutination Inhibition Test

The HI test was performed to evaluate the reactivity of ferret antiserum against 2008–09 vaccine strain A/Brisbane/59/2009, as described by the WHO manual (*17*). Antiserum was treated by receptor-destroying enzyme II (Denka Seiken, Tokyo, Japan) and adsorbed with packed turkey erythrocytes before testing to prevent nonspecific reaction. A 0.5% suspension of turkey erythrocytes was used for the HI test. Viruses with >8-fold reduced HI titer to the homologous titer of A/Brisbane/59/2009 antiserum were regarded as antigenic variants.

Statistical Analysis

To determine the cutoff value between NAI-resistant (outlier) and -sensitive viruses, box-and-whisker plots were used. The cutoff value was defined as upper quartile + 5.0×interquartile range from the 25th to 75th percentile. In this study, ORVs with H275Y were excluded from the overall population for statistical analysis. Outliers were excluded from the calculation of mean values and standard deviations for IC_{50}.

Results

Geographic Distribution of ORVs during the 2007–08 and 2008–09 Influenza Seasons

To estimate the frequency of influenza A (H1N1) ORVs in each prefecture of Japan, 1,734 isolates during the 2007–08 season and 1,482 isolates during the 2008–09 season were collected from all prefectures and examined by NA sequencing to detect the H275Y mutation in NA protein. In the 2007–08 season, 45 viruses possessing H275Y mutation (total frequency of ORVs 2.6%; Figure 2, panel A) were observed in 10 prefectures, indicating that the frequency of ORVs was significantly lower than that in countries in Europe and the United States (*8,11–14*). In Tottori prefecture, however, 22 of 68 influenza viruses A (H1N1) tested possessed H275Y, showing a markedly higher frequency (32.4%) than that in other prefectures. In the 2008–09 season, however, ORVs were observed nationwide. Of 1,482 influenza viruses A (H1N1), 1,477 viruses possessed a H275Y mutation, for a total frequency of 99.7% (Figure 2, panel B). These data show that ORVs increased dramatically in Japan from the 2007–08 season to the 2008–09 season.

Genetic Analysis

Influenza viruses A (H1N1) during 2007–08 fell into either clade 2B, including the current vaccine strain A/

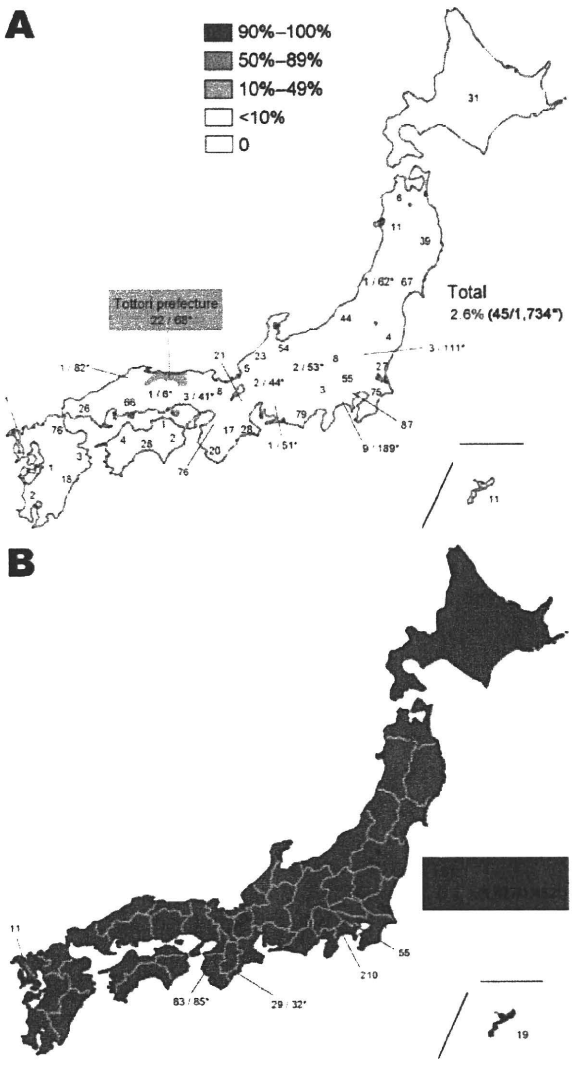

Figure 2. Geographic distribution of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) with H275Y in Japan during the 2007–08 and 2008–09 seasons. The total number of influenza A (H1N1) isolates tested is described inside each prefecture. Total frequency in Japan was 2.6% (45/1,734) during the 2007–08 season, although a high frequency (32.4%) of ORVs was observed in Tottori prefecture (A). On the other hand, total frequency was 99.7% (1,477/1,482) during the 2008–09 season (B), indicating a drastic increase in ORVs in Japan from the 2007–08 season to the 2008–09 season. *Number of ORVs/number of subtype H1N1 isolates tested.

Brisbane/59/2007, or clade 2C, and almost all influenza viruses A (H1N1) during 2008–09 fell into clade 2B. Most ORVs with H275Y belong to clade 2B, which can be further divided into 2 distinct lineages by an aspartic acid to glycine substitution at aa 354: a Northern-Eu lineage sharing 354G, which was first isolated from countries in northern Europe and now represents most ORVs worldwide; and a Hawaii lineage sharing 354D, which was first detected in Hawaii and was rarely isolated in a few countries during the 2007–08 season (online Appendix Figure, www.cdc.gov/EID/content/16/6/926-appF.htm). In the 2007–08 season, of 45 ORVs, 1 virus (A/Yokohama/91/2007) isolated in November 2007 belonged to clade 2C, and 44 viruses fell into either the Hawaii lineage or Northern-Eu lineage. Conversely, in the 2008–09 season, all ORVs belonged to the Northern-Eu lineage, indicating that ORVs of the Northern-Eu lineage dominated in the 2008–09 season in Japan.

In the Hawaii lineage, OSVs genetically close to ORVs were observed. The NA gene of some ORVs had only 1 nucleotide difference from that of OSV counterparts (i.e., A/Tochigi/8/2008 and A/Tochigi/9/2008, A/Nagano/1100/2008 and A/Nagano/1071/2008, A/Yamagata/68/2008 and A/Yamagata/41/2008, respectively), and the other ORVs are also genetically close to OSVs from Japan (online Appendix Figure). These HA genes were also genetically identical or close together (online Appendix Figure), suggesting that almost all ORVs from Japan with the Hawaii lineage are derived from OSVs from Japan. On the other hand, in the Northern-Eu lineage, OSV counterparts were not observed, but foreign ORVs genetically close to ORVs from Japan were observed. During the 2007–08 season, the NA gene of ORVs from Japan was close to that of ORVs isolated from countries in Europe (i.e., A/Paris/0341/2007 and A/England/26/2008). During the 2008–09 season, the ORVs from Japan, which shared A189T on HA protein, were further divided into 4 subclades (C-1 to C-4) by common amino acid changes on HA and/or NA (online Appendix Figure). ORVs from Japan in C-2 and C-3 were genetically close to the ORVs isolated from North America or Hawaii (e.g., A/Memphis/03/2008 and A/Hawaii/19/2008), whereas ORVs in C-1, representing most influenza A (H1N1) viruses from the 2008–09 season in Japan, and ORVs in C-4 were close to ORVs isolated from South Africa and Australia in the Southern Hemisphere (e.g., A/Kenya/1432/2008 and A/Victoria/501/2008). All ORVs except C-3 were isolated before the emergence of ORVs from Japan in each subclade. These findings suggest that ORVs from Japan within a Northern-Eu lineage would not have emerged domestically but instead may have been introduced from various countries.

Antiviral Drug Susceptibility

Of the 364 viruses (306 isolates in the 2007–08 season and 58 isolates in the 2008–09 season) tested by NA inhibition assay, 101 possessed a H275Y substitution. With the NA inhibition assay, although precise IC_{50} values were calculated from a normal sigmoid curve (Figure 3, panels A and B), some viruses generated 2 types of unusual sigmoid curves (Figure 3, panels C and D) resulting from the mixed population of NAI-resistant and -sensitive viruses, as previously reported (*18*). Tentative IC_{50} values were calculated from type A curves (Figure 3, panel C) and included in overall statistical analysis, but values could not be calculated from type B curves (Figure 3, panel D). Later viruses were regarded as resistant candidates.

In the NA inhibition assay for oseltamivir, OSVs showed a mean IC_{50} ± SD of 0.10 ± 0.05 nmol/L (range 0.01–0.35 nmol/L), and ORVs had a mean ± SD IC_{50} of 67.7 ± 44.1 nmol/L (range 26.1–239.2 nmol/L), showing a reduction of >260-fold in susceptibility to oseltamivir. One OSV identified as a statistical outlier (cutoff IC_{50}, >0.40 nmol/L; upper quartile + 5.0× interquartile range) showed a D151E substitution on the NA protein (Table 1).

In the NA inhibition assay for zanamivir, statistical analysis showed that 341 viruses were regarded as the zanamivir-sensitive viruses, with a mean ± SD IC_{50} of 0.40 ± 0.26 nmol/L (range 0.01–1.92 nmol/L), and 16 viruses (10 ORVs and 6 OSVs) were identified as outliers (cutoff IC_{50}, >1.99 nmol/L) (Table 1). Seven viruses (2 ORVs and 5 OSVs) were regarded as resistant candidates from curve fit patterns. NA-sequencing for these 23 viruses (12 ORVs and 11 OSVs) showed 2 types of amino acid changes on the NA protein. One virus, A/Tottori/16/2008 (OSV), possessed a Q136K substitution, showing a high IC_{50} (41.89 nmol/L), and 19 of the other 22 viruses displayed an amino acid change G, N, or V at the D151 position (Table 1). These data suggest that D151 changes have a substantial effect on sensitivity to zanamivir (and oseltamivir). Moreover, A/Tottori/44/2008 with H275Y and D151D/G substitutions conferred high-level resistance to both NAIs (Figure 3, panels A and B). However, a recent study reported that a D151E change was detected only after virus propagation in cell culture, but not in the original clinical specimen (*19*), suggesting a possible role of cell culture in selecting these D151 variant viruses. To further investigate D151 variants, available original clinical specimens of viruses with D151 variation were subjected to NA sequencing, so that all D151 variations (D151G/E/N) were not detected in the original clinical specimens (Table 1). We thus concluded that D151 variants might not have emerged as a natural occurrence and all recent ORVs would retain sensitivity to zanamivir.

Susceptibility to M2 inhibitors was determined to find established-resistant markers by M2-sequencing. The 367

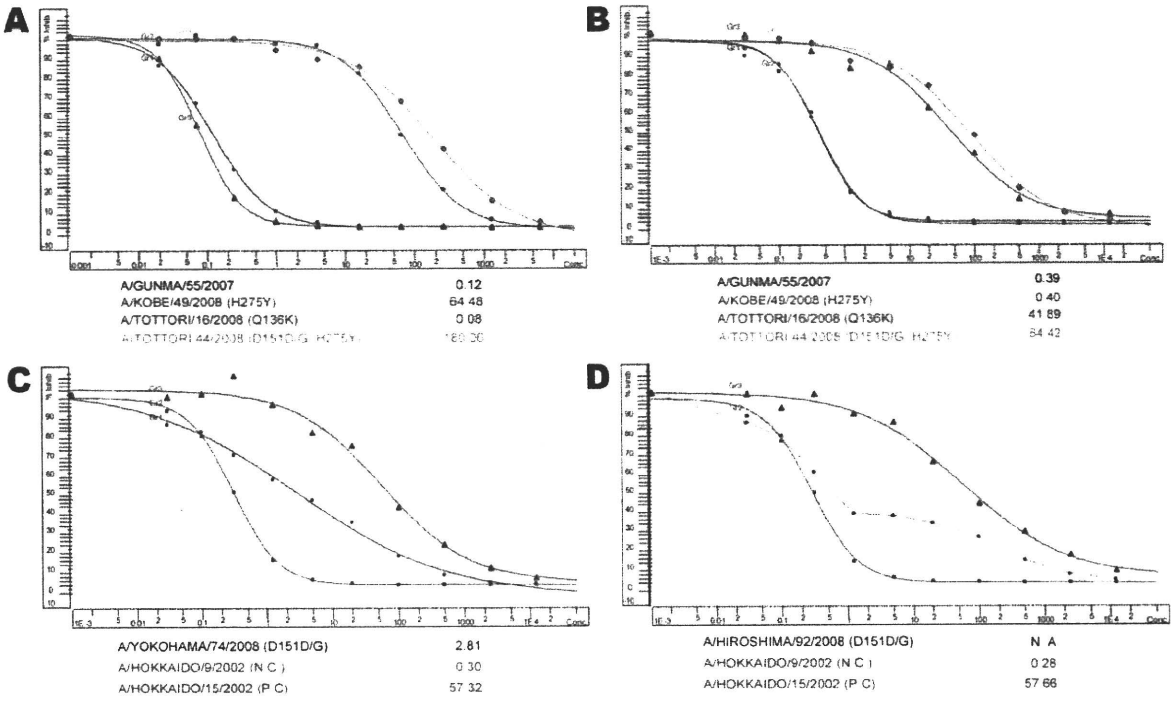

Figure 3. Assessment of drug concentrations required to inhibit neuraminidase activity by 50% (IC_{50}) for neuramindase inhibitors (NAIs). Normal sigmoid curves were generated for most tested viruses by a neuraminidase inhibition assay for oseltamivir (A) and zanamivir (B). Sensitive A/Gunma/55/2007 (blue), oseltamivir-resistant A/Kobe/49/2008 (red) with H275Y, zanamivir-resistant A/Tottori/16/2008 (green) with Q136K, and oseltamivir/zanamivir-resistant A/Tottori/44/2008 (orange) with H275Y and D151D/G are shown. Unusual sigmoid curves were sometimes generated by the mixed population of NAI-resistant and -sensitive viruses for zanamivir: A/Yokohama/74/2008 with D151D/G (C, type A curve); and A/Hiroshima/92/2008 with D151D/G (D, type B curve). Tentative IC_{50} values (nM), shown below each panel, were obtained from type A curves but not from type B curves. NA, not available; NC, negative control; PC, positive control.

viruses (288 isolates in the 2007–08 season; 79 isolates in the 2008–09 season) including 123 ORVs (45 and 78, respectively) and 244 OSVs (243 and 1, respectively) were tested. Viruses belonging to clade 2B were sensitive to M2 inhibitors, and viruses belonging to clade 2C were resistant to M2 inhibitors, so all ORVs except A/Yokohama/91/2007 were sensitive to M2 inhibitors. A/Yokohama/91/2007 belonged to clade 2C and was the only virus resistant to both oseltamivir and M2 inhibitors.

Antigenic Characteristics

To estimate the reactivity of ORVs and OSVs to ferret antiserum against 2008–09 vaccine strain A/Brisbane/59/2009, the HI test was performed. Good inhibition was achieved in 76% of OSVs and 69% of ORVs by A/Brisbane/59/2009 ferret antiserum, and 22% of OSVs and 28% of ORVs showed a 4-fold reduction in HI titer to the homologous titer, respectively (Table 2). Only 2% and 3% of OSVs and ORVs showed a >8-fold reduction in HI titer to A/Brisbane/59/2009 ferret antiserum. These data demonstrated that OSVs and ORVs were anitigenitically indistinguishable from each other and were similar to the 2008–09 vaccine strain A/Brisbane/59/2009.

High Frequency of ORVs in Tottori Prefecture during the 2007–08 Season

Tottori Prefecture is located in the western part of the main island of Japan. Comprising 19 cities and geographically divided into 3 areas, this prefecture has the lowest population in Japan (Figure 4, panel B). Despite a low frequency of only 2.6% in Japan during 2007–08 season, an unexpectedly high frequency (32.4%) of ORVs was observed in Tottori prefecture (Figure 2, panel A). ORVs from Tottori were collected from 4 cities in 2 areas with no systematic bias apparent in the sampling process (Figure 4, panel B).

Phylogenetic analysis of NA genes showed that these ORVs formed 3 subclades (Figure 4, panel A): the first with a Northern-Eu lineage sharing V75A and D354G (T-1); the second with a Hawaii lineage without common changes (T-2); and the third with a Hawaii lineage and sharing M188L (T-3). Among these, only OSVs genetically close

to ORVs were observed in T-2, suggesting that ORVs in T-2 would be derived from OSVs in Tottori prefecture.

A mapping study for ORVs showed that all ORVs in the Hawaii lineage were collected from Tottori city only, primarily at the end of January, whereas ORVs with the Northern-Eu lineage were collected from 4 cities, including Tottori city, during February and March. Genetically diverse ORVs belonging to T1-T3 were cocirculating only in Tottori city in the eastern area (Figure 4, panel B). The Tottori case raised concern about the possibility that these Tottori ORVs could survive to become an origin ORV for the 2008–09 season in Japan. However, phylogenetic analysis showed that all ORVs isolated during the 2008–09 season were not genetically close to ORVs from Tottori (online Appendix Figure). As a result, all ORVs from Tottori seemed to have been eliminated in the 2007–08 season, and ORVs that may have been introduced from other counties were circulating during 2008–09 in Japan.

Discussion

Our study demonstrated that ORVs dramatically increased in Japan from the 2007–08 season (2.6%) to the 2008–09 season (99.7%). All tested ORVs showed a reduction of >260-fold in susceptibility to oseltamivir by NA inhibition assay. On the other hand, almost all ORVs remained sensitive to the other antiviral-drugs, e.g., zanamivir, and M2 inhibitors. HI testing suggested that the current vaccine, A/Brisbane/59/2008, would be effective against recent ORVs. In addition, recent studies have reported that symptoms and hospitalization rates of patients infected with ORVs are no different from those seen with OSVs (*14*,*20*).

Table 1. Influenza virus A (H1N1) outliers to oseltamivir and/or zanamivir, Japan*

Strain	Sequence change(s)			Clinical specimen	Curve fit‡	IC_{50}, nmol/L	
	D151	H275	Q136	D151		Oseltamivir	Zanamivir
Oseltamivir outlier							
A/YAMAGATA/28/2008	D/E†	H	Q	NA	Normal	0.55	0.60
Zanamivir outlier candidates							
A/TOTTORI/16/2008	D	H	K	NA	Normal	0.08	41.89
A/TOTTORI/60/2008	D	Y	Q	NA	Normal	113.86	3.64
A/KOBE/31/2008	D	Y	Q	NA	Type A	26.05	2.75
A/KOBE/32/2008	D	Y	Q	NA	Type A	135.85	3.56
A/MIE/13/2008	D/G	H	Q	D	Type A	0.18	14.80
A/YOKOHAMA/75/2007	D/G	H	Q	NA	Type A	0.13	6.53
A/HAMAMATSU/33/2008	D/G	H	Q	NA	Type A	0.13	6.15
A/TOCHIGI/30/2008	D/G	H	Q	NA	Type A	0.13	4.32
A/YOKOHAMA/74/2007	D/G	H	Q	NA	Type A	0.12	2.81
A/HIROSHIMA/92/2007	D/G	H	Q	NA	Type B	0.07	NA
A/MIE/9/2008	D/G	H	Q	D	Type B	0.08	NA
A/MIE/1/2008	D/G	H	Q	D	Type B	0.16	NA
A/MIE/14/2008	D/G	H	Q	D	Type B	0.08	NA
A/YAMAGATA/60/2008	D/G	H	Q	NA	Type B	0.19	NA
A/SAPPORO/64/2008	D/G	Y	Q	NA	Type B	147.90	NA
A/TOTTORI/44/2008	D/G	Y	Q	NA	Normal§	180.06	84.42
A/HIROSHIMA/44/2008	D/N	Y	Q	NA	Type A	239.23	2.26
A/YOKOHAMA/79/2008	D/N	Y	Q	NA	Type A	167.66	2.28
A/HIROSHIMA/46/2008	D/N	Y	Q	NA	Type A	190.35	2.40
A/HIROSHIMA/45/2008	D/N	Y	Q	NA	Type A	169.92	3.34
A/MIE/1/2009	D/N	Y	Q	NA	Type A	231.78	3.55
A/HIROSHIMA/47/2008	D/N	Y	Q	NA	Type A	106.19	4.24
A/YOKOHAMA/96/2008	D/V	Y	Q	NA	Type B	126.50	NA
Zanamivir sensitive							
A/MIE/18/2008	D/E¶	H	Q	D	Normal	0.35	1.06
A/MIE/21/2008	D/N	H	Q	D	Normal	0.22	1.18
IC_{50} mean of sensitive viruses						0.10 ± 0.05	0.40 ± 0.26
Cutoff IC_{50} values (UQ +5.0 IQR)						0.40	1.99

*IC_{50}, drug concentrations required to inhibit neuraminidase activity by 50%; UQ, upper quartile; IQR, interquartile range; NA, not available. Oseltamivir-resistant viruses with H275Y were excluded from overall population in statistical analysis of oseltamivir.
†Mixture of D151 and D151 variants.
‡Curve fit patterns were evaluated based on Figure 3, panels C (Type A) and D (Type B). Although the viruses with D151D/G tend to generate both patterns from repeat testing for the same samples, type B was selected in this case.
§Although A/TOTTORI/44/2008 showed mixed population of D151D/G, it tended to show a normal curve fit (Figure 3, panel B).
¶The IC_{50} values of most viruses with D151D/E tend to be higher than mean IC_{50} values but do not exceed the cutoff value.

Table 2. Antigenic characterization of oseltamivir-resistant and oseltamivir-sensitive influenza virus A (H1N1), Japan, 2007–2009

Antiserum	Low to homologous titer, -fold*	No. (%) sensitive, n = 169	No. (%) resistant, n = 77
A/Brisbane/59/2007	<2	128 (76)	53 (69)
	4	36 (22)	22 (28)
	>8	3 (2)	2 (3)

*Viruses with >8-fold reduced hemagglutinin inhibition titer to homologous titer were regarded as an antigenic variant.

Japan has the largest per capita use of oseltamivir (>70%) in the world (*10*). Because this use could cause efficient selection of ORVs in individual patients, Japan might be the initial site of worldwide spread of ORVs. However, long-term NAI surveillance in Japan during 1996–2007 and recent surveillance showed a low frequency of NAI-resistant viruses for any strains and subtypes (*10,21,22*), suggesting that transmissibility of ORVs selected by drug pressure was remarkably decreased. In addition, previous NAI surveillance (*5–10*) and several animal studies (*23–26*) also suggested that NAI-resistant viruses would become defective viruses with attenuated infectivity and transmissibility to human. In contrast, despite little NAI use, a high emergence of ORVs has been detected in several countries in Europe since November 2007. These ORVs had as efficient transmissibility as OSVs in human-to-human transmission, resulting in worldwide spread in a short period of time. Although whether the initial ORV detected in Norway in the 2007–08 season appeared because of NAI drug pressure is unknown, those ORVs may have obtained amino acid changes on NA and/or other proteins to compensate for the defect, in addition to the H275Y substitution on the NA protein. Most ORVs belong genetically to the Northern-Eu lineage in clade 2B, suggesting that the gene constellation may contain a big advantage to retain infectivity and transmissibility.

An interesting question arose as to where the ORVs in Japan originated. In the Hawaii lineage, almost all ORVs in Japan would be derived from OSVs in Japan because the NA gene of ORVs was similar to OSV counterparts isolated at similar times or from similar regions (online Appendix Figure). On the other hand, in the Northern-Eu lineage, ORVs in Japan would have been introduced from other countries. In 2007–08, almost all ORVs would be imported from countries in Europe. In 2008–09, the ORVs in C-1, which comprised most isolates in 2008–09, and ORVs in C-4 were genetically similar to ORVs isolated from the Southern Hemisphere. Because influenza activity in the Southern Hemisphere occurs half a year earlier than that in the Northern Hemisphere, most ORVs in Japan conceivably could have been imported from the Southern Hemisphere. ORVs in C-2 and C-3 were genetically similar to ORVs isolated in North America and Hawaii, but the collection month of ORVs in C-3 were similar to each other, suggesting that ORVs in C-3 might be derived from an unknown common origin ORV. The ORVs obtained during 2008–09 were not genetically similar to any ORVs isolated in Tottori during 2007–08, indicating that ORVs from Tottori had been eliminated and had not formed the origin ORVs for the 2008–09 season in Japan. As for A/Yokohama/91/2007 belonging to clade 2C, the patient from which this virus was isolated was known to have taken oseltamivir before sampling (*22*), indicating that selective drug pressure in this person might have selected for this ORV.

In the NA inhibition assay for zanamivir, some viruses, including ORVs and OSVs, showed reduced sensitivity to zanamivir. NA sequencing of these viruses showed 2 types of amino acid changes. One virus, A/Tottori/16/2008 (OSV), possessed a Q136K substitution, which reportedly confers resistance to zanamivir (*27,28*). Conversely, most of the other viruses possessed D151 G/V/N. The amino acid changes D151 to N or E among subtype H1N1 viruses and to A, G, E, N, or V among H3N2 have been reported (*7,8,19*), and viruses with D151 substitutions often exhibit reduced sensitivity to NAIs (*8,19,29*). However, a recent study reported a possible role for cell culture in selecting these D151 variant viruses (*19*). In the present study, D151 variations (D151G/E/N) also were not detected from available original clinical specimens (Table 1), supporting the previous finding. We thus concluded that viruses with D151 variations would not have emerged naturally, and all ORVs would remain sensitive to zanamivir.

By sequencing of M2 gene, we confirmed that almost all Japanese ORVs belonging to clade 2B retained sensitive genotype to M2 inhibitors, consistent with previously reports that recent clade 2B viruses are sensitive to M2 inhibitors, but clade 2C viruses are resistant (*27*).

During the 2007–09 seasons, we also addressed NAI surveillance for A/H3N2 and type B circulating in Japan and identified no viruses resistant to both NAIs. Conversely, in March and early April 2009, a new swine-origin influenza virus A (H1N1) (now known as pandemic [H1N1] 2009 virus) emerged in Mexico and the United States and spread rapidly to many countries, including Japan (*30–33*). In June 2009, detection of pandemic (H1N1) 2009 virus with H275Y on the NA protein was reported from Denmark, Hong Kong Special Administrative Region, People's Republic of China, and Japan, but all ORVs of pandemic (H1N1) 2009 virus emerged as sporadic cases with no evidence of efficient human-to-human transmission (*34*). Although oseltamivir remains a valuable drug for treatment of pandemic (H1N1) 2009, many ORVs were isolated after prophylaxis with a half dose of the drug. Therefore, prophylaxis with oseltamivir may not be recommended as

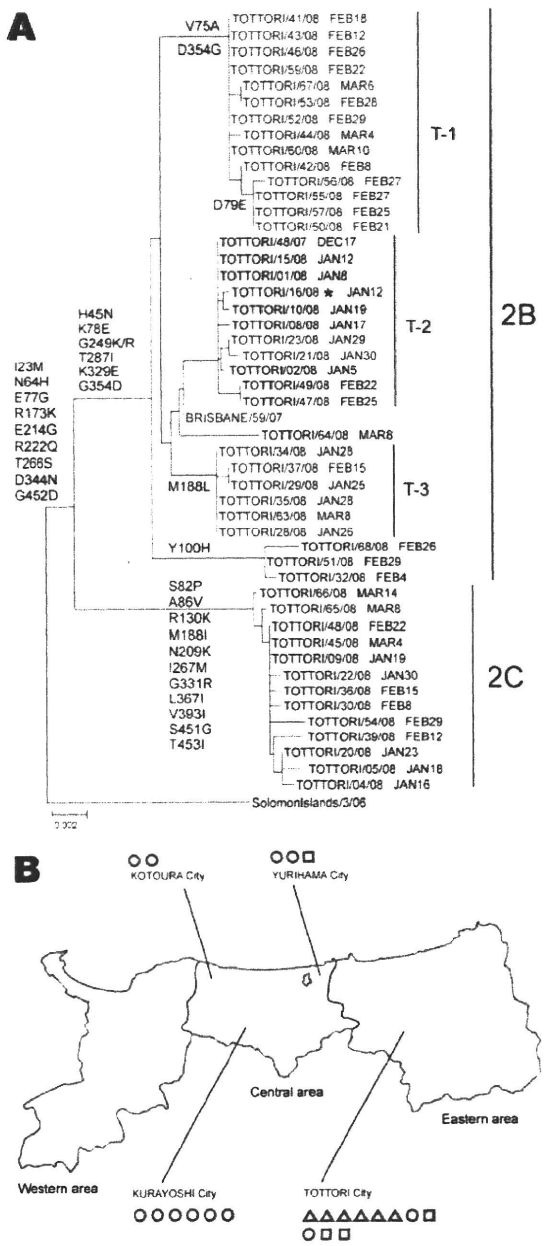

Figure 4. Phylogenetic analysis of influenza A(H1N1) neuraminidase genes (A) and geographic distribution of oseltamivir-resistant viruses (ORVs) (B) isolated from Tottori Prefecture, Japan, 2007–08. ORVs fell into either Northern-Eu lineage (red) or Hawaii lineage (blue); Tottori ORVs and current vaccine strains are indicated by black and purple, respectively. A) ORVs formed 3 subclades: T-1, sharing V75A and D354G; T-2, without common changes; and T-3, sharing M188L. Sampling dates are given after each strain name. Scale bars indicates nucleotide substitutions per site. B) Tottori Prefecture is geographically divided into 3 areas, comprising 19 cities. ORVs from Tottori were collected from 4 cities over 2 areas. The sampling month for each ORV is indicated by a triangle (January), circle (February), or square (March). *Zanamivir resistant.

stated by WHO (*35*). Rapid and continuous monitoring of NAI-resistant viruses, including pandemic (H1N1) 2009 virus, and dissemination of the findings in timely manner remains essential.

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