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### III. 研究成果の刊行物・別刷



# Intranasal vaccination with an inactivated whole influenza virus vaccine induces strong antibody responses in serum and nasal mucus of healthy adults

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**Keywords:** influenza virus, intranasal vaccination, neutralizing antibody, haemagglutination-inhibiting antibody, healthy adult volunteer

**Abbreviations:** S-IgA, secretory IgA; HA, haemagglutinin; NT titer, neutralization titer; HI assay, haemagglutination inhibition assay; HI titer, haemagglutination inhibition titer; HI antibody response, haemagglutination-inhibiting antibody response; GMT, geometric mean titer; ELISA, enzyme-linked immunosorbent assay

Haemagglutination inhibition (HI) and neutralization (NT) titers as well as haemagglutinin (HA) specific antibody responses were examined in 50 healthy adults aged between 22 and 69 y old after two intranasal administrations of an inactivated whole virus vaccine derived from A/Victoria/210/2009 virus (45 µg HA per dose) at 3 week intervals. Serum HI titers after two-doses of the nasal vaccine showed >2.5-fold rise in the ratio of geometric mean titer upon vaccination, >40% of subjects with a ≥4-fold increase in titer and >70% of subjects with a titer of ≥1:40, all parameters associated with an effective outcome of vaccination in the criteria defined by the European Medicines Agency. Serum neutralizing antibody responses correlated with HI antibody responses, although NT titers were about 2-fold higher than HI titers. These high levels of serum responses were accompanied by high levels of HI and neutralizing antibody responses in nasal mucus as measured in concentrated nasal wash samples that were about 10 times diluted compared with natural nasal mucus. Serum and nasal HI and neutralizing antibody responses consisted of HA-specific IgG and IgA antibody responses, with IgG and IgA antibodies being dominant in serum and nasal responses, respectively.

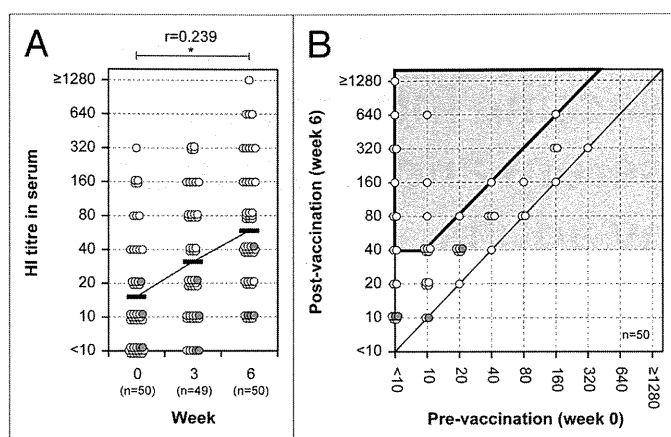
## Introduction

Currently available inactivated vaccines, usually whole virus vaccines or sub-virion vaccines, such as detergent-disrupted split-viruses or purified surface glycoprotein vaccines, are injected via the non-mucosal route.<sup>1</sup> These vaccines induce serum IgG antibodies, which are highly protective against homologous virus infections but less effective against heterologous virus infections. However, it has been shown that secretory IgA (S-IgA) and IgG antibodies in the respiratory tract largely contribute to the protective immunity induced by influenza virus infection.<sup>2,3</sup> Moreover, S-IgA antibodies are more cross-reactive against variant influenza viruses than serum IgG antibodies and therefore provide more effective protection against a heterologous virus.<sup>4–9</sup> Thus, intranasal administration of an inactivated influenza vaccine that induces both S-IgA and IgG antibody responses is expected to outperform the protective efficacy of intramuscular or subcutaneous vaccines.<sup>10</sup> Of note, intranasal vaccination would have

several additional advantages, since this type of vaccination is needle-free which enables easier administration, is more readily accepted by the recipients, reduces the problems associated with needle waste and prevents the risk of disease transmission through needle reuse.<sup>11</sup>

Several trials have been conducted to augment the induction of both S-IgA and IgG antibodies using intranasal administration of an inactivated influenza vaccine, either with or without an extrinsic adjuvant.<sup>12–21</sup> In several clinical trials, antibody responses were mainly evaluated based on haemagglutination inhibition (HI) titers of the serum and haemagglutinin (HA)-specific IgA and IgG antibody titers estimated by enzyme-linked immunosorbent assay (ELISA) in nasal wash samples. Currently, serum HI titers are used for the evaluation of the efficacy of the seasonal influenza vaccines,<sup>22–24</sup> because levels of protection against viruses that are homologous to the vaccine strain, correlate well with these serum HI titers.<sup>25</sup> However, both serum and nasal antibody responses are involved in protection,<sup>26,27</sup> and together

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**Figure 1.** HI antibody responses in serum. (A) HI antibody responses before and after primary and secondary vaccination are shown for serum. A paired t test was performed to compare data from week 0 (pre) and 6 (post). Correlation coefficient ( $r$ ) and  $p$  value were calculated. \*,  $p < 0.05$ . (B) The relationship of serum HI antibody responses before and after secondary vaccination. The abscissa and ordinate show the pre- and post-vaccination HI titers, respectively. Further, it is shown how these relate to conversion rate and protection rate, which are on the border or within the area marked by the bold line and by the light gray background, respectively. Each circle represents an individual and shows the relation between the pre- and post-vaccination titers. Gray circles indicate subjects between 60 and 69 y-of-age.

might result in better correlates for protection against heterologous influenza strains. Therefore, it would be useful to measure HI titers in both serum and nasal mucus in humans. In addition, although neutralizing capacity is considered to be a more functional criterion for protection than HI or HA-specific binding, neutralizing antibody responses in nasal wash samples have rarely been assessed. Previous studies show that HI titers may be lower or higher than the corresponding neutralization (NT) titers, depending on the strain of influenza A or B virus used.<sup>28</sup> Other studies show that HI assays using anti-sera failed to detect the H5N1 virus.<sup>29,30</sup> Thus, the efficacy of antibody responses following nasal vaccination should preferentially be assessed by characterizing the HI and NT titers in serum and nasal mucus.

In a previous study, it was shown that neutralizing antibody responses in both serum and nasal mucus were induced in five healthy adults after intranasal administration of a split-virus vaccine derived from A/Uruguay/716/2007 (H3N2) virus (45  $\mu$ g HA per dose).<sup>31</sup> Neutralizing antibody titers were measured in nasal wash samples, which typically contain about 1/10 the amount of IgA antibody found in natural nasal mucus.<sup>31,32</sup> Virus-specific neutralizing antibody responses were detected in nasal mucus samples from 4 out of 5 subjects, with a rise in NT titer of  $\geq 4$ -fold after the second vaccination.<sup>31</sup> Nasal mucus NT titers appeared to reflect the absolute titers of nasal mucus antibodies and these titers were not affected by the slight variability in the recovery of total antibodies from nasal mucus of different subjects.

Inactivated influenza whole virus vaccines are more immunogenic than split-product vaccines when administered intranasally to mice.<sup>33,34</sup> Similar results were found for humans in

clinical trials showing that intranasally administered whole virus vaccines cause enhanced production of both local HA-specific IgA antibodies and serum HI antibodies.<sup>14-16</sup> The higher immunogenicity of the whole virus vaccine may be explained by the adjuvant action of single-stranded viral RNAs that activate toll-like receptor 7. Viral RNA is present in the inactivated virus particles, but is absent in split-product vaccine formulations.<sup>35-38</sup> Together, these reports suggest that inactivated whole virus vaccines can induce more effective immune responses than split-virus vaccines after intranasal vaccination in healthy adults.

In the present study HI and neutralizing antibody responses were examined in serum and nasal mucus samples from 50 healthy adults after two intranasal vaccinations with an inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus (A/Victoria vaccine; 45  $\mu$ g HA per dose) with a 3-week interval. Antibody responses in nasal mucus were assayed using concentrated nasal wash (containing 1 mg/ml total protein).<sup>31,32</sup> HA-specific IgA and IgG antibodies were also examined to characterize the immune response. It was found that two doses of the nasal vaccine induced high levels of HI and neutralizing antibody responses in both serum and nasal mucus. These responses were accompanied by major changes in HA-specific serum IgG and nasal IgA antibody responses, respectively. In addition, the vaccination with the A/Victoria vaccine resulted in a slight increase in HI and NT titers, which were cross-reactive to the A/Sydney/05/1997 (H3N2) virus.

## Results

**HI and neutralizing antibody responses in serum.** HI antibody responses, currently in use as a correlate of protection for the evaluation of vaccine efficacy, were examined in serum samples from the volunteers aged between 22 and 69 y old who received two intranasal vaccinations with an inactivated whole virus vaccine derived from A/Victoria virus strain (containing 45  $\mu$ g HA) with a 3-week interval. Serum samples were obtained both before vaccination (week 0) and 3 weeks after primary (week 3) and secondary vaccination (week 6). HI titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (Fig. 1A). The serum HI titers were evaluated using the mean geometric increase between week 0 and 6, the conversion rate as well as the protection rate, which are also used by the EMA and the FDA to review the efficacy of seasonal influenza vaccines (Table 1). After two doses of the intranasal vaccination (week 6), serum HI titers fulfilled the criteria of the mean geometric increase (4.25-fold) and the conversion rate (43.5%), as defined by the EMA for the vaccine efficacy in people aged 18–60 y. In addition, serum samples also reached protective levels (76.1%), which were defined as the protection rate by the EMA (Fig. 1B and Table 2). The subjects over 60 y old produced only low levels of HI titers. These results show that serum HI titers induced by administration of two doses of the nasal A/Victoria vaccine exceeded three of the EMA criteria in people aged 18–60 y.

**Table 1.** Tools currently in use to evaluate vaccine-induced changes in serum HI titer

Name	Description	EMA criteria <sup>a</sup>	FDA criteria <sup>b</sup>
Mean geometric increase	Ratio of the geometric mean titer (GMT) post vaccination to that pre-vaccination	>2.5	No standard
Conversion rate or significant increase in titer [n/N (%; 95% CI)] <sup>c</sup>	Conversion rate: Proportion of subjects showing an increase from a pre-vaccination titer of < 1:10 (non-immune) to a post-vaccination titer of ≥ 1:40 (designated immune state). Significant increase in titer: Proportion of subjects showing a 4-fold or greater increase from a pre-vaccination titer of ≠ 1:10 (designated as a significant increase in titer).	>40%	LL of 95% CI > 40% <sup>d</sup>
Protection rate [n/N (%; 95% CI)] <sup>c</sup>	Proportion of subjects showing a post-vaccination titer of ≠ 1:40	>70%	LL of 95% CI > 70% <sup>e</sup>

<sup>a</sup>European Medicines Agency (EMA) criteria for serum HI antibody responses in people aged 18–60 y. <sup>b</sup>US Food and Drug Administration (FDA) criteria for serum HI antibody responses in adults < 65 y. <sup>c</sup>n = the number of subjects who meet a requirement; n = total number of subjects; 95% CI = the 95% Confidence Interval. <sup>d</sup>Lower limit (LL) of the 95% CI should exceed 40%. <sup>e</sup>Lower limit (LL) of the 95% CI should exceed 70%.

**Table 2.** Serum HI antibody responses after two doses of the nasal A/Victoria vaccine

	EMA criteria	FDA criteria	Week	Serum <sup>a</sup>
GMT (mean geometric increase)	>2.5	No standard	0	16.2 (1.00)
			6	<b>68.8 (4.25<sup>b</sup>)</b>
Conversion rate or significant increase in titer [n/N (%; 95% CI)]	>40%	LL of 95% CI > 40%	6	20/46 ( <b>43.5</b> , 28.5–58.4)
Protection rate [n/N (%; 95% CI)]	>70%	LL of 95% CI > 70%	0	13/46 (28.3, 14.7–41.8)
			6	35/46 ( <b>76.1</b> , 63.2–88.9)

<sup>a</sup>Serum HI titers of < 1:10 were considered negative and were arbitrarily assigned a titer of 1:5. <sup>b</sup>In bold values that exceed EMA criteria as defined for HI titers.

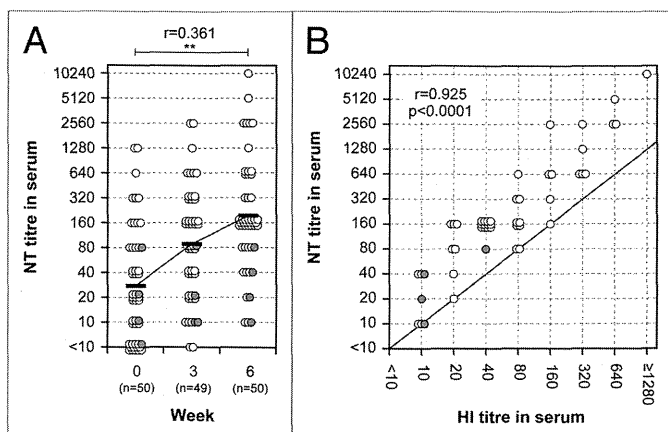
In addition, neutralizing antibody responses were also examined in the serum samples, since those responses are considered to be more functional in the protection against influenza viruses than HI antibody responses. NT titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (Fig. 2A). Serum HI titers correlated strongly with serum NT titers ( $r = 0.925$ ,  $p < 0.0001$ ). Among 46 volunteers below 60 y of age, the ratio of GMTs between week 0 and 6 (the mean geometric increase after two doses of the nasal A/Victoria vaccine) in serum NT titers was 8.00 (Fig. 2A and Table 3), whereas this ratio in serum HI titers was 4.25 (Table 2). These results show that the increase in NT titers is about 2-fold higher than the increase in HI titers (Fig. 2B).

These data suggest that a titer of 1:80 for neutralizing antibodies would correspond to a titer of 1:40 for HI antibodies, which is defined as the minimal HI titer providing protection. Using an NT titer of 1:80 temporarily as the lower limit of protection, the enhanced sensitivity of neutralization assay could be corrected so as to tentatively estimate the vaccine efficacy. As shown in Table 3, serum NT titers at week 6 showed a 63.0% conversion rate, and an 87.0% protection rate. These results roughly correspond to those obtained in the serum HI titers (Table 2), suggesting that NT titers after correcting for the enhanced sensitivity of the NT assay could be used as an indicator to evaluate protective efficacy of the vaccines.

**HI and neutralizing antibody responses in nasal mucus.** HI and neutralizing antibody responses were also examined in nasal mucus samples from the volunteers who received two intranasal vaccinations with the inactivated vaccine. Nasal mucus samples

(containing 1 mg/ml total protein), prepared by concentrating nasal wash samples so that the total amount of IgA was equivalent to about 1/10 of that in natural nasal mucus, were obtained both before vaccination (week 0) and 3 weeks after primary (week 3) and secondary vaccination (week 6). HI and NT titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (Fig. 3A and B). Among 46 volunteers below 60 y of age, the ratio of GMTs between week 0 and 6 were 3.13 and 5.88 for HI (Fig. 3A and Table 4) and NT (Fig. 3B and Table 4) titers, respectively. These results show that NT titers in nasal mucus samples are about 2-fold higher than the HI titers (Fig. 3C), similar to the relationship between HI and NT titers in serum samples (Fig. 2B). Thus, the volunteers who received two doses of intranasal inactivated vaccine induced high levels of HI and neutralizing antibody responses both in nasal mucus and serum.

**HA-specific IgA and IgG ELISA antibody responses in serum and nasal mucus.** The HA-specific IgA and IgG responses in serum from subjects before and after two intranasal vaccinations with the A/Victoria vaccine were determined by ELISA (Table 5). Serum HA-specific IgG and IgA titers were obtained using serum samples starting from a 1:10 dilution. The mean geometric increase of IgG and IgA antibodies after two doses of nasal vaccine (week 6) was estimated to be 2.96-fold and 2.47-fold, respectively (Fig. S1A and B; Table 5). The GMTs of IgG responses in serum samples were higher than those of IgA responses at each time point. This indicates that HA-specific IgG, rather than IgA, is likely to be the major isotype responsible for haemagglutination inhibiting and neutralizing activity in serum samples.



**Figure 2.** Neutralizing antibody responses and correlation between HI and NT titers in serum. (A) Neutralizing antibody responses before and after primary and secondary vaccination are shown. A paired t test was performed to compare data from week 0 (pre) and 6 (post). The correlation coefficient ( $r$ ) and  $p$  value were calculated.  $**$ ;  $p < 0.01$ . (B) Correlation between HI and NT titers in serum 3 weeks after the secondary nasal vaccination. The abscissa and ordinate show HI and NT titers, respectively. Pearson  $r$  value and  $p$  value were calculated. Each individual is represented by a circle showing corresponding HI and NT titers. Gray circles indicate subjects between 60 and 69 y-of-age.

The HA-specific IgA and IgG responses from subjects before and after the two intranasal vaccinations were also determined in nasal mucus (Table 5). HA-specific IgG and IgA titers in nasal mucus were assayed using concentrated nasal wash samples (containing 1 mg/ml total protein) which were measured by ELISA starting with a 1:160 dilution. The GMTs of HA-specific IgA responses were higher than those of HA-specific IgG responses at each time point. The mean geometric increase of IgA and IgG antibody after two doses of nasal vaccine (week 6) was estimated to be 3.88-fold and 1.37-fold, respectively (Fig. S1C and D; Table 5). This indicates that HA-specific IgA antibody is most likely the predominant antibody isotype responsible for the haemagglutination inhibiting and neutralizing activity in the nasal mucus. Thus, predominant changes in HA-specific IgA and IgG titers were found in the nasal mucus and serum, respectively.

In addition, relationships between NT titer and HA-specific antibody titer in serum or nasal wash were evaluated. In serum, NT titers correlated well with HA-specific IgG titers ( $r = 0.778$ ,  $p < 0.0001$ ), but not with IgA titers (Fig. S2A and B). Nasal NT titers show a weak correlation with HA-specific IgA titers ( $r = 0.473$ ,  $p < 0.001$ ), but not IgG titers (Fig. S2C and D).

**HI and neutralizing antibody responses show cross-reactivity with the A/Sydney/05/1997 virus.** Cross-reactivity of HI and neutralizing antibody responses that were induced upon vaccination with A/Victoria (H3N2) with the A/Sydney/05/1997 (A/Sydney, H3N2) virus was examined in serum (Table 6). Before vaccination, moderate HI and very high neutralizing antibody responses, were found to be cross-reactive to A/Sydney virus (week 0, GMTs in Table 6), when compared with responses to the homologous A/Victoria virus (Table 2 and 3). The mean geometric increase in the cross-reactive HI and NT titers were

very similar and showed a 1.44-fold and a 1.46-fold, respectively. This increase was lower than that directed against the homologous A/Victoria virus (Tables 2, 3, and 6).

The cross-reactive HI and neutralizing antibody responses in nasal mucus samples were also examined using concentrated nasal wash samples. They showed a 1.63-fold and a 2.12 mean geometric increase, respectively. Similar to the responses in serum, this increase was lower than that directed against the homologous A/Victoria virus (Tables 4 and 6).

**Clinical observation for adverse reactions.** Before and after each vaccination clinical data were compiled from the health check records and personal interviews. None of the subjects experienced systemic adverse effects after the nasal vaccination. Minor complaints included light local reactions (mainly runny nose and nasal congestion) that resolved spontaneously within a few days (data not shown). Thus, the intranasal vaccine was considered to be well tolerated.

## Discussion

The present study examined HI antibodies as well as neutralizing antibodies in serum and nasal mucus of 50 healthy adults who received two intranasal doses of an inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus (45  $\mu$ g HA per dose) with an interval of 3 weeks. The two doses of nasal vaccine induced serum HI titers, demonstrated by a  $>4$ -fold mean geometric increase, a  $>40\%$  conversion rate or significant increase in titer, and a  $>70\%$  protection rate in subjects aged 18–60 y (Table 2 and Fig. 1). When the efficacy of vaccination was evaluated using the serum HI antibody responses observed in the present experiments, the serum HI titers exceeded three of the criteria used by the EMA, however, they did not meet the FDA criteria. These results suggest that two intranasal administrations of inactivated whole virus vaccine (45  $\mu$ g HA per dose) could be a candidate treatment regimen. This vaccination procedure is simple, safe and effective, at least for adults with some immunological memory induced by previous infection or vaccination.

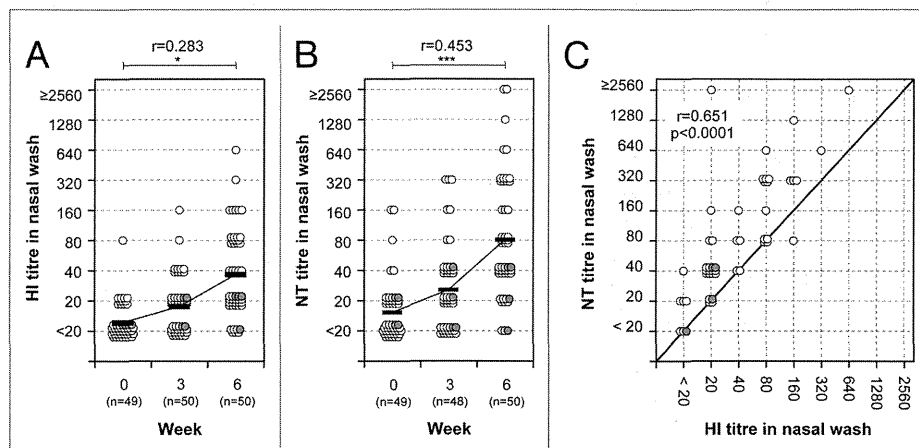
The intranasal A/Victoria vaccination also induced high levels of serum neutralizing antibody responses, measured by the microneutralization assay, of which the sensitivity was about 2-fold higher than that of the HI assay (Table 2 and Fig. 2). When the enhanced sensitivity of the NT assays was corrected in such a way that it could be compared with the antibody response obtained by the HI assays, the neutralizing antibody responses at week 6 showed a mean geometric increase, a conversion rate, and a protection rate which corresponded to those obtained in the serum HI antibody responses in subjects aged 18–60 y (Tables 2 and 3). These results suggest that serum NT titers could be used as an indicator to evaluate protective efficacy of the vaccines.

The present study also examined HI, NT and HA-specific antibody titers in nasal mucus samples, prepared by concentrating nasal wash samples resulting in a total amount of IgA that was equivalent to about 1/10 of that in undiluted nasal mucus. In our previous study, the total protein level and the levels of IgA, IgG and IgM and human serum albumin before and after

**Table 3.** Serum neutralizing antibody responses after two doses of the nasal vaccine

	Time after first vaccination (week)	Serum <sup>a</sup>
GMT (mean geometric increase)	0	28.7 (1.00)
	6	229.7 (8.00)
Conversion rate or significant increase in titer [n/N (% , 95% CI)] <sup>b</sup>	6	29/46 (63.0, 48.5–77.6)
Protection rate [n/N (% , 95% CI)] <sup>b</sup>	0	14/46 (30.4, 16.6–44.3)
	6	40/46 (87.0, 76.8–97.1)

<sup>a</sup>Serum NT titers of < 1:10 were considered negative and were arbitrarily assigned a titer of 1:5. <sup>b</sup>Conversion rate and protection rate were tentatively estimated by correcting for the relative ratio between GMT of NT titers and that of HI titers; a serum NT titer of 1:80 was considered to be a 1:40 of the lowest protection level in HI titers.



**Figure 3.** HI and neutralizing antibody responses in nasal mucus. HI (A) and NT (B) titers before and after primary and secondary vaccination. A paired t test was performed to compare data from week 0 (pre) and 6 (post). The correlation coefficient (r) and p value were calculated. \*, p < 0.05, \*\*\*, p < 0.001. (C) Correlation between HI and NT titers in nasal wash 3 weeks after the secondary nasal vaccination. The abscissa and ordinate show HI and NT titers, respectively. Pearson r value and p value were calculated. Each circle represents an individual showing corresponding HI and NT titers. Gray circles indicate subjects between 60 and 69 y-of-age.

concentration of nasal wash samples from several participants were examined.<sup>31</sup> About 70% of the total nasal wash proteins, 67% of IgA and 26% of IgG were lost during the concentration processes. These decreases might be caused by degradation by proteolytic enzymes or the aggregation of immunoglobulin complexes with other materials in the process of concentration. The amount of total IgA and total IgG recovered from each participant varied slightly at each sampling time; however, the average amount was comparable and constant. This means that the concentrated nasal material is sufficiently comparable within different isolations to be used to express relative antibody responses in the nasal wash. Under the described experimental conditions, allowing for small variations in the recovery of total IgA and IgG from the nasal mucus, the specific antibody titers in the nasal wash samples could be considered to be suitable to compare absolute antibody titers in the nasal mucus before and after vaccination.

The two doses of intranasal vaccine induced both nasal HI and NT titers, demonstrated by a 3.13-fold and 5.88-fold mean geometric increase (the ratio of GMTs between week 0 and 6 after the nasal A/Victoria vaccine), respectively (Fig. 3 and Table 4).

In addition, the nasal vaccine induced HA-specific IgA and IgG antibodies in nasal mucus, demonstrated by a 3.88-fold and 1.37-fold mean geometric increase, respectively (Table 5). Thus, the nasal inactivated vaccine induced high levels of nasal HI and neutralizing antibody responses with dominant HA-specific IgA antibody responses, in parallel with high levels of serum HI and neutralizing antibody responses dominated by HA-specific IgG antibody responses (Table 5; Fig. S2). It is currently unknown how long these antibody responses are maintained in nasal mucus. Nasal HA-specific IgA and IgG antibody responses induced by live attenuated influenza A virus vaccine were shown to persist for at least 12 mo after inoculation in children who had not previously been infected by influenza A virus.<sup>2</sup> However, the duration of antibody responses in nasal mucus induced by intranasal vaccination with an inactivated whole virus vaccine in healthy adults remains to be examined.

Regarding antibody responses, Clements et al. compared the correlation between antibody responses and the degree of protection in adults who received a live attenuated intranasal vaccine followed by a challenge with wild-type influenza virus with those in adults who received an inactivated parenteral vaccine prior to

**Table 4.** Nasal HI and neutralizing antibody responses after two doses of the nasal vaccine

	Week	HI titers <sup>a</sup>	NT titers <sup>a</sup>
GMT (Mean geometric increase)	0	12.4 (1.00)	15.4 (1.00)
	6	38.8 (3.13)	90.5 (5.88)

<sup>a</sup>Nasal HI and neutralization titers were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HI and neutralization titers of < 1:20 were considered negative and were arbitrarily assigned a titer of 1:10.

**Table 5.** Serum and nasal HA-specific antibody responses after two doses of the nasal vaccine

	Isotype	Week	Serum	Nasal wash
GMT (Mean geometric increase)	IgG	0	83.7 (1.00)	80.0 (1.00)
		6	247.7 (2.96)	109.8 (1.37)
	IgA	0	14.6 (1.00)	122.0 (1.00)
		6	36.0 (2.47)	473.5 (3.88)

<sup>a</sup>Serum HA-specific antibody titers of <1:10 were considered negative and arbitrarily assigned a titer of 1:5. <sup>b</sup>Nasal HA-specific antibody responses were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HA-specific IgG and IgA titers of <1:160 were considered negative and arbitrarily assigned a titer of 1:80.

**Table 6.** Cross-reactive HI and neutralizing antibody responses to A/Sydney virus after two doses of the A/Victoria vaccine

Item	Antibody responses	Week	Serum <sup>a</sup>	Nasal wash <sup>b</sup>
GMT (Mean geometric increase)	HI antibody	0	21.2 (1.00)	13.6 (1.00)
		6	30.5 (1.44)	22.2 (1.63)
	Neutralizing antibody	0	160.0 (1.00)	23.0 (1.00)
		6	233.2 (1.46)	48.7 (2.12)

<sup>a</sup>Serum HI and neutralization titers of <1:10 were considered negative and arbitrarily assigned a titer of 1:5. <sup>b</sup>Nasal wash HI and neutralization titers were determined using concentrated nasal wash samples containing 1 mg/ml total protein. Nasal HI and neutralization titers of <1:20 were considered negative and arbitrarily assigned a titer of 1:10.

challenge.<sup>39,40</sup> They found that serum HI titers correlated with protection against viral replication after parenteral vaccination, but not after intranasal vaccination. In contrast, intranasal vaccination induced nasal HA-specific IgA antibodies that correlated with the degree of protection. It was shown that in the upper respiratory tract the majority of the protective immunity induced by influenza virus infection is mainly due to S-IgA antibodies.<sup>2,3</sup> In this study, the nasal inactivated vaccine induced high levels of HI, NT and HA-specific IgA antibody responses in nasal mucus (Fig. 3, Table 4, and 5). Although several reports show a good correlation between HA-specific antibody titers and HI or NT titers in serum,<sup>41,42</sup> HA-specific antibody responses seem

to be more sensitive than microneutralization assay in this study (Fig. S2A and D). In addition, NT titers were well correlated with HI titers in both serum and nasal wash (Fig. 2B and 3C). Among three antibody measurements (HI, microneutralization and HA-specific antibody ELISA), ELISA may be the best choice for achieving sensitivity, but microneutralization has the advantage of representing not only antibody responses against HA like HI and ELISA, but also those against neuraminidase (NA), since it was already shown that NA-specific antibody can contribute partially to the virus neutralization.<sup>43</sup> Therefore, neutralizing antibody might be more useful for the evaluation of protective antibody response.<sup>10</sup> Together with the fact that at present there are no guidelines available for the evaluation of antibodies in nasal mucus, we chose to focus on the neutralizing antibodies. In the present study, HI, neutralizing and HA-specific antibody titers in nasal mucus, as well as in serum, showed synchronous changes in many of the subjects following nasal vaccination, although the degree of the respective responses and the type of dominant neutralizing antibody varied slightly from subject to subject (data not shown). In addition, antibody responses decreased with age; subjects aged > 60 y produced only low levels of HI and neutralizing antibodies (Figs. 1, 2, and 3). Thus, as reported previously, the magnitude of the antibody response in each subject appeared to change depending on parameters that affect the immune responses of the host, such as pre-vaccination antibody levels, sex and age.<sup>34,44</sup>

HI and neutralizing antibodies cross-reactive with the A/Sydney virus were detected in pre-vaccination serum and nasal wash, and were enhanced after two doses of the nasal A/Victoria vaccine, although the mean geometric increase in the cross-reactive antibody responses increased only about 1.5- to 2-fold (Table 6). These results suggest that cross-protection against infection with different strains of influenza virus could be enhanced by an intranasal inactivated whole virus vaccine. Since this field is relatively unexplored yet, it will be interesting to find to which extent this cross-protection can be enhanced by optimizing intranasal vaccination.

The present study first demonstrated that intranasal vaccination with an inactivated whole virus vaccine alone (45 µg HA per dose) could induce serum HI antibody responses which exceeded the EMA criteria for serum HI antibody titers as well as serum neutralizing antibody responses, and that high levels of nasal HI and neutralizing antibody responses were detected using concentrated nasal wash samples. Thus, at least among adults with some immunological memory induced by previous infection or vaccination, the intranasal inactivated whole virus vaccine could be a promising candidate for a needle-free mucosal vaccine. Antibody responses induced by intranasal vaccination in individuals who are naïve to influenza virus antigens remain to be determined. In addition, further studies which compare the HI and NT antibody responses between groups treated with intranasal and existing non-mucosal vaccines are needed to gain more insight in the possible benefits of intranasal vaccination. Some clinical trials have already shown that trivalent inactivated whole virus vaccines induce significantly higher vaccine-specific IgA antibody responses in intranasally immunized subjects than in

intramuscularly-immunized elderly subjects, with no significant differences in serum HI antibody responses between the groups.<sup>16</sup> A drawback for analyses of nasal antibody responses is that there are currently no criteria to evaluate these type of responses. In this study it is shown that the measurement of nasal HI and NT titers can provide important data in addition to the serum antibody titers. Improved knowledge on these different types of antibody responses in different types of samples will be useful to increase the understanding of immune responses to both vaccination and infection and will be of help for defining the criteria for evaluation of responses in nasal mucus. In order to optimize the accuracy of the data acquired from nasal mucus, the techniques for recovery of nasal antibodies from nasal wash samples might need further improvement.

In conclusion, high levels of serum HI antibody responses, which exceeded all criteria used by the EMA for the evaluation of vaccine efficacy, could be induced by intranasal administration of an inactivated whole A/Victoria virus vaccine containing 45 µg HA in healthy adults. The serum antibody responses were accompanied by high levels of HI, NT and HA-specific IgA antibodies in nasal mucus. The results show that the intranasal vaccination induces both high levels of serum and nasal antibody responses that may be involved in mounting effective protective responses, including cross-protection, against both upper and lower respiratory tract infection by various influenza viruses. The inactivated whole virus vaccine therefore appears to be a promising candidate for intranasal vaccination.

## Materials and Methods

**Subjects.** The study subjects comprised 50 healthy volunteers aged between 22 and 69 y (average 36.5 ± 12.8 y); 14 (28%) were female and four subjects (8%) were over 60 y of age. None of the subjects had to be excluded due a history of allergy to eggs, past or current neurological conditions, or respiratory illness or fever at the time of vaccination. Based on the answers of the volunteers on questions regarding their history of influenza infection and vaccination during the past 5 y, almost all subjects were considered to have acquired at least some degree of immunity to influenza viruses. Written informed consent was obtained from each subject before the onset of the trial. The protocol and other relevant study documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

**Viruses and vaccines.** Influenza viruses, A/Victoria/210/2009 (H3N2; A/Victoria) and A/Sydney/05/1997 (H3N2; A/Sydney) strains, were obtained from the National Institute of Infectious Diseases (Tokyo, Japan), propagated in the allantoic cavity of 10-d-old embryonated hen's eggs, and purified from the allantoic fluid. The TCID<sub>50</sub> (50% infectious dose in tissue culture) of the virus was estimated using a previously described method.<sup>45,46</sup> In brief, 10-fold serial dilutions of allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney cells (MDCK: ATCC No. CCL-34) in a 96-well culture plate and incubated for 3 d at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The cytopathic effect observed in the virus-containing wells was evaluated using

a microscope and the TCID<sub>50</sub> was calculated using the Reed-Muench method.<sup>47</sup> An inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus, containing 45 µg HA per dose, was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Kagawa, Japan). The vaccine was prepared from the purified viruses, which were sedimented through a linear sucrose gradient and treated with formalin by the method of Davenport et al.<sup>48</sup>

**Vaccinations and adverse effects.** Intranasal vaccination with an inactivated whole virus vaccine (45 µg HA/dose) was performed twice, with a 3-week interval (week 0 and 3), by spraying 0.25 ml of vaccine into each nostril (0.5 ml total) using an atomizer (Keytron; Ichikawa, Chiba, Japan). The mean droplet diameter of the mist generated by the atomizer was 56.5 µm (range: 10 µm to 90 µm). Blood and nasal wash samples were taken from each of the subjects prior to vaccination and three weeks after each vaccination (week 0, 3, and 6).

A health check sheet was given to each subject to record any symptoms after the nasal vaccination as well as their answers to questions regarding their medical history. Medical examinations were held every 3 weeks after the primary and secondary vaccinations to assess their medical condition. Adverse effects were evaluated in terms of local reactions (discomfort, pain in the nose, sneezing, stuffiness and/or running nose, throat pain, or cough) along with systemic reactions, such as malaise, headache, fever and abdominal pain, by means of health check sheets and personal interviews.

**Nasal wash specimens.** About 80 ml of nasal wash was collected by washing the nasal cavity several times with a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) according to the manufacturer's instructions.<sup>31</sup> The collected nasal wash samples were filtered using bottle top filters (Nalgene Nunc International, Chiba, Japan) with membranes covered with a cotton mat to remove mucopolysaccharides and other debris. The pooled cleaned nasal wash samples were then concentrated to a final volume of approximately 1 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO 30.000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash samples were stored at -80°C until use. The protein concentration in the concentrated nasal wash was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

**Neutralization and haemagglutination inhibition assays.** NT titers were examined using microneutralization assays as previously described with minor modifications.<sup>41,46</sup> Briefly, serum samples were treated with a receptor-destroying enzyme (RDE: Denka Seiken, Niigata, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C, and then diluted 1:10 before the assay. Nasal wash samples were adjusted to 1 mg/ml total protein, and the final samples contained about 1/10 of the total IgA found in nasal mucus (0.22 mg/ml; original concentration in nasal mucus is 2.20 mg/ml).<sup>31</sup> These standardized nasal wash samples were treated with RDE and heat-inactivated similar to the serum samples, and then diluted 1:20 for use in the assays.

2-fold serial dilutions of samples were mixed with an equal volume of diluent containing influenza virus equivalent to 100

TCID<sub>50</sub> and added to the wells of a 96-well plate containing a monolayer culture of MDCK cells. Four control wells containing virus or diluent alone were included on each plate. The plates were incubated for 3 or 4 d at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. All wells were observed for the presence or absence of cytopathic effects and then fixed with 10% formalin phosphate buffer for more than 5 min at room temperature and stained with Naphthol blue black. After washing and drying, cells were solubilized with 0.1 M NaOH and the absorbance (A) was read at 630 nm. The average A<sub>630</sub> value was determined from virus-only controls (A<sub>virus</sub>) and medium-only controls (A<sub>cell</sub>). All values above 50% of the specific signal, calculated using the formula  $X = (A_{\text{cell}} - A_{\text{virus}})/2 + A_{\text{virus}}$ , were considered positive for neutralization. The titers recorded were the reciprocal of the highest dilution, where A<sub>630</sub> was > X.

HI titers were examined using a microtitration method as previously described.<sup>49</sup> Serum and nasal wash samples were prepared in the same manner as for the neutralization assay and treated with packed red blood cells to remove non-specific haemagglutination-inhibiting materials. The starting dilutions for the HI assay were 1:10 and 1:20 for the serum and standardized nasal wash samples, respectively.

**Evaluation of serum HI titers.** Serum HI titers were evaluated using the following three parameters: the ratio of the geometric mean titer (GMT) in post-vaccination to that in pre-vaccination (the mean geometric increase); the percentage of subjects showing an increase from a pre-vaccination titer of < 1:10 to a post-vaccination titer of ≥ 1:40 (the conversion rate) or showing a ≥ 4-fold increase from a pre-vaccination titer of ≥ 1:10 (significant increase in titer); and the percentage of subjects with a post-vaccination titer of ≥ 1:40 (the protection rate). Hereafter, the conversion rate or significant increase in titer is indicated as conversion rate. As shown in Table 1, the parameters for serum HI titers after vaccination are currently used as criteria to evaluate the vaccine efficacy by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA).<sup>22-24</sup>

**Determination of HA-specific IgA and IgG antibody titers.** The titers of IgA and IgG antibodies specific for the HA molecule of the A/Victoria virus (HA-specific IgA titer and HA-specific IgG titer, respectively) in the serum and standardized nasal wash samples were determined by ELISA. The ELISA assay was performed in microtiter plates (Costar, Cambridge, MA) using the following procedure. First, wells of microtiter plates were coated with HA molecules purified from the A/Victoria virus according to the procedure of Phelan et al.<sup>50</sup> Second, the HA molecules were incubated with 2-fold serial dilutions of serum or standardized nasal wash samples followed by detection with

goat anti-human IgA (α-chain specific) or goat anti-human IgG (γ-chain specific) (BETHYL Laboratories, Montgomery, AL) antibodies conjugated to alkaline phosphatase. Third, the enzymatic reaction was started by adding 1 mg/ml of p-nitrophenyl-phosphate as the substrate. Color development was measured at 405 nm using a microplate reader (Model 680: Bio-Rad Laboratories, Hercules, CA). The antibody titer for a given sample was calculated as the reciprocal of the highest dilution of the test sample that gave an absorbance read at 405 nm (A<sub>405</sub>) greater than a cut-off value equal to the mean A<sub>405</sub> + 2SD of 11 2-fold serial dilutions (starting at 1:10 for serum and at 1:160 for nasal sample due to sample limitation) of the negative control samples (NT titer, < 10 × 2<sup>0</sup>; HI titer, < 10 × 2<sup>0</sup>; HA-specific antibody titer, < 10 × 2<sup>0</sup> for serum and < 10 × 2<sup>4</sup> for nasal wash) selected from the pre-vaccination serum and nasal wash samples of 50 subjects.

**Statistical analysis.** Statistical analysis was performed using the GraphPad Prism statistical software package (Version 5.0c: Graph Pad Software Inc., CA USA). The threshold of statistical significance was set at 5% (p < 0.05).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Materials

Supplemental materials may be found here:  
[www.landesbioscience.com/journals/vaccines/article/25458](http://www.landesbioscience.com/journals/vaccines/article/25458)

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# Pathological study of archival lung tissues from five fatal cases of avian H5N1 influenza in Vietnam

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Highly pathogenic avian H5N1 influenza virus (H5N1) infection in humans causes acute respiratory distress syndrome, leading to multiple organ failure. Five fatal cases of H5N1 infection in Vietnam were analyzed pathologically to reveal virus distribution, and local proinflammatory cytokine and chemokine expression profiles in formalin-fixed, paraffin-embedded lung tissues. Our main histopathological findings showed diffuse alveolar damage in the lungs. The infiltration of myeloperoxidase-positive and/or CD68 (clone KP-1)-positive neutrophils and monocytes/macrophages was remarkable in the alveolar septa and alveolar spaces. Immunohistochemistry revealed that H5N1 mainly infected alveolar epithelial cells and monocytes/macrophages in lungs. H5N1 replication was confirmed by detecting H5N1 mRNA in epithelial cells using *in situ* hybridization. Quantitation of H5N1 RNA using quantitative reverse transcription PCR assays revealed that the level of H5N1 RNA was increased in cases during early phases of the disease. We quantified the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, IL-8, regulated on activation normal T-cell expressed and secreted (commonly known as RANTES), and interferon- $\gamma$ -inducible protein of 10 kDa (IP-10) in formalin-fixed, paraffin-embedded lung sections. Their expression levels correlated with H5N1 RNA copy numbers detected in the same lung region. Double immunofluorescence staining revealed that TNF- $\alpha$ , IL-6, IL-8 and IP-10 were expressed in epithelial cells and/or monocytes/macrophages. In particular, IL-6 was also expressed in endothelial cells. The dissemination of H5N1 beyond respiratory organs was not confirmed in two cases examined in this study.

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Since the outbreak of highly pathogenic avian H5N1 influenza virus infection in humans in 2003, the WHO has reported 608 confirmed cases from 15 countries, with a mortality rate of about 60% by August 2012 ([http://www.who.int/influenza/human\\_animal\\_interface/EN\\_GIP\\_20120810CumulativeNumberH5N1cases.pdf](http://www.who.int/influenza/human_animal_interface/EN_GIP_20120810CumulativeNumberH5N1cases.pdf)). The potential for mutation

and reassortment of the viral genome, which may be responsible for human-to-human transmission, has increased the threat of an influenza pandemic.

Many H5N1-infected patients develop acute respiratory distress syndrome and die because of respiratory failure or multiple organ failure.<sup>1,2</sup> Pathological study of autopsied cases revealed that the H5N1 virus infected type I and type II pneumocytes and caused primary viral pneumonia, which further developed into acute respiratory distress syndrome.<sup>3–18</sup> Although seasonal influenza virus infection is also associated with pneumonia, the virus mainly infects epithelial cells of the upper respiratory tract.<sup>19–21</sup> Complications involving

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pneumonia are often due to bacterial infection. In the case of the influenza pandemic that occurred in 2009, the virus, designated A(H1N1)pdm09, infected pneumocytes in some patients. It also caused severe respiratory failure, similar to that seen for H5N1 infection, during the first year of the outbreak.<sup>22–26</sup> The analysis of 20 autopsied A(H1N1)pdm09 infection cases in Japan revealed that diffuse alveolar damage, similar to that seen in a case of H5N1 infection, was present in the lungs of 5 cases (25%).<sup>27</sup>

The pathological study of human tissues can be restricted because of the difficulty in obtaining samples, but it is crucial for elucidating pathogenesis of the disease. There have been only 16 reports of pathological studies carried out on fatal cases of H5N1 infection. These have been reported from Hong Kong,<sup>3–6</sup> Thailand,<sup>7–13</sup> China<sup>14–17</sup> and Vietnam.<sup>18</sup> Lungs infected with H5N1 presented with diffuse alveolar damage in all cases. In most cases, H5N1 antigens and RNA were detected in extrapulmonary organs, suggesting disseminated systemic H5N1 infection and viremia.

Nearly 77% (10/13 patients) of H5N1 infection cases have resulted in death at the National Hospital of Pediatrics Hanoi, since December 2003. In this study, post-mortem biopsied or autopsied tissues from five fatal cases were analyzed pathologically to reveal virus distribution and the expression levels of cytokines and chemokines in lungs. Immunohistochemistry, *in situ* hybridization, double immunofluorescence staining and quantitative reverse transcription PCR (qRT-PCR) methods were used in our studies.

## Materials and methods

### Patients and Formalin-Fixed, Paraffin-Embedded Tissues

H5N1 infection of the five cases was confirmed by detecting viral RNA in bronchiolar aspirate using RT-PCR. The numbering of the case was in order of the shortness of the disease duration. In three cases (cases 1, 4 and 5; Table 1), post-mortem biopsied lung tissues were examined; these cases have been reported previously in a short communication.<sup>18</sup> In the other two cases, patients were autopsied at National Hospital of Pediatrics; in addition to lung tissue, liver, heart, kidney, intestine, spleen and pancreas were examined in case 2. For case 3, liver, heart and kidney tissue were also examined in addition to lung tissue (Table 1). Only formalin-fixed, paraffin-embedded tissues were available for this study. This study was approved by the institutional medical ethical committee of the National Institute of Infectious Diseases, Japan (approval no. 320) and National Hospital of Pediatrics Hanoi.

### Histopathological Studies and Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were cut (3- $\mu$ m thick sections) and mounted on silane-coated glass slides. Histopathological studies were performed on all samples using hematoxylin–eosin (HE) staining. For lung sections, Elastica–Masson Goldner staining was also done. Immunohistochemistry for influenza A nucleoprotein antigen (InfA-NP) was performed to evaluate the distribution of H5N1 antigens with a mouse monoclonal antibody against InfA-NP<sup>28</sup> as previously described.<sup>18,23,27</sup>

Immunohistochemistry for cell type-specific marker proteins, cytokines and chemokines was performed using primary antibodies against the following proteins: myeloperoxidase (MPO; Nichirei Bioscience, Tokyo, Japan); neutrophil elastase (DAKO Cytomation, Copenhagen, Denmark); CD68 (KP-1 or PGM-1; DAKO); CD8 (Novocastra Laboratories, Newcastle, UK); tumor necrosis factor-alpha (TNF- $\alpha$ ; BD Pharmingen, San Diego, CA, USA); interleukin (IL)-6 (R&D Systems, Minneapolis, MN, USA); IL-8 (R&D Systems); regulated on activation normal T-cell expressed and secreted (RANTES; R&D Systems); and interferon-gamma-inducible protein of 10 kDa (IP-10; R&D Systems). After incubation with these primary antibodies, signals were detected using the avidin–biotin complex immunoperoxidase method (LSAB2, DAKO). A biotin-free catalyzed signal amplification system (CSAII, DAKO) was used for the detection of TNF- $\alpha$ , IL-6 and CD8. The EnVision system (DAKO) was used for the detection of neutrophil elastase. As a negative control, an irrelevant antibody was used in place of the primary antibody.

### In Situ Hybridization

Influenza virus genomic RNA and mRNA were detected in formalin-fixed, paraffin-embedded lung sections by *in situ* hybridization AT tailing combined with a catalyzed signal amplification method (ISH-AT) as described previously.<sup>29,30</sup> Anti-sense and sense ISH-AT probes against H5N1 nucleoprotein (NP) genes were prepared and used to detect H5N1 mRNA and genomic RNA. The anti-sense ISH-AT probe against the rabies virus NP gene was used as an irrelevant negative control (Table 2).

### Double Immunofluorescence Staining

Double immunofluorescence staining for InfA-NP, cytokines, chemokines or for cell type-specific marker proteins was performed. We used antibodies against the following marker proteins to determine cell type: MPO (DAKO); surfactant apoprotein D (SP-D; Chemicon, Temecula, CA, USA); CD68 (KP-1 or PGM-1; DAKO); cytokeratin AE1/AE3 (DAKO); epithelial membrane antigen (EMA; DAKO); and CD34 (Novocastra). AlexaFluor 488-conjugated anti-

**Table 1** Summary of the five H5N1 infection cases examined in this study

Case	Date of admission (month, year)	Age (year)/sex	Duration of illness (days)	Day of admission	Sample examined	Lung histology	Laboratory data (on admission)			Treatment			Detection of viral-RNA or antigen			
							WBC (/mm <sup>3</sup> )	Plt (/mm <sup>3</sup> )	CRP (mg/dl)	Antibiotics	Oseltamivir	MSL	qRT-PCR			
													Bronchial aspirate	FFPE lung copies per cell	IHC	ISH
1	December, 2003	12/F	8	Day 6	Lung	DAD (exudative phase)	2100	45 000	3.1	Yes	No	Yes	+	256	+	+
2	March, 2008	11/M	10	Day 7	Lung, liver, heart, kidney, spleen, intestine, pancreas	DAD (exudative phase)	1700	207 000	6.8	Yes	Yes	Yes	+	4.89	+	+
3	December, 2007	4/M	13	Day 11	Lung, liver, heart, kidney	DAD (exudative phase)	2300	154 000	2.4	Yes	Yes	Yes	+	23.1	+	+
4	July, 2004	4/M	16	Day 8	Lung	DAD (proliferation phase)	2300	150 000	0.59	Yes	Yes	Yes	+	1.05	—	—
5	December, 2003	5/M	18	Day 8	Lung	DAD (proliferation phase)	3400	174 000	0.59	Yes	No	Yes	+	UDL	—	—

Abbreviations: CRP, C-reactive protein; DAD, diffuse alveolar damage; FFPE, formalin-fixed, paraffin-embedded; IHC, immunohistochemistry; ISH, *in situ* hybridization; MSL, methylprednisolone(10 mg/kg/day); Plt, Platelet; qRT-PCR, quantitative real-time RT-PCR; UDL, under detection limit; WBC, white blood cell; +, positive; —, negative.