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CLINICAL IMMUNOLOGY

# Kinetics, Longevity, and Cross-Reactivity of Antineuraminidase Antibody after Natural Infection with Influenza A Viruses

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**ABSTRACT** The kinetics, longevity, and breadth of antibodies to influenza virus neuraminidase (NA) in archival, sequential serum/plasma samples from influenza A virus (IAV) H5N1 infection survivors and from patients infected with the 2009 pandemic IAV (H1N1) virus were determined using an enzyme-linked lectin-based assay. The reverse-genetics-derived H4N1 viruses harboring a hemagglutinin (HA) segment from A/duck/Shan Tou/461/2000 (H4N9) and an NA segment derived from either IAV H5N1 clade 1, IAV H5N1 clade 2.3.4, the 2009 pandemic IAV (H1N1) (H1N1pdm), or A/Puerto Rico/8/1934 (H1N1) virus were used as the test antigens. These serum/plasma samples were also investigated by microneutralization (MN) and/or hemagglutination inhibition (HI) assays. Neuraminidase-inhibiting (NI) antibodies against N1 NA of both homologous and heterologous viruses were observed in H5N1 survivors and H1N1pdm patients. H5N1 survivors who were never exposed to H1N1pdm virus developed NI antibodies to H1N1pdm NA. Seroconversion of NI antibodies was observed in 65% of the H1N1pdm patients at day 7 after disease onset, but an increase in titer was not observed in serum samples obtained late in infection. On the other hand, an increase in seroconversion rate with the HI assay was observed in the follow-up series of sera obtained on days 7, 14, 28, and 90 after infection. The study also showed that NI antibodies are broadly reactive, while MN and HI antibodies are more strain specific.

**KEYWORDS** H5N1 avian influenza virus, pandemic influenza A (H1N1) 2009 virus, neuraminidase, neuraminidase inhibition assay, reverse-genetics-derived virus, microneutralization assay, hemagglutination inhibition assay

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Influenza is a major public health problem worldwide. The disease is mainly caused by influenza type A and type B viruses, although influenza type A virus causes a more severe disease and is the only type that has caused pandemics in the past (1). Currently, influenza type A virus is further classified into 18 HA and 11 NA subtypes. Of these identified type A subtypes, only H1N1 and H3N2 are the major circulating viruses causing human influenza. There are 16 H and 9 N subtypes that cause infections in aquatic birds, and recently discovered H17N10 and H18N11 are bat influenza-like viruses (2–4). Many avian influenza viruses spread and cause large-scale outbreaks among domestic poultry and even cross the species barrier to infect humans (5). Among the avian viruses reported, the highly pathogenic avian influenza (HPAI) H5N1 virus is the most virulent. The first outbreak of H5N1 HPAI virus in humans was reported in Hong Kong in 1997 and had a mortality rate of about 30% (6). A new strain of H5N1

HPAI virus with higher virulence reemerged in late 2003. The virus spread globally and infected humans in 16 countries with a mortality rate of about 53% (7). Thailand reported a total of 25 human cases with 17 deaths between January 2004 and July 2006. The kinetics and longevity of the antibody response for some of these patients as determined by hemagglutination inhibition (HI), microneutralization (MN) (8), and indirect immunofluorescence (IF) assays (9) have been previously reported.

The antihemagglutinin (anti-HA) antibodies play a dominant role in protection against HA-matched influenza virus, while anti-NA antibodies may also be protective in the case of mismatched HA (10–16). HA antibodies block viral attachment to the target cell surface, and if the virus enters into the endolysosome, these antibodies also block viral uncoating and release of the nucleoprotein into cytoplasm. On the other hand, neuraminidase (NA)-inhibiting (NI) antibodies inhibit viral release, preventing viral spread from infected cells and viral invasion (17). To study the antibody responses to natural influenza infection or influenza vaccination, most investigators have employed HI and MN assays that are strain specific. However, to help improve vaccine potency, it is useful for anti-NA antibodies to be measured as well, although there are fewer reports of the antibody response against NA than against HA. This study therefore explored the kinetics and longevity of NI antibodies in archival serum samples from H5N1 infection survivors and H1N1pdm patients. Moreover, cross-reactive antibodies to N1 NA from heterologous viruses were also determined. To accomplish this aim, reverse-genetics-derived H4N1 viruses with N1 genomic segments from various origins (rgH4N1 viruses) were constructed and used as test antigens for NI antibody measurement in an enzyme-linked lectin assay (ELLA).

## RESULTS

**Detection of NI antibody in H5N1 infection survivors.** NI antibodies against rgH4N1 viruses carrying homologous NA from HPAI H5N1 A/Thailand/1(KAN-1)/04 (KAN-1 NA) or heterologous NA from A/Laos/Nong Khai 1/2007 (H5N1) (NK-1 NA), A/Thailand/104/2009 (H1N1) (pdm NA), or A/Puerto Rico/8/1934 (H1N1) (PR8 NA) were determined by ELLA in 26 sequential serum samples collected from 4 H5N1 survivors over the period 2005 to 2008. The results of MN and HI assays of these sera have been previously reported (8). This study found that 3 of the H5N1 survivors had mounted an NI antibody response against the homologous and all of the heterologous NAs used (Table 1). Survivor 1 developed a 64-fold increase in NI antibody titer in the serum samples collected at 2 years 3 months and 2 years 9 months after onset of disease, while the MN antibody titer was relatively stable in both serum samples. Survivor 2 had a high NI antibody titer as well as high MN and HI antibody titers in all serum samples tested. Survivor 3 had no MN antibody in the first blood sample collected at 10 days after onset of disease, while this sample contained high NI antibody titers against all of the NAs investigated, suggesting that these might be preexisting NI antibodies that developed in response to seasonal influenza A (H1N1) viruses. Survivor 4 did not develop NI antibodies at all, even though the patient produced high and persistent levels of MN and HI antibodies to KAN-1 virus over the 3 years of follow-up. This set of H5N1 sera demonstrated broad reactivity of NI antibodies across homologous and heterologous NAs belonging to the same NA subtype, while HA antibodies are more specific than NI antibodies (18).

**Detection of NI and HI antibodies in H1N1pdm patients.** Determination of NI antibodies against 3 rgH4N1 viruses with pdm NA, KAN-1 NA, or PR8 NA was carried out in sequential serum samples collected at days 0, 7, 14, 28, and 90 from 20 H1N1pdm patients. A 4-fold or greater rise in NI antibody titer to these rgH4N1 viruses was observed at day 7 (the seroconversion rate ranged from 55 to 65%), rose to a peak at day 14 or 28 (the seroconversion rate ranged from 60 to 65%), and then declined as observed at day 90 (Fig. 1A, B, and C and Table 2). The levels of NI antibodies against rgH4N1 viruses with pdm NA (homologous NA) were significantly higher than those against KAN-1 NA and PR8 NA (heterologous NA) (analysis of variance [ANOVA], $P < 0.05$) (Fig. 1D).

**TABLE 1** MN, HI, and NI antibody titers in H5N1 survivors

| Subject no. (gender/age in yr) | Time of collection after disease onset | Antibody titer to KAN-1 virus by:[a] | | NI antibody titer to rgH4N1 carrying: | | | |
|---|---|---|---|---|---|---|---|
| | | MN assay | HI assay | KAN-1 NA | NK-1 NA | pdm NA | PR8 NA |
| 1 (male/2) | 2 yr 3 mo | 80 | 80 | 80 | 40 | 40 | <10 |
| | 2 yr 9 mo | 160 | 80 | 5,120 | 1,280 | 2,560 | 320 |
| | 3 yr 3 mo | 80 | 80 | 1,280 | 640 | 1,280 | 160 |
| | 3 yr 11 mo | 80 | 80 | 640 | 320 | 640 | 80 |
| | 4 yr 5 mo | 40 | 40 | 640 | 320 | 640 | 80 |
| | 4 yr 11 mo | 40 | 40 | 640 | 320 | 640 | 40 |
| 2 (male/29) | 2 yr 2 mo | 160 | 80 | 640 | 160 | 160 | 40 |
| | 2 yr 8 mo | 160 | 80 | 640 | 160 | 160 | 40 |
| | 3 yr 2 mo | 160 | 80 | 640 | 160 | 160 | 40 |
| | 3 yr 10 mo | 160 | 80 | 640 | 160 | 160 | 40 |
| | 4 yr 3 mo | 80 | 80 | 320 | 80 | 160 | 40 |
| | 4 yr 10 mo | 80 | 80 | 640 | 160 | 160 | 40 |
| 3 (female/32) | 10 days | <5 | 20 | 2,560 | 640 | 640 | 640 |
| | 1 yr 6 mo | 160 | 80 | 320 | 640 | 320 | 1,280 |
| | 2 yr | 160 | 80 | 320 | 320 | 320 | 1,280 |
| | 2 yr 6 mo | 160 | 80 | 320 | 640 | 320 | 1,280 |
| | 3 yr 3 mo | 80 | 80 | 320 | 320 | 320 | 1,280 |
| | 3 yr 8 mo | 80 | 80 | 320 | 320 | 320 | 1,280 |
| | 4 yr 2 mo | 80 | 80 | 320 | 320 | 160 | 1,280 |
| 4 (male/7) | 20 days | 640 | 160 | <10 | <10 | <10 | <10 |
| | 5 mo | 80 | 80 | <10 | <10 | <10 | <10 |
| | 11 mo | 80 | 80 | 10 | 10 | 10 | <10 |
| | 1 yr 5 mo | 40 | 80 | <10 | <10 | <10 | <10 |
| | 2 yr 2 mo | 40 | 40 | <10 | <10 | <10 | <10 |
| | 2 yr 7 mo | 40 | 40 | <10 | <10 | <10 | <10 |
| | 3 yr 1 mo | 40 | 40 | <10 | <10 | <10 | <10 |

[a]Previously reported by Kitphati et al. (8).

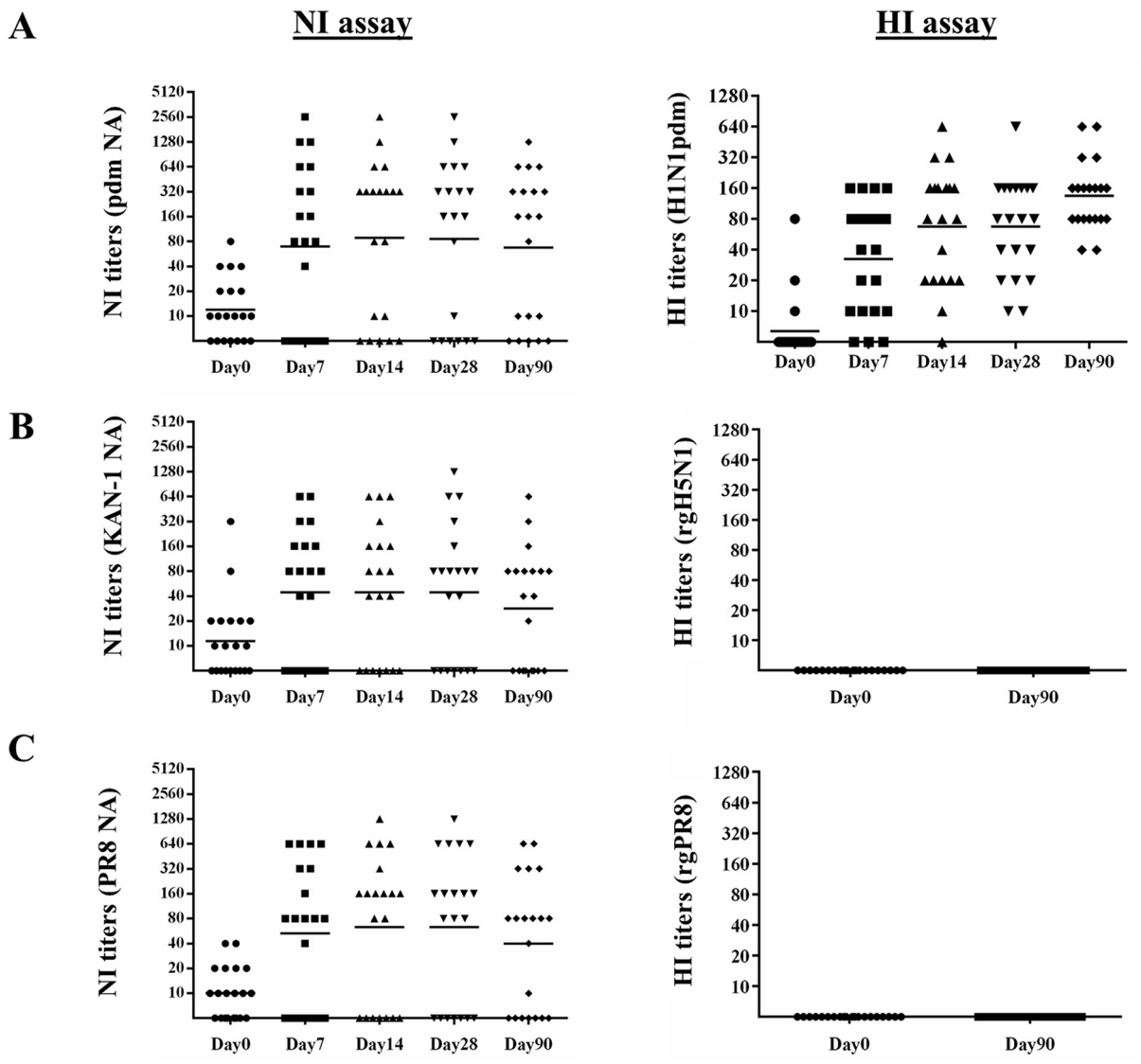
The geometric mean titers (GMTs) and 95% confidence interval of NI antibodies against the rgH4N1 viruses are shown in Fig. 1D.

The results of the NI antibody assays were different from those of HI assays in that a gradual increase in HI antibody titers was observed over time. The seroconversion rate of HI antibodies to wild-type H1N1pdm virus increased from 50 to 65 to 75 and to 100% at days 7, 14, 28, and 90, respectively (Fig. 1A and Table 2). However, no HI antibody titers against rgH5N1 and rgPR8 viruses were observed in any of the serum samples tested (Fig. 1B and C). Taken together, the results show that HI antibodies were strain specific, while NI antibody broadly reacted across homologous and heterologous NA belonging to the N1 subtype. Nevertheless, homologous NA yielded significantly higher NI antibody titers than the heterologous NA.

**Conservation of amino acid residues involved in the NI antibody assay.** Amino acid sequences of various influenza viruses belonging to subtype N1 were aligned for residues in the NA catalytic site as follows, using N1 numbering (with N2 numbering shown in parentheses), as previously reported (19): R118, D151, R152, R225 (224), E277 (276), R293 (292), R368 (371), and Y402 (406); for the framework, the residues were E119, R156, W179 (178), S180 (179), D199 (198), I223 (222), E228 (227), H275 (274), E278 (277), and E425. An analysis shows that the catalytic site and framework of all NAs belonging to subtype N1 are 100% identical. These residues did not change over time as observed with N1 NA of the influenza virus strains circulating in 1934, 1999, 2004, 2006, and 2009 (Fig. 2). In other words, the catalytic site and framework of the NA proteins of influenza viruses belonging to the same NA subtype are extremely conserved.

## DISCUSSION

Several lines of direct and indirect evidence support an important role for anti-NA antibodies in protecting against influenza. Levels of NI antibodies induced by virus-




| Day after onset | GMT (95% CI) to | | |
|---|---|---|---|
| | pdm NA | KAN-1 NA | PR8 NA |
| Day 0 | 11.9 (8-18) | 11.5 (7-19) | 10.0 (7-14) |
| Day 7 | 69.6 (24-198) | 44.4 (19-103) | 52.8 (21-131) |
| Day 14 | 88.8 (32-244) | 44.4 (19-105) | 62.8 (24-162) |
| Day 28 | 85.7 (31-240) | 44.4 (18-107) | 62.8 (24-164) |
| Day 90 | 67.3 (26-176) | 28.3 (13-60) | 40.0 (17-94) |

**FIG 1** (A to C) Kinetics and longevity of HI and NI antibodies in 20 H1N1pdm patients against rgH4N1 viruses with pdm NA (A), KAN-1 NA (B), and PR8 NA (C). (D) Geometric mean titers (GMT) of NI antibody against rgH4N1 with pdm NA, KAN-1 NA, and PR8 NA are significantly different (ANOVA, $P < 0.05$); CI, confidence interval.

**TABLE 2** Seroconversion rates by HI versus NI antibody assays in H1N1pdm patients at various time points

| Assay and test virus | No. (%) of serum samples with seroconversion ($n$ = 20) on day: | | | |
|---|---|---|---|---|
| | 7 | 14 | 28 | 90 |
| HI | | | | |
| A/Thailand/104/2009 (H1N1) | 10 (50) | 13 (65) | 15 (75) | 20 (100) |
| rgH5N1 | | | | 0 |
| rgPR8 | | | | 0 |
| NI | | | | |
| rgH4N1 (pdm NA) | 13 (65) | 13 (65) | 13 (65) | 12 (60) |
| rgH4N1 (KAN-1 NA) | 11 (55) | 11 (55) | 12 (60) | 9 (45) |
| rgH4N1 (PR8 NA) | 12 (60) | 13 (65) | 13 (65) | 12 (60) |

like-particles (VLPs) containing H1N1pdm NA were found to correlate with protection against H1N1pdm virus infection (20). Evidence that anti-NA antibodies might exert protective immunity had been presented in our previous study, which showed that antisera from mice immunized with recombinant vaccinia virus carrying an NA gene insert derived from an H1N1pdm virus could inhibit H1N1pdm NA enzymatic activity as well as reduce plaque formation and virus replication (21).

On the surface of the influenza virion, the level of HA molecules is 4 to 5 times higher than that of NA molecules. Binding of anti-HA antibodies to the HA antigen on the virion surface may cause steric hindrance, which prevents anti-NA antibodies from getting access to the NA antigen in ELLA for detection of NI antibodies. To avoid this interference effect, the rg-viruses carrying the HA genomic segment of nonhuman influenza virus were constructed and used as the test antigen in the NI antibody assay. This study chose Shan Tou virus from duck as the H4 HA gene donor, while the HA6 HA gene was chosen to construct the rg-viruses used as the test antigen in the other studies (22, 23). Nevertheless, our rgH4N1 viruses with the NA genomic segment derived from KAN-1 (H5N1 clade 1), NK-1 (H5N1 clade 2.3.4), H1N1pdm, or PR8 virus grew well in MDCK cells.

The present study showed that all sequential serum samples from all 4 influenza A virus (IAV) H5N1 survivors who were infected with H5N1 clade 1 virus developed MN and HI antibodies to KAN-1 virus, while 3 (75%) of them were positive for NI antibodies against all rg-viruses with NA from KAN-1, NK-1, H1N1pdm, or PR8 virus. The result showed that IAV H5N1 survivors had anti-NI antibodies that reacted with PR8 NA and pdm NA, even though they had never been exposed to these two viruses, suggesting that NI antibodies are broadly reactive. However, it cannot be excluded that the cross-reactivity could be due to previous infections by other seasonal influenza viruses. Subject 3 had a cross-reactive NI antibody against rgH4N1 PR8 NA in high titers, which could be a result of recent infection with a seasonal influenza A H1N1 virus. When the patient got infected with H5N1 virus, the antibody response to viral NA was boosted and resulted in a high titer of NI antibody. Interestingly, one survivor did not mount an anti-NA antibody response to any of the rg-viruses investigated. Cross-reactive NI antibodies to H1N1pdm virus have been detected in individuals who were immunized with a live-attenuated seasonal influenza vaccine (24). Cross-reactivity between NI antibodies against H1N1pdm and H5N1 viruses has also been reported (21, 25–30). NI antibodies to seasonal influenza H1N1 virus provided immunity to mice against H5N1 virus challenge (12). Moreover, immunity mounted in mice immunized with seasonal influenza virus H1N1 strains from the 2006 and 2007 seasons contributed to protection against H1N1pdm virus challenge (31). This suggests that at the time of an influenza outbreak, preexisting NA antibodies might contribute to partial protection in the absence of homologous HA antibodies (13). NA antibodies might directly exert protective activity through binding with the catalytic site or framework of NA and then block the NA enzymatic activity and the progeny viral release. On the other hand, it

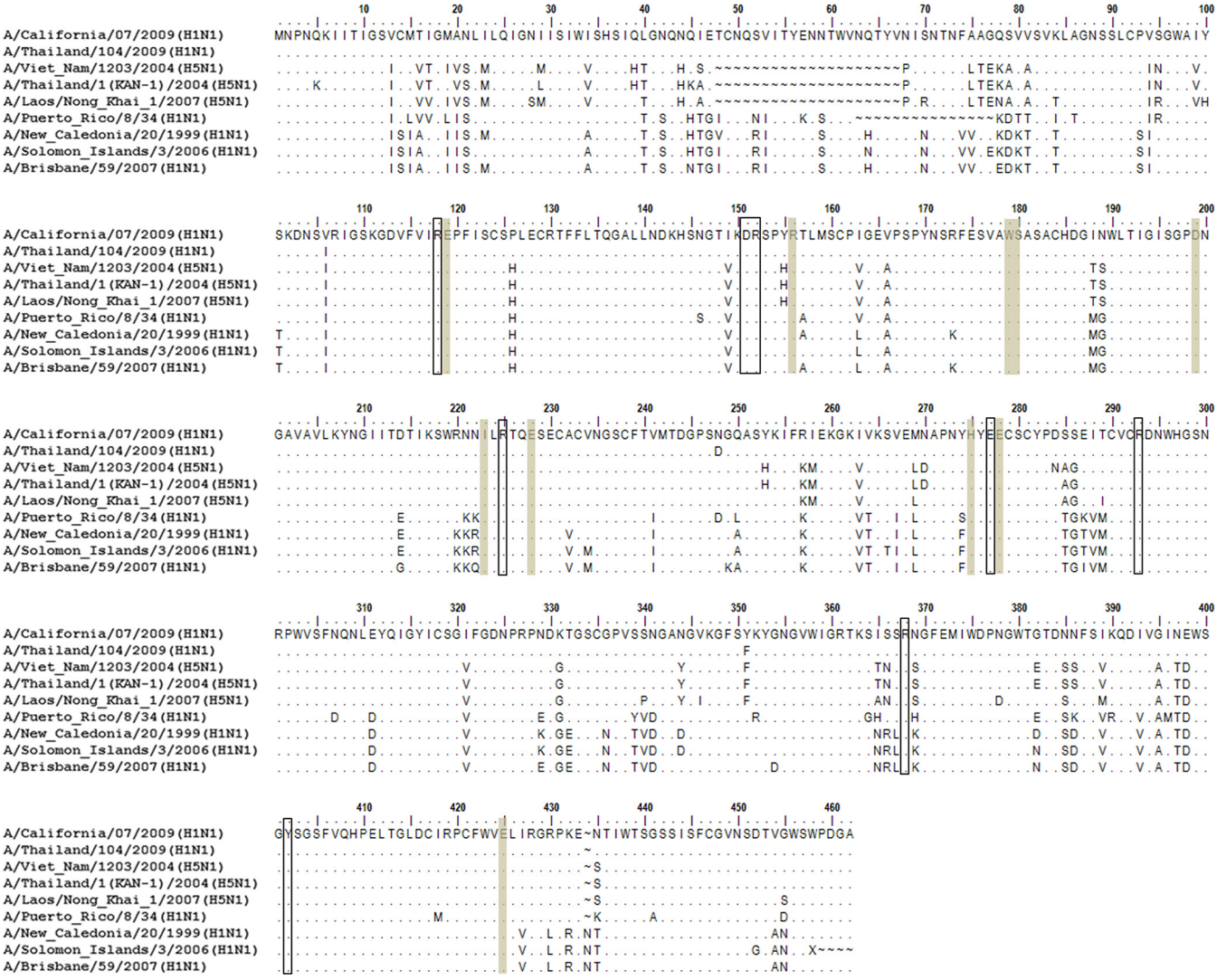



**FIG 2** Alignment of NA amino acid sequences of influenza virus subtype N1. Analysis shows that the catalytic site (boxed) and framework (highlighted) of NA proteins of H1N1pdm, HPAI H5N1, and seasonal H1N1 viruses are 100% identical. The viruses included in the alignment are A/California/07/2009 (H1N1), A/Thailand/104/2009 (H1N1), A/Viet Nam/1203/2004 (H5N1), A/Thailand/1(KAN-1)/2004 (H5N1), A/Laos/Nong Khai 1/2007 (H5N1), A/Puerto Rico/8/1934 (H1N1), A/New Caledonia/20/1999 (H1N1), A/Solomon Islands/3/2006 (H1N1), and A/Brisbane/59/2007 (H1N1) viruses (GenBank accession numbers GQ377078, GQ169381, HM006761, AY555151, EU499378, NC002018, CY033624, EU124136, and CY058489, respectively).

might be possible that NA antibodies bind the antigenic sites on NA and form antigen-antibody complexes, which cause a steric hindrance effect to block the catalytic site of NA from accessing the sialic acid receptor.

The study in H1N1pdm patients demonstrated that seroconversion of NI antibodies could be observed at day 7. Levels of NI antibodies to rgH4N1 viruses with various NAs rose to peaks at days 7 to 28 and had declined by day 90. The NI titer against rg-virus with pdm NA (homologous strain) was significantly higher than those against KAN-1 NA and PR8 NA (heterologous strains). Nevertheless, only 60 to 65% of the patients developed NI antibody. In contrast, the levels of HI antibody titers gradually increased over time and resulted in seroconversion rates of 50, 65, 75, and 100% at days 7, 14, 30, and 90, respectively. This result suggests that the kinetics of NA antibody development is different from the HA antibody response by showing faster peaking time and shorter duration in H1N1pdm-infected patients. Our study results were in line with previous reports that showed lower seroconversion rates for NI antibodies than for HI antibodies in the vaccinees who received trivalent inactivated influenza vaccines (23, 32). This could result from antigenic competition between the HA and NA proteins when both

antigens are presented together, as the HA protein is more abundant than the NA protein on the viral surface (33, 34). It is possible that the anti-NA antibody response might be increased if the HA and NA proteins were administered separately (35). Our previous reports showed that BALB/c mice immunized with recombinant vaccinia virus harboring an NA genomic segment derived from A/Thailand/1(KAN-1)/04 (H5N1), pandemic A/Thailand/104/2009 (H1N1), or A/Anhui/1/2013 (H7N9) virus contained NI antibodies and also neutralizing antibody activities against wild-type virus or reverse-genetics-derived viruses carrying the same NA subtype (21, 36).

Immunity mediated by anti-NA antibodies is a promising area for development, but the problem is to develop a procedure for eliciting a strong NI antibody response. The amount of NA protein content in vaccines varies by production lot, and thus the degree of NA antibody response may be affected (34, 37, 38). The data on the kinetics and longevity of the NI antibody response from this study provide useful information for future vaccine design, in which a standard level of NA protein content is an integral component.

## MATERIALS AND METHODS

**Ethical issues.** This study was approved by the Institutional Review Board at the Faculty of Medicine Siriraj Hospital, Mahidol University, and also at the Ministry of Public Health, Thailand. Subjects or parents gave consent to participate in the follow-up blood collection.

**Viruses.** The viruses used in this study included highly pathogenic avian influenza virus A/Thailand/1(KAN-1)/04 (H5N1) clade 1 (KAN-1 virus), A/Laos/Nong Khai 1/2007 (H5N1) clade 2.3.4 (NK-1 virus), pandemic A/Thailand/104/2009 (H1N1) (H1N1pdm virus), and A/duck/Shan Tou/461/2000 (H4N9) (Shan Tou virus), kindly provided by Robert G. Webster, St. Jude Children Research Hospital, Memphis, TN. Shan Tou virus was grown in embryonic chicken eggs, while the other viruses were propagated in Madin-Darby canine kidney (MDCK) cell monolayers maintained in viral growth medium (VGM) containing minimal essential medium (MEM) (Gibco, Thermo Fisher Scientific, Waltham, MA) without fetal bovine serum supplement. The VGM for propagation of human virus also contained tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St. Louis, MO), while the VGM for HPAI viruses did not. Experiments related to HPAI viruses were conducted in a biosafety level 3 facility.

**Subjects.** The subjects in this study comprised 4 H5N1 survivors and 20 H1N1pdm influenza patients. The 4 survivors, aged 2, 29, 32, and 7 years, were infected with the H5N1 HPAI virus in 2004 and 2005. A total of 26 sequential serum or plasma samples were collected from these subjects at approximately 6-month intervals. Serum/plasma samples were aliquoted and kept frozen at −20°C until tested. These samples were previously investigated by HI and microNT assays using KAN-1 virus as the test antigen, and the results were reported (8). Archival serum samples were obtained from 20 H1N1pdm-infected patients with a median age of 20 years (range, 18 to 42 years). These samples were collected at 0, 7, 14, 28, and 90 days after onset of disease from patients who were diagnosed with H1N1pdm infection in 2011.

**Reverse-genetics-derived influenza viruses.** In this study, 4 rgH4N1 viruses, including a reassorted PR8 virus (control), were constructed. The rg-viruses harbored an H4 HA genomic segment from Shan Tou virus and an N1 NA segment from KAN-1, NK-1, or H1N1pdm virus in the backbone of the PR8 virus. The pHW-2000 recombinant plasmids with inserts derived from each of the eight genomic segments of PR8 virus were kindly provided by Robert G. Webster, and the reverse genetics was undertaken as described by Hoffmann and colleagues (39). Briefly, complete HA and NA segments were amplified by PCR using universal primers (40). Thereafter, the amplified DNA products were cloned into the pGEM-T easy vector (Promega Corporation, Fitchburg, WI) and subcloned into the pHW-2000 plasmids. Subsequently, the recombinant plasmids with the HA and NA segments together with the other 6 internal segments from the PR8 virus in TransLT solution (MirusBio, Madison, WI) were used to transfect MDCK and HEK-293T cocultures (39) maintained in Opti-MEM (Gibco). The inoculated cell monolayers were incubated at 37°C in a $CO_2$ incubator and observed daily for cytopathic effects. The recovered rg-viruses were propagated in MDCK cell monolayers maintained in VGM containing TPCK-trypsin.

**Microneutralization assay.** An enzyme-linked immunosorbent microneutralization assay was conducted for detection of neutralizing antibodies to H5N1 virus in H5N1 survivors, using a test protocol previously described (8, 41). The assay was performed in duplicate in MDCK cell monolayers using the test virus at a concentration of 100 50% tissue culture infective doses ($TCID_{50}$) per reaction mixture. Influenza virus infection in MDCK cells was detected by indirect enzyme-linked immunosorbent assay (ELISA) using a mouse monoclonal antibody specific to influenza A viral nucleoprotein (Merck Millipore, Billerica, MA) and a goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Southern Biotech Associates, Birmingham, AL) as the secondary antibody. The antibody titer was defined as the reciprocal of the highest serum dilution that gave ≥50% neutralization of the test virus.

**Hemagglutination inhibition assay.** A hemagglutination inhibition (HI) assay was performed using a previously described protocol (8). Briefly, the test serum was treated with receptor-destroying enzyme (Denka Seiken, Japan) for removal of nonspecific serum inhibitors and adsorbed with packed goose red blood cells (RBC) for removal of nonspecific agglutinators. A/Thailand/104/2009 (H1N1) at a concentration of 4 HA units

was used as the test virus. The assay was performed in duplicate. The HI antibody titer was defined as the reciprocal of the highest serum dilution that gave complete inhibition of hemagglutination.

**Enzyme-linked lectin assay.** An enzyme-linked lectin assay (ELLA) was undertaken to determine the presence of neuraminidase-inhibiting (NI) antibodies that block the enzymatic activity of influenza virus NA using a previously described protocol (21, 22, 25). Briefly, the optimal concentration of the test virus (rgH4N1 virus) was determined by NA assay prior to performing ELLA. For the NA assay, the rgH4N1 virus was serially 2-fold diluted with the sample diluent containing 1% bovine serum albumin and 0.5% Tween 20 in phosphate-buffered saline, and then 50 $\mu$l of each virus dilution was added into a well of a 96-well microtiter plate precoated with fetuin (Sigma-Aldrich) in duplicate. The reaction plate was incubated for 16 to 18 h at 37°C to allow the viral NA to cleave the sialic acid side chains of fetuin and yield the carbohydrate moieties, which were subsequently detected by horseradish peroxidase conjugated-peanut lectin (PNA) using *o*-phenylenediamine dihydrochloride (OPD) as the chromogenic substrate. The reaction plate was read under a spectrophotometer at a wavelength of 492 nm. Each virus dilution was plotted against its optical density (OD) value to establish a titration curve. An OD value of about 2.0 was extrapolated against the titration curve to determine the working virus dilution for further use in ELLA. In this study, the working concentrations of H4N1pdm, H4N1 H5 KAN-1, H4N1 H5 NK-1, and H4N1 PR8 NA in NI assay were equivalent to approximately 0.5, 2.3, 1.6, and 0.6 HA units, respectively. To determine the NI antibody titer, the test serum/plasma was pretreated with receptor-destroying enzyme (Denka Seiken) at 37°C for 18 h, followed by heat inactivation at 56°C for 45 min. The treated serum/plasma sample at a dilution of 1:10 was 2-fold serially diluted with sample diluent, and then a 50-$\mu$l volume was added into a fetuin-coated plate in duplicate, together with 50 $\mu$l of the test virus at the working concentration. After overnight incubation, the amount of carbohydrate moieties remaining after viral NA digestion of sialic acid was determined as described above for the NA assay. Each experiment included at least 4 wells of the virus control and 4 wells of the sample diluent as the background control. The mean OD value of the test wells was subtracted from the mean OD value of the background control wells in order to obtain the corrected OD value of the test serum/plasma. The corrected OD value of the virus control was similarly obtained. The NI antibody titer was defined as the highest serum/plasma dilution that yielded a 50% reduction of the corrected OD value compared with the virus controls.

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We declare that we have no conflict of interest.

D. Changsom and P. Puthavathana conceived and designed the experiments. D. Changsom, L. Jiang, H. Lerdsamran, and P. Pooruk performed the experiments. D. Changsom and P. Puthavathana analyzed the data. S. Iamsirithaworn, R. Kitphati, and P. Puthavathana contributed reagents and materials. D. Changsom and P. Puthavathana wrote the manuscript.

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