MAJOR ARTICLE

Effect of Clinical and Virological Parameters on the Level of Neutralizing Antibody against Pandemic Influenza A Virus H1N1 2009

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Background. Little is known about the antibody response in natural infection by the novel 2009 influenza A (H1N1) virus and its relationship with clinical and virological parameters. The relative lack of background neutralizing antibody against this novel virus provides a unique opportunity for understanding this issue.

Methods. Case patients presenting with influenza-like illness who were positive for the pandemic H1 gene by reverse transcription polymerase chain reaction were identified. The serum antibody response was assayed by neutralizing antibody titer (NAT) against the virus in 881 convalescent donors. We retrospectively analyzed clinical parameters and viral load.

Results. Ninety percent of the 881 convalescent donors had seroprotective titer of 1:40 or greater. The geometric mean titer of donors with convalescent NAT measured between day 21 and 42 was 1:101.1. Multivariate analysis by ordinal regression showed that pneumonia (odds ratio, 3.39; 95% confidence interval, 1.49–7.61; $P = .004$) and sputum production (odds ratio, 1.75; 95% CI, 1.01–3.01; $P = .046$) were the 2 independent factors associated with a higher level of convalescent NAT. Being afebrile on influenza presentation was associated with subsequent poor NAT (<1:40) response ($P = .04$). A positive correlation between the nasopharyngeal viral load on presentation and the convalescent NAT was demonstrated (Spearman correlation ρ, 0.238; $P = .026$).

Conclusions. About 10% of these convalescent patients do not have a seroprotective NAT and may benefit from vaccination to prevent reinfection. The convalescent NAT correlated well with the initial viral load and was independently associated with severity of the viral illness, including pneumonia. The findings provide both the clinical and virological markers for identifying potential convalescent plasma donors with high serum NAT, which can be used to produce hyperimmune intravenous immunoglobulin in a randomized treatment trial for patients with severe pandemic H1N1 infection.

Since the emergence of the novel 2009 influenza A (H1N1) virus in March 2009, the virus has caused a pandemic affecting >213 countries, with at least 17,700 deaths [1]. In Hong Kong, the number of laboratory-confirmed cases has exceeded 30,000, resulting in >50 deaths [2]. Although oseltamivir appeared to be useful if instituted within 48 h of onset of symptom in mild cases, the viral load and clinical outcome in severe cases with respiratory failure, especially those who presented late, did not respond well to antiviral therapy [3]. Thus, other modalities of therapy including intravenous neuraminidase inhibitors [4, 5] and convalescent plasma or intravenous immunoglobulin with high neutralizing antibody titer (NAT) are investigated as salvage therapy for this particular group of patients. Because premarketing trials with the monovalent 2009 influenza A (H1N1) vaccine demonstrated good NAT after a single dose of 15 μg hemagglutinin given to adults [6–8], a mass vaccination program has commenced in most de-

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veloped areas [9], including Hong Kong, which started in late December 2009. This offers protection to at-risk individuals with pregnancy, chronic illness, or extremes of age against the infection. Unlike vaccination studies, parameters affecting the level of neutralizing antibody after natural influenza infection are quite unknown. The difficulty of getting such data lies mainly with repeated exposure to different strains of antigenically drifted influenza A virus. Nevertheless, recent studies have demonstrated that most patients born after 1950 have no preexisting crossreactive antibodies against this novel virus [10]. This provided us with a unique opportunity to investigate the parameters which can affect the specific NAT in individuals aged <60 years, who have recovered from the virologically documented infection before the vaccination program began. Findings will improve our understanding of the seroepidemiology for vaccination strategy and facilitate the identification of potential donors for convalescent plasma with higher NAT [11], which may be suitable for production of hyperimmune intravenous immunoglobulin. Such a product can be tested in a randomized control treatment trial for severe cases of infection.

METHODS

This study was approved by the institutional review board of the Hospital Authority of Hong Kong. From 1 September through 30 October 2009, patients aged <60 years who have recovered from 2009 influenza A (H1N1) infection, including both hospitalized patients and outpatients, were invited by the Hong Kong Red Cross Blood Transfusion Service to give informed consent for donation of their convalescent plasma voluntarily to treat patients with severe disease due to this novel virus. All potential donors had the diagnosis of 2009 influenza A (H1N1) infection confirmed by positive reverse transcription polymerase chain reaction (RT-PCR) results for the influenza A virus M and pandemic H1 genes and negative RT-PCR results for the seasonal influenza A virus H1 and H3 genes in nasopharyngeal specimens. Clinical information for these potential donors was retrieved from the hospital Computer Medical System, including baseline demographic data, date of symptoms onset, date of the first positive virological test, presenting symptoms including fever, cough, sputum production and sore throat, past medical and smoking history, the viral load from nasopharyngeal specimens on diagnosis, detail of antiviral treatment including oral oseltamivir and inhaled zanamivir, hospitalization, radiological evidence of complication by pneumonia, laboratory results, and clinical outcome. All presenting symptoms except fever were defined by patients' report to standard questions asked by the clinicians. Fever was defined as a single reading of the patient's tympanic temperature of >37.8°C, measured on presentation at the outpatient clinic or hospital. Laboratory parameters in our analysis included blood lymphocyte count (standard range, 1.2×10^9 to 3.4×10^9 lymphocytes/L), alanine transaminase level (standard range, 5–31 U/L), lactate dehydrogenase level (standard range, 91–232 U/L), and creatine kinase level (standard range, 26–192 U/L). Laboratory parameters with values outside the standard range were defined as abnormal. Donors who volunteered for a second convalescent plasma donation had their paired convalescent serum samples tested again for NAT before the second donation. The criteria for the diagnosis of pneumonia included fever, cough or sputum, crepitations on auscultation of the chest, and radiological finding of unexplained pulmonary infiltrate.

Nasopharyngeal specimens obtained on admission were sent to the laboratory in viral transport medium. Total nucleic acid extraction was performed using NucliSens easyMAG instrument (bioMerieux), and RT-PCR was performed with modification, as described elsewhere [12, 13]. All procedures involving clinical specimens and influenza virus were performed in a biosafety level 2 laboratory with biosafety level 3 practices.

Serum samples from all potential donors were tested for NAT by the microneutralization assay inside a type II Biosafety Cabinet in a Biosafety Containment level III facility. The 2009 influenza A (H1N1) virus strain A/HK/415742/09 (H1N1) was used. The virus stocks were aliquoted and stored at −70°C until use. The 50% tissue culture infectious dose ($TCID_{50}$) of each virus was determined by titration in Madin Darby canine kidney cells. One hundred $TCID_{50}$ was used as inoculum in 100 μL of cell culture medium. The $TCID_{50}$ of each stock virus was calculated by the method of Reed and Muench. Serum samples were 2-fold diluted in Eagle's minimum essential medium. Each 50-μL volume of virus inoculum was mixed with equal volume of serum dilution (starting dilution of 1:10). After a 2 h incubation at 37°C in a 5% CO_2 humidified atmosphere, the mixture was added to the seeded microtiter plate. The microtiter plates were seeded with a confluent layer of Madin Darby canine kidney cells. Each flat-bottom 96-well microtiter plate contained 12,500 cells per well, which was incubated in a 5% CO_2 atmosphere at 37°C. After 1 h of infection, virus-serum mixtures were removed, and Eagle's minimum essential medium with the addition of exogenous L-1-tosylamide-2-phenylethyl chloromethyl ketone–treated trypsin (TPCK-trypsin; Sigma Immunochemical) was added to each well. A duplicate for each serum dilution was included on the same microtiter plate. The cells were examined for cytopathic effect at 72–96 h. The microneutralization titer is considered to be the highest serum dilution at which the percentage of cytopathic effect is $\leqslant$50%. All tests included a positive control of standard serum, as well as a negative serum.

We used the Mann-Whitney *U* tests for comparison of the median antibody level between the 2 groups of patients, with previously defined presenting symptoms, baseline demographic characteristics, smoking history, detail of antiviral treatment, radiological evidence of complication by pneumonia, and lab-

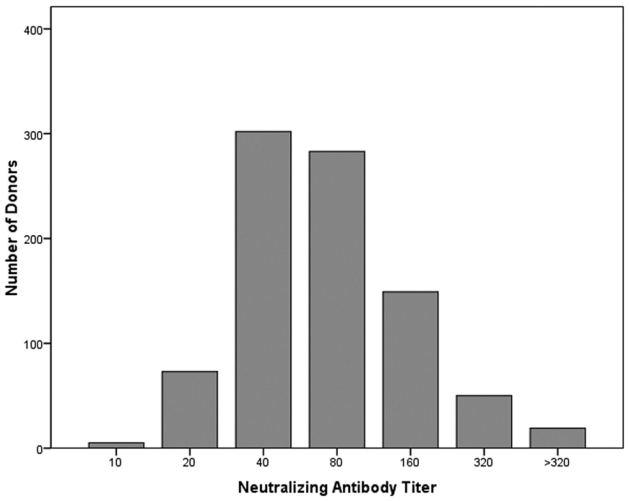

Figure 1. Distribution of serum neutralizing antibody titer of the 881 convalescent donors.

oratory results by univariate analysis. Factors that were significantly different statistically (as defined by P value <.05) in the univariate analysis (except laboratory results with incomplete sampling) were entered into the multivariate analysis by ordinal regression, to determine independent factors associated with high NAT. Correlation between nasopharyngeal viral load and NAT was calculated by Spearman's rank correlation coefficient test. The aforementioned clinical factors were further assessed by binary logistic regression to identify factors associated with poor NAT response (<1:40). SPSS, version 16.0 for Windows (SPSS), was used for statistical computation.

RESULTS

Four hundred twenty-nine of the 881 potential donors were male, and the median age was 27 years (interquartile range [IQR], 22–38 years). A Gaussian distribution of the NAT from the donors was illustrated (Figure 1). All donors had their convalescent NAT measured at day 21 or later after symptom onset. A majority (67.4%) of the donors had the NAT measured between days 21 and 42 after symptom onset (median, 35 days; IQR, 29–46 days). The overall median NAT level was 1:80 (IQR, 1:40–1:80). Ninety percent of the 881 convalescent donors had seroprotective titers of 1:40 or greater. The geometric mean titers (GMTs) of donors with convalescent NAT measured between days 21 and 42 was 1:101.1 (95% confidence interval, 1:20–1:320). None of the donors had a history of underlying medical illness. Sixty-five donors were hospitalized, of which 19 had a complication of pneumonia. All patients in our study

Table 1. Comparison of the Median Serum Neutralizing Antibody Level with Clinical and Laboratory Parameters of the 881 Convalescent Donors

	Patients with variable or with abnormal result		Patients without variable or with normal result		
Variable or measurement	No (%) of patients	Median antibody level (range)	No (%) of patients	Median antibody level (range)	*P*
Antiviral treatment	575 (65.3)	80 (40–160)	306 (34.7)	80 (40–80)	.64
Complication with pneumonia	19 (2.2)	160 (80–320)	862 (97.8)	80 (40–80)	.003
Fever	793 (90)	80 (40–160)	88 (10)	80 (40–80)	.66
Cough	722 (82)	80 (40–160)	159 (18)	80 (40–80)	.37
Sputum production	108 (12.3)	80 (40–160)	773 (87.7)	80 (40–80)	.005
Sore throat	531 (60.3)	80 (40–80)	350 (39.7)	80 (40–160)	.29
Smoker	60 (6.8)	80 (40–160)	821 (93.2)	80 (40–80)	.75
Lymphocyte count (n = 76)	37 (43.4)	80 (40–160)	39 (56.6)	80 (40–160)	.583
ALT level (n = 76)	18 (23.7)	80 (40–160)	58 (76.3)	80 (70–200)	.120
LDH level (n = 24)	13 (54.2)	80 (40–160)	11 (45.8)	80 (80–160)	.494
CK level (n = 27)	6 (22.2)	120 (40–200)	21 (77.8)	80 (80–160)	.887
Sex					
Male	429 (49)	80 (40–160)	…	…	.337
Female	452 (51)	80 (40–80)	…	…	
Age, years					
<20	76 (8.6)	80 (40–160)	…	…	.34
20–29	437 (49.6)	80 (40–160)	…	…	
30–39	187 (21.2)	80 (40–80)	…	…	
40–49	132 (15)	80 (40–80)	…	…	
50–55	49 (5.6)	40 (40–80)	…	…	

NOTE. Lymphocyte count normal range, 1.2×10^9 to 3.4×10^9 lymphocytes/L; alanine transaminase (ALT) normal range, 5–31 U/L; lactate dehydrogenase (LDH) normal range, 91–232 U/L; creatine kinase (CK) normal range, 26–192 U/L.

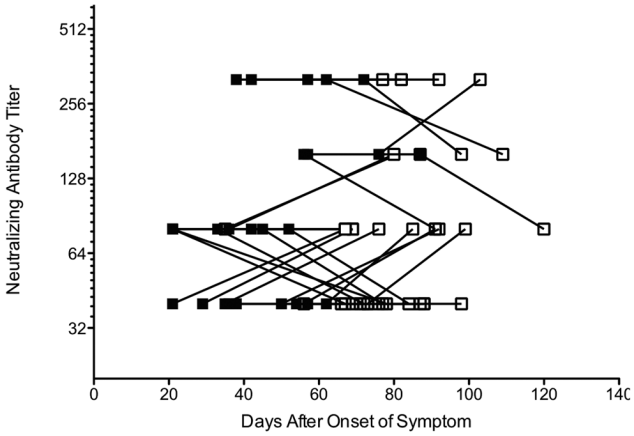

Figure 2. Change of serum neutralizing antibody titer of 33 convalescent donors with paired samples. *Closed squares,* first neutralizing antibody titers; *open squares,* second neutralizing antibody titers.

survived and recovered from the viral illness with no complications, such as respiratory failure or extrapulmonary spread. A majority of the donors received antiviral treatment during their infections (575 donors received oral oseltamivir 75 mg twice daily for 5 days; none received inhaled zanamivir). Details of the clinical and laboratory parameters of the 881 donors are illustrated in Table 1. Thirty-three donors returned for consideration of a second donation and had a paired convalescent NAT measurement (Figure 2).

Univariate analysis showed that only complication by pneumonia and clinical presentation of sputum production were associated with a higher level of NAT. Receipt of antiviral treatment, sex, age, interval between presentation and blood taking (Spearman correlation ρ, 0.02; $P = .59$), and other parameters were not associated with a lower level of NAT. Multivariate analysis by ordinal regression showed that complication of pneumonia (odds ratio, 3.39; 95% confidence interval, 1.49–7.61; $P = .004$) and sputum production (odds ratio, 1.75; 95% confidence interval, 1.01–3.01; $P = .046$) were independent factors for a higher level of NAT (Table 2). Binary logistic regression demonstrated that being afebrile on influenza presentation was the only factor associated with subsequent failure to achieve a protective NAT $\geqslant$1:40 ($P = .04$) (data not shown).

Eighty-seven donors had their initial nasopharyngeal specimen viral load determined at the time of diagnosis (Figure 3). All nasopharyngeal specimens were obtained within 7 days after onset of symptoms. The median viral load was 4.3×10^5 copies/mL (IQR, 7×10^4 to 1.02×10^7 copies/mL). There is a statistically significant positive correlation between the nasopharyngeal specimen viral load and the NAT (Spearman correlation ρ, 0.238; $P = .026$), demonstrating that a high initial viral load was associated with a high NAT. However, nasopharyngeal viral load did not show any correlation with pneumonia (Spearman correlation ρ, 0.056; $P = .61$) or sputum production (Spearman correlation ρ, -0.087; $P = .42$).

Thirty-three donors who had second convalescent serum samples obtained for consideration of a second donation and therefore had paired NAT measured (Figure 2). The median time interval between the paired NAT measurements was 32 days (IQR, 17–42 days). None of the donors had a change in NAT of >2 fold for the paired NAT tests. Thirteen donors (39.4%) had static paired NAT measurements, 10 donors (30.3%) had a 2-fold increase in NAT between measurements, and 10 donors (30.3%) had a 2-fold decrease in NAT between paired measurements. No recurrence or reinfection was documented in this case series.

DISCUSSION

This is the first study, to our knowledge, that has comprehensively investigated the relationship of the NAT after 2009 influenza A (H1N1) infection with other clinical parameters in a young and healthy population. This group of donors is unlikely to have been exposed to 2009 influenza A (H1N1) infection before, as opposed to people born before 1957 [10]. As expected, the NAT assumed a Gaussian distribution slightly skewed to the left, with most donors having a NAT of 1:40 and 1:80. There was also no significant difference in the NAT across the different age groups. Only 2 factors were independently associated with high NAT: the complication of pneumonia and clinical presentation of sputum production. Though we cannot measure the viral load in bronchoalveolar lavage specimens, both factors indicated lower respiratory tract involvement and, therefore, more severe disease, resulting in a more prominent immune response and a higher NAT.

Viral load reflects the dynamic interaction between viral replication and clearance by body defense mechanisms. In this study, we also demonstrated a positive correlation between the initial 2009 influenza A (H1N1) viral load and NAT. It is likely that exposure of the immune system to a higher dose of antigen and the associated higher viral load would result in a higher NAT. Nevertheless, a high viral load does not necessarily mean a more severe infection because the cytokine response of each individual to the viral infection differs, leading to a variable spectrum of clinical presentation. To our surprise, antiviral treatment that suppressed the initial viral load and attenuated

Table 2. Multivariate Analysis by Ordinal Regression of Parameters Independently Associated with High Serum Neutralizing Antibody Level

Variable	Odds ratio (95% confidence interval)	*P*
Complication with pneumonia	3.39 (1.49–7.61)	.004
Sputum production	1.75 (1.01–3.01)	.046

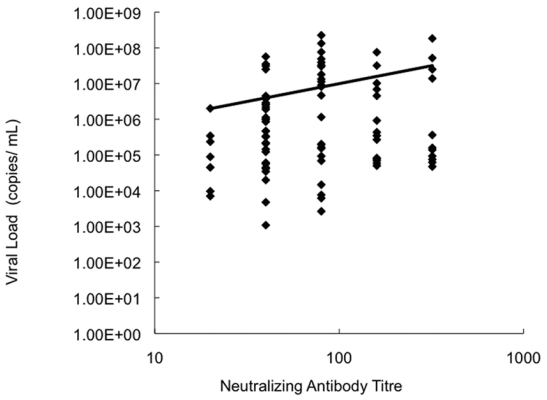

Figure 3. Correlation of viral load with serum neutralizing antibody titer of 87 convalescent donors. Spearman correlation ρ, 0.238; $P = .026$.

the clinical severity was not associated with a lower NAT, as suggested in textbooks for some of the viral infections [14]. It was believed that antiviral agents might blunt host immune responses by altering humoral and cellular immune responses indirectly through reductions in the dose of viral antigen exposure, resulting in a subsequent lower NAT. However, it is important to note that many viral respiratory diseases, such as influenza A and respiratory syncytial virus infection, are associated with the early peaking of the viral load, which leaves a narrow window of opportunity for antiviral treatment. In both human influenza A and respiratory syncytial virus infection, the viral loads peaked at 2–3 days after symptom onset in natural and experimental infections of healthy adults [15]. The early control of viral load in influenza A may be explained by the onset of innate immune response and the early recruitment of viral nucleoprotein specific cytotoxic T lymphocyte response, which will start to clear virus infected cells because of previous antigenic exposure to the influenza A viral matrix 1 and nucleoprotein, which are highly conserved for all subtypes and strains. The apparent lack of effect of antiviral therapy may be further confounded by the interval between the commencement of oseltamivir treatment and the rapidity of oseltamivir in the suppression of viral load. However, at least in this study and in the mouse model [16], there was no evidence that receipt of antiviral treatment would affect the subsequent humoral immune response of the individual to the viral infection, and it is therefore unlikely to be associated with a higher rate of recurrence or reinfection.

From the test result of donors with paired convalescent NAT measurements, most donors (70%) are able to sustain the NAT level over a period of 1–4 months. Thirty percent of the donors had a 2-fold increase in the NAT. This may represent an acceptable dilution error, rather than a reinfection. Similarly, the remaining 30% donors had a 2-fold decrease in the NAT but still maintained a NAT level of 1:40. This again may be a result of dilution difference and may not represent an actual decrease of the NAT. A number of donors demonstrated that they were able to maintain a high NAT of 1:160, even after a period of >100 days after symptom onset.

The NAT profile against influenza virus after natural infection has not been clearly reported in the literature, mainly because of the confounding effect of crossreactivity between different seasonal influenza virus strains. There were little data on convalescent 2009 influenza A (H1N1), but in the recent months, 3 randomized clinical trials [6–8] were performed to assess the safety and immunogenicity of the monovalent, inactivated, split-virus H1N1 2009 vaccine in adults. In all 3 clinical trials, a majority (≥95%) of the subjects aged 18–50 years achieved a seroprotection hemagglutination-inhibition titer of at least 1:40 by day 21 after administration of a single 15-μg nonadjuvanted vaccine or a 7.5-μg MF59-adjuvanted vaccine. A similar result was achieved by microneutralization antibody testing. This is comparable to the seroprotection rate of 90% of the convalescent donors recovered from natural infection by day 35 (median day from symptom onset) in our study. However, the GMT achieved by vaccination with a single 15-μg nonadjuvanted vaccine ranged from 237.8 to 1:277.3 (hemagglutination inhibition) and 1:651.6 (microneutralization) by day 21 after vaccination. A similar GMT (1:288.7) was achieved with the 7.5-μg MF59-adjuvanted vaccine. By day 35 and 42 after vaccination, the GMT reached 1:212.9 and 1:321.3 with the nonadjuvanted and adjuvanted vaccines, respectively. This level of GMT is much higher, compared with the GMT of 1:101.1 achieved by natural infection in our study. Previous studies on inactivated influenza vaccines in healthy adults and young children have suggested that the seroprotection rate could decrease by 30%–60% by 6 months after vaccination [17–19]. This relatively low level of GMT achieved by natural infection raised a concern that a significant proportion of the patients who have recovered from the 2009 influenza A (H1N1) infection in the summer of 2009 may experience a second attack as their NAT falls below the 1:40 seroprotection level, unless they receive the 2009 influenza A (H1N1) vaccine.

One limitation of the present study is its retrospective nature and that it relies on the good will of patients to return and provide a blood sample. Also, the nasopharyngeal and serum samples for viral load and other laboratory investigations were incomplete for all 881 donors. The number of paired titer measurements was limited to 33 donors who returned for a second convalescent donation. The strength of this study was that a majority (67.4%) of the donors had their NAT measured within 42 days after symptom onset, allowing comparison with the antibody response after vaccination. Correlation between NAT, initial viral load, and treatment with antivirals to the subsequent NAT was also analyzed in this study.

In conclusion, the 2009 influenza A (H1N1) antibody titers

after natural infection were maintained at a sufficiently high level to offer adequate seroprotection for the first few months. However, apart from those patients recovered from severe viral illness, the low GMT resulting from natural infection raised a concern that a significant proportion of the patients may experience a second attack after their NAT decreases below the 1:40 seroprotection level. About 10% of these convalescent patients with low NAT may still benefit from vaccination. The NAT correlated well with the initial viral load and is independently associated with the severity of the viral illness, including complication by pneumonia. The findings provide both the clinical and virological markers for potential donors of convalescent plasma with high serum NAT, which can be used to produce hyperimmune intravenous immunoglobulin. This will maximize the time effectiveness of the limited plasmaphoresis capacity in most blood transfusion services [11] and, therefore, allow the production of sufficient doses within a shorter period of time for randomized treatment trials on severe cases during a pandemic. Longer follow-up is necessary to determine the endurance of the antibody level.

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