Serum Antibody Response to Matrix Protein 2 Following Natural Infection With 2009 Pandemic Influenza A(H1N1) Virus in Humans

Weimin Zhong,1 Carrie Reed,1 Patrick J. Blair,2,a Jacqueline M. Katz,1 and Kathy Hancock,1 for the Influenza Serology Working Groupb

1Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia and 2Naval Health Research Center, San Diego, California

(See the editorial commentary by Epstein and García on pages 975–7.)

Natural infection–induced humoral immunity to matrix protein 2 (M2) of influenza A viruses in humans is not fully understood. Evidence suggests that anti-M2 antibody responses following influenza A virus infection are weak and/or transient. We show that the seroprevalence of anti-M2 antibodies increased with age in 317 serum samples from healthy individuals in the United States in 2007–2008. Infection with 2009 pandemic H1N1 influenza A virus (A[H1N1]pdm09) elicited a recall serum antibody response to M2 protein of A(H1N1)pdm09 in 47% of the affected 118 individuals tested. Anti-M2 antibody responses were more robust among individuals with preexisting antibodies to M2 protein. Moreover, the antibodies induced as a result of infection with A(H1N1)pdm09 were cross-reactive with M2 protein of seasonal influenza A viruses. These results emphasize the need to further investigate the possible roles of anti-M2 antibodies in human influenza A virus infection.

Keywords. Influenza A virus; Matrix protein 2; Antibody; Human.

Influenza A viruses possess 3 surface proteins, hemagglutinin (HA), neuraminidase (NA), and matrix protein 2 (M2). The HA and NA are glycoproteins that are abundantly expressed on the surface of virions [1]. Natural infection of humans with influenza A viruses results in a robust antibody response that, in most infected individuals, is primarily directed toward the HA glycoprotein of the virus [2]. Serum and mucosal neutralizing HA–specific antibodies provide strong protection against infection by blocking attachment of viral particles to host cell membranes. In addition, NA-inhibiting antibodies are detectable, albeit with lower frequency and reduced magnitude, compared with anti-HA antibodies [3, 4]. Unlike HA–specific neutralizing antibodies, NA–specific antibodies function by preventing the release of newly synthesized virions from infected epithelial cells. Because of the high mutation rate of HA [1], HA–specific antibodies directed against the globular head of HA generally can provide optimal protection against antigenically closely related strains of influenza A viruses and are inefficient against hetero-subtypic viruses [5, 6]. In the absence of antigenic drift or shift, protective immunity conferred by HA- and NA–specific antibodies may persist for many years [7].

M2 is an integral transmembrane protein present at low density on the surface of the virus particles but abundantly expressed at the surface of infected cells [8]. M2 forms an ion channel, exhibiting proton transport activity that regulates the pH of the viral core during viral entry into host cells and assembly of virion particles [9]. The amino acid sequences of the ectodomain of M2 protein (M2e) are highly conserved across all human influenza A virus isolates, making this region an attractive target for broadly cross-reactive influenza vaccines and therapeutic antibodies [10, 11]. Natural
infection–induced immunity to M2 protein in humans is poorly understood. Although previous studies have detected antibodies specific for M2e in convalescent-phase serum samples from influenza A virus–infected persons, only a minority of recently infected adults exhibited anti-M2 antibodies, which appeared to be transient in nature [11, 12]. Furthermore, some of these studies used an M2e peptide–specific enzyme-linked immunosorbent assay, which may not detect all antibodies capable of binding to M2 [11, 13, 14]. In contrast, M2 vaccine–induced immunity has been studied extensively in the mouse model. Both passive and active immunization with M2 can protect mice from lethal challenge with homologous and heterosubtypic influenza A viruses [15–21].

The 2009 influenza A(H1N1) virus (A[H1N1]pdm09) pandemic has provided us with a unique opportunity to revisit the issue of infection-induced immunity to M2 in humans. In the United States, an estimated 20% of the population of 310 million was naturally infected by A(H1N1)pdm09 [22]. Although the illness caused by A(H1N1)pdm09 was mild to moderate in severity and self-limited in most cases, severe and fatal infections were reported, with an estimated 265,000 hospitalizations and 12,000 deaths in the United States [23, 24]. Age-stratified rates of A(H1N1)pdm09 infection reported in the United States by 23 July 2009 were highest among children and young adults; >70% of the reported cases were in persons ≤24 years of age, while only 1% were in adults ≥65 years of age [25]. Early serological studies suggested that approximately one third of adults aged >60 years had preexisting serum neutralizing antibodies cross-reactive to the HA glycoprotein of A(H1N1)pdm09 [26], which is one possible explanation for the relative sparing of this age group. More recently, memory T lymphocytes to the viruses have been identified [27–29], suggesting that other forms of preexisting immunity may contribute to cross-protection against new emerging influenza A viruses such as A(H1N1)pdm09.

Recently, we developed a sensitive M2-293FT cell–based flow cytometric assay (M2-FCA) to detect serum antibodies to M2 of influenza A viruses [30]. Results obtained in the mouse model demonstrate that M2-FCA is highly efficient in quantifying serum antibodies to the natural tetrameric form of M2 antigen. In the present study, we report the results obtained using this new method to assess serum antibody response to M2 following natural infection with A(H1N1)pdm09 in humans.

**MATERIALS AND METHODS**

**Source of Serum Samples**

To assess the baseline prevalence of preexisting immunity to M2 in different age groups before onset of the A(H1N1)pdm09 pandemic, we tested a panel of 317 serum samples collected in 2007–2008 from healthy individuals aged 3 to ≥80 years. The serum panel was a subsample of the banked serum specimens randomly selected from different age groups during the National Health and Nutrition Examination Survey (NHANES), 2007–2008, in the United States [31]. Use of the serum samples in the present study was approved by the National Center for Health Statistics Research Ethics Review Board. The serum panel was selected to investigate the age-specific anti-M2 antibody response and was not representative of the total 2007–2008 NHANES serum panel or the US population.

To study the serum antibody response to M2 in humans naturally infected with A(H1N1)pdm09, a panel of 217 serum samples from 118 patients in the United States was assembled (Table 1). A(H1N1)pdm09 infection was confirmed either by real-time reverse-transcription polymerase chain reaction (RT-PCR) or by detection of seroconversion in paired serum samples by either hemagglutination inhibition assays (HIAs) or microneutralization assays as described elsewhere [32]. The collection of these serum samples was part of the Centers for Disease Control and Prevention (CDC) public health emergency response to the A(H1N1)pdm09 pandemic and considered a nonresearch activity that did not require CDC institutional review board review.

**Hemagglutination inhibition assay (HIA)**

The HIA was performed according to standard procedures, using 4 hemagglutinating units of virus and 0.5% turkey red blood cells [33]. The novel A(H1N1)pdm09 used in the study was an A(California/07/2009-like virus, A/Mexico/4108/2009, grown in 10–11-day-old embryonated chicken eggs. All serum samples were treated with receptor-destroying enzyme to remove the nonspecific inhibitors of agglutination. Those containing nonspecific agglutinins were preadsorbed with turkey erythrocytes. Serum specimens were tested at an initial dilution of 1:10.

**M2-293FT cell-based flow cytometric assay (M2-FCA)**

Antibodies against M2 of influenza A viruses were quantified by using the M2-FCA as described previously in detail [30], with the following modifications for human serum samples. Briefly, human serum samples were diluted at a single dilution, 1:40, for testing in the M2-FCA as justified in the Supplementary Materials. A total of 50 μL of each diluted serum sample was incubated with 1 × 10^6 cells of a M2 transfectant in U-bottomed 96-well plates at 4°C for 45 minutes. Two M2 transfectants, human M2 (HM2) and A(H1N1)pdm09 M2 (SWM2), were used as M2 antigen sources [30]. After washing, cells were incubated with 55 μL of diluted fluorescein isothiocyanate–conjugated, goat anti-human immunoglobulin G antibody (Cappel). For all experiments performed in the present study, the same anti-M2–positive and anti-M2–negative control human serum samples, pooled from multiple serum samples, were run in parallel with test serum samples. Data acquisition was performed on a Becton Dickenson LRS II flow cytometer. A representative staining profile of human serum samples in M2-FCA is shown...
in Supplementary Figure 1. The results were expressed as units (U), calculated as follows:

\[(\text{percentage of test serum samples} - \text{percentage of negative control serum})/(\text{percentage of positive control serum} - \text{percentage of negative control serum}) \times 100\]

where “percentage” refers to the percentage of M2-positive cells obtained by staining with each respective serum sample as indicated in the formula. The positivity threshold of the M2-FCA was experimentally determined to be ≥3 U for human serum samples.

RESULTS

Preexisting Serum Antibodies to M2 in Different Age Groups

We first measured serum antibodies to M2 of human influenza A viruses in a total of 317 serum samples obtained in 2007–2008 from healthy individuals residing in the United States. Overall, 33.4% of the serum samples tested contained a low level of anti-M2 antibodies (median level in anti-M2-positive serum samples, 9.3 U). However, the frequencies and the levels of the preexisting anti-M2 antibodies varied considerably among different age groups. As shown in Figure 1, anti-M2 antibodies were detectable in 9.7% (95% confidence interval [CI], 3.4%–24.9%) of children aged 3–10 years. When serum samples from children aged 10–18 years and adults aged 19–39 years were examined, only 11%–21% of the serum samples contained a low level of anti-M2 antibodies (median level in anti-M2-positive serum samples, 6.9 U). With the exception of adults between 60–69 years, frequencies and magnitudes of preexisting anti-M2 antibodies were considerably higher in adults aged ≥40 years (38%–50%; median level of anti-M2-positive serum samples, 13.0 U) than in children and younger adults aged 20–39 years.

We examined the cross-reactivity of preexisting anti-M2 antibodies in a subset of 45 serum samples randomly selected from each of the 3 age groups of the 317 NHANES serum samples. The M gene that encodes M2 of A(H1N1)pdm09 is of Eurasian avian-like swine lineage origin [34, 35]. Overall, results obtained with HM2 correlated well to those obtained using SWM2 antigen in the M2-FCA (Pearson $r$, 0.9647; $P < .0001$; Supplementary Table 1).

Human Serum Antibody Response to M2 Following Infection With A(H1N1)pdm09

Next, we monitored the serum antibody response to M2 of A(H1N1)pdm09 in 217 serum samples collected from 118 patients with confirmed A(H1N1)pdm09 infection. Overall, serum anti-M2 antibodies were detected in 47% of the serum samples tested, with a median anti-M2 antibody titer of 30.0 U. The timing of the immune response is an important consideration for the detection of antibody responses to influenza virus infection. Therefore, paired serum samples collected over a range of intervals after symptom onset were selected from the serum panel (Table 1) and stratified in 5-day intervals according to the timing of serum sampling relative to the symptom onset. As shown in Figure 2A, only 19.2% of the 26 serum
samples collected 1–5 days after symptom onset had low levels of anti-M2 antibodies (median level in anti-M2–positive serum samples, 6.9 U). The median levels of anti-M2 positive antibodies in M2 responders rapidly increased to 43.5 U in 26.7% of the 30 serum samples obtained between days 6 and 10 and reached a maximum of 57.7 U in 53.3% of the 15 serum samples collected between days 11 and 16 after symptom onset. The levels of anti-M2 antibodies remained at approximately 30 U in approximately 50% of the serum samples obtained ≥20 days after symptom onset. In contrast, HI antibodies specific for A(H1N1)pdm09 were not detectable in the same set of serum samples collected before day 11 after symptom onset (Figure 2B).

Titers of ≥20 of A(H1N1)pdm09–specific HI antibodies (geometric mean titer, 36.6) were first detectable in 87% of the 15 serum samples obtained ≥20 days after symptom onset. The levels of the strain-specific HI antibodies continued to increase throughout the convalescent phase of A(H1N1)pdm09 infection and reached a geometric mean titer of 78.8 in 97% of the 32 serum samples obtained around 30 days after symptom onset.

This finding suggests that antibody responses to M2 may be more robust in individuals with preexisting immunity to M2. To test this hypothesis, 42 paired serum specimens from subgroup 2 in Table 1 were divided further into 3 subgroups, based on different degrees of preexisting immunity and different responses to M2, for further analysis. Figure 3A shows that anti-M2 antibodies were not detectable in the acute-phase serum samples from these 13 patients, and only low levels of anti-M2 antibodies (median level in anti-M2–positive serum samples, 12.9 U [95% CI, 9.6–45.8]) were detected in the paired convalescent-phase serum samples. In contrast, anti-M2 antibodies were detected in all of the acute-phase serum samples from the second subgroup of 9 patients (median level in anti-M2–positive serum samples, 22.2 U [95% CI, 10.1–39.9]; Figure 3B). The median levels of anti-M2 antibodies in the paired convalescent-phase serum samples increased to 48.0 U (95% CI, 23.8–66.8). In the third subgroup of 20 patients, neither the acute-phase serum samples nor the convalescent-phase serum samples contained positive levels of anti-M2 antibodies (Figure 3E). All of the 3 patient subgroups compared mounted similar magnitudes of antibody response to the HA of A(H1N1)pdm09 (Figure 3B, 3D, and 3F).

Next, we tested responsiveness to M2 antigen in 11 young children who were born in 2008 and had RT-PCR–confirmed infection with A(H1N1)pdm09. As shown in Table 2, A(H1N1)pdm09–specific HI antibodies were detected in all 11 children. In contrast, none of the 11 children had detectable anti-M2 antibodies.
Last, we analyzed the cross-reactive nature of the anti-M2 antibodies induced following A(H1N1)pdm09 infection. To this end, 16 paired serum samples with anti-HM2 antibodies were selected from subgroup 3 patients described in Table 1. The first serum samples were collected 3–11 days after symptom onset, whereas the second serum samples were collected 21–32 days after symptom onset. As shown in Figure 4, patient serum samples obtained at either time reacted similarly with either SWM2 or HM2. Although the median titers of acute-phase serum samples were higher against SWM2 and the median titers of the convalescent-phase serum samples were higher against HM2, the differences were not statistically significant.

**DISCUSSION**

M2 of influenza A viruses is an attractive target of “universal” influenza vaccine. However, knowledge of natural immunity to M2 antigen is limited. To address this, we assessed the status of preexisting serum antibodies to M2 in the US residents before onset of the A(H1N1)pdm09 pandemic and characterized serum antibody response to M2 in patients with laboratory-confirmed infection with A(H1N1)pdm09.

We found that, overall, approximately 33% of the 317 serum samples collected from U.S. residents in 2007–2008 had low levels of antibodies that were reactive to M2 of human H1N1 viruses and cross-reactive with the M2 of the A(H1N1)pdm09 (SWM2). The results were expressed as units (U), with a U of ≥3 considered positive. The lines denote the median levels of anti-M2–positive serum samples. B, Titers of serum hemagglutination inhibition (HI) antibodies were determined by a HI assay as described in Materials and Methods, using A/Mexico/4108/2009 (H1N1) as antigen. The results are expressed as the reciprocal of the highest dilution of a serum sample that inhibits agglutination of turkey red blood cells, with an HI of ≥20 considered positive [32]. The lines denote the geometric mean titer of serum HI antibodies transformed to log2 scale.
preexisting antibodies to M2 by age may reflect increasing numbers of exposures to seasonal influenza A viruses with increasing age. Thus, in addition to serum cross-reactive neutralizing antibodies to HA [26], NA [36], and cross-reactive memory T cells [27–29], our study reveals that preexisting serum antibodies to M2 may represent a previously undocumented component of cross-reactive immunity to influenza A viruses in humans.

Infection with A(H1N1)pdm09 elicited an anti-M2 antibody response in approximately 50% of the 118 patients examined. This rate of response to M2 antigen appears to be higher than those reported previously (35%–40%) that were based on the studies of serum samples from seasonal influenza A virus–infected individuals [11–14]. This difference may be attributed to either an improved sensitivity of our M2-FCA to detect serum antibodies to the natural tetrameric form of M2 [30] and/or to strain differences between A(H1N1)pdm09 and seasonal influenza A viruses in their ability to induce an anti-M2 antibody response (discussed further below).

Anti-M2 antibodies were detected in serum samples from some A(H1N1)pdm09–infected patients within the first 10 days after symptom onset and before A(H1N1)pdm09-specific HI antibodies (Figure 2B), suggesting a recall serum antibody response to M2 following A(H1N1)pdm09 infection. Because

Table 2. Serum Antibody Response to Matrix Protein 2 (M2) in Children born in 2008 Who Were Infected With 2009 Pandemic Influenza A(H1N1) Virus (A[H1N1]pdm09)

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<th>M2-FCA Finding, U</th>
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Single serum samples were derived from patients in subgroup 1, described in Table 1.

Abbreviations: HI, hemagglutination inhibition; HM2, human M2 transfectant; ILI, influenza-like illness; M2-FCA, M-293FT cell-based flow cytometric assay; SWM2, A(H1N1)pdm09 M2 transfectant.

* Time of serum collection relative to symptom onset.
baseline serum samples collected from patients before infection with A(H1N1)pdm09 were not available, we cannot formally rule out the possibility that the characteristic kinetics of the anti-M2 antibody response observed was caused by anti-M2 antibodies present in these individuals before A(H1N1)pdm09 infection. However, the fact that the proportion of the anti-M2–positive serum samples stratified on days 6–10 after symptom onset was more than twice that from the age-matched normal human serum samples (26.7% vs 11.1%; Supplementary Table 2) suggests that it is less likely that this is the case.

At present, we do not know the extent to which infection-induced anti-M2 antibodies are protective in humans. However, it is intriguing to observe that preexisting humoral immunity to M2 showed a clear tendency toward a negative correlation with the incidence of A(H1N1)pdm09 infection by age group in the US population (Figure 1). Because of limited information on the clinical illness of the patients tested, it was not feasible to assess a possible link between preexisting anti-M2 antibodies and the clinical course of influenza after A(H1N1)pdm09 infection in the present study. Recent studies in mice have revealed that polyclonal and monoclonal mouse antibodies to M2 were able to reduce viral load and influenza severity when passively transferred into naive recipient animals [19, 37, 38]. It is conceivable that infection-induced anti-M2 antibodies that reach a critical threshold for clinical protection may contribute to the mitigation of influenza. On the other hand, a possible detrimental effect of preexisting anti-M2 antibodies has been proposed. Vaccination of pigs with a DNA-based M2 vaccine exacerbated influenza after challenge with a swine influenza A virus [39].

We noted that only approximately half of the patients examined mounted a detectable antibody response to M2 following infection with A(H1N1)pdm09. Age was not a factor contributing to the different responsiveness to M2 antigen among the patients tested (median age, 23 years [range, 3–53 years] for M2 responders and 23 years [range, 0.5–43 years] for M2 nonresponders). In our opinion, a number of confounding factors may have contributed to the positivity or negativity of anti-M2 humoral immunity in these individuals. One possible confounder is prior exposure to live influenza A viruses. In the present study, anti-M2 antibodies were not detectable in any of the 11 children who were born in 2008 and had no serologic evidence of infection with seasonal influenza A viruses, suggesting that infection with A(H1N1)pdm09 in 2009 was the first encounter with influenza virus infection (Table 2). This observation further supports the idea that multiple infections with influenza A viruses may be required to stimulate a detectable antibody response to M2 [11]. A second potential confounder involves a possible difference between pandemic and seasonal strains of influenza A viruses in induction of anti-M2 antibodies. In the present study, we found that healthy individuals who were aged ≥40 years (eg, born before the 1968 influenza A[H3N2] pandemic) had considerably higher frequency of anti-M2 antibodies than younger adults and children (approximately 50% vs 20%; Figure 1). Moreover, approximately 50% of the 118 A(H1N1)pdm09-infected patients had a detectable anti-M2 antibody response, higher than the previously reported 35%–40% with seasonal influenza virus infection [11–14]. Together, these data suggest a higher rate of response to M2 antigen in humans following exposure to pandemic influenza A viruses. The observation that different strains of influenza A viruses varied considerably in their ability to induce a detectable anti-M2 response after primary infection in mice ([30],

Figure 4. Antibodies induced in response to 2009 pandemic influenza A(H1N1) virus (A[H1N1]pdm09) infection were cross-reactive with matrix protein 2 (M2) of recent seasonal influenza A viruses. Sixteen paired serum samples from A[H1N1]pdm09-infected patients were selected from patient subgroup 3 described in Table 1. Serum anti-M2 antibodies were quantified in parallel by M2-293FT cell–based flow cytometric assay, using A[H1N1]pdm09 M2 transfectant (SWM2) and human M2 transfectant (HM2) as M2 antigens. The lines denote the median levels of anti-M2–positive serum samples. A, The first serum samples collected 3–11 days after symptom onset. B, The second serum samples collected 21–32 days after symptom onset.
unpublished data) supports this idea. A third possible confounder involves previous history of influenza vaccination. Inactivated split influenza vaccine (TIV) and live attenuated influenza vaccines (LAIVs) are 2 trivalent influenza vaccines currently licensed in the United States [40]. The former is primarily composed of the HA and NA glycoproteins and is unlikely to contain M2. Therefore, it is unlikely that preexisting anti-M2 antibodies detected in the present study were a result of vaccination with TIV before the A(H1N1)pdm09 pandemic. LAIV, on the other hand, relies on replication of the vaccine virus and M2 would be expressed to some degree in infected epithelial cells. However, the ability of LAIV to induce or boost anti-M2 immunity remains unknown.

M2e sequences derived from human influenza A viruses and A(H1N1)pdm09 differ by 4 amino acids [30]. Analyses with monoclonal antibodies and polyclonal mouse serum samples have revealed that both M2e sequence–specific and cross-reactive B-cell epitopes were present in the M2e molecule [16]. The finding that anti-M2 antibodies present in the serum samples from healthy individuals before onset of the A(H1N1)pdm09 pandemic cross-reacted strongly with M2 of the A(H1N1)pdm09 (Supplementary Table 1) suggests that a substantial portion of human anti-M2 antibodies induced after infection may recognize the conserved N-terminal part of the M2e molecule. Indeed, infection with A(H1N1)pdm09 resulted in a strong cross-reactive antibody response to M2 of human influenza A viruses (Figure 4). Therefore, infection with A(H1N1)pdm09 may have boosted humoral immunity to M2 of human influenza A viruses in a substantial proportion of the affected individuals in the United States. Taken together, our study reveals that natural infection with A(H1N1)pdm09 elicited an M2 antibody response in approximately 50% of affected individuals. Given that anti-M2 antibodies are protective in mice, primarily via FcYR-dependent mechanisms such as antibody-dependent cell-mediated cytotoxicity [18, 41], our data emphasize the need to further explore the possible roles of anti-M2 antibodies in modulating the outcome of influenza following influenza A virus infection in humans. Furthermore, our results suggest that induction of immunity to M2 by vaccination may be worthwhile to prime for anti-M2 antibody responses to subsequent natural infections with new emerging influenza A viruses. Together with other components of cross-protective immunity to influenza viruses, such as cross-reactive memory CD8 T cells, anti-M2 antibodies may provide cross-protection against heterosubtypic influenza A viruses, as observed recently in the mouse model [42]. This may have important public health implications in the event of another influenza pandemic.

STUDY GROUP MEMBERS

The members of the CDC Influenza Serology Working Group are as follows, in alphabetical order: Darbi Aranio, Yaohui Bai, Peter Browning, Alicia Branch, Evelene Steward-Clark, Li Cronin, Hanan Dababneh, Eric Gillis, Crystal Holiday, Feng Liu, Xiuhua Lu, Heather R. Tatum, Conrad Quinn, Stephen Soroka, Jarad Schiffer, Hong Sun, Leilani Thomas, Byron Tsang, Vic Veguilla, David Wang, and Melissa Whaley.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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