

Survey of Human Antibody Responses to Influenza Virus Matrix Protein 2 by Use of a Sensitive Flow Cytometric Method

Suzanne L. Epstein and Mayra García

Division of Cellular and Gene Therapies, Office of Cellular, Tissue, and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland

(See the major article by Zhong et al on pages 986–94.)

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Conventional influenza vaccines are designed to elicit antibodies to strain-specific antigens, leaving a public health gap when novel viruses break out unexpectedly. As examples, strain-matched vaccine became available too late in the pandemic of 2009 to protect against the fall wave, and drift viruses are sometimes divergent enough to cause vaccine failure (eg, A/Sydney in 1997). Available vaccines are also inadequate to protect against various zoonotic strains, including avian influenza A(H5N1), influenza A(H7N7), and most recently influenza A(H7N9). For this reason, there is much interest in developing vaccines based on conserved influenza virus features that can provide protection regardless of strain. These are usually designed for influenza A virus, but such vaccines can also be made for influenza B virus. Universal influenza vaccines

can induce immune protection dependent upon antibody or T-cell responses or both, and the target antigens explored have included nucleoprotein, matrix proteins (M1 and M2), the hemagglutinin (HA) stem, polymerase PB1, and other antigens, as reviewed previously [1].

M2, the focus of Zhong et al in the current issue of the *Journal*, has long been known as a target of antibodies that reduce viral replication and spread [2, 3]. Vaccines based on many forms of M2 (fusion proteins, M2 multiple antigenic peptides, peptide conjugates, and M2 expressed from viral vectors) provide protective immunity in animals [4–10], and some have been tested in humans. A clinical trial of recombinant M2 shows that this antigen is immunogenic when administered with adjuvant [11].

Human anti-M2 antibodies induced by natural infection have been reported, but data are scanty. Black et al reported that 6 of 17 pairs of acute-phase and convalescent-phase serum specimens showed increased anti-M2 activity by enzyme-linked immunosorbent assay (ELISA), while 12 of 17 convalescent-phase serum specimens demonstrated some signal by Western blot [12]. In a study by Feng et al, an increase in anti-M2 activity was found for 11 of 24

such serum pairs [13]. In that study, of the antibodies detectable by assay on cell surface tetrameric M2, only a minority also recognized M2e peptide. Thus, the majority of antibodies appeared to be conformational [13]. These antibodies may be biologically very important, so assays on native M2 are needed. However, measuring antibodies to native M2 has presented technical problems, with high background encountered in cell surface ELISA.

To overcome these problems, Zhong et al developed a flow cytometric assay (M2-FCA) using a panel of 293FT transfected cell lines (M2-293FT) stably expressing full-length tetrameric forms of M2 from various viral strains [14]. With this sensitive assay, some mouse antibodies recognize strain-specific epitopes and some see epitopes cross-reactive among viral strains [14]. The present article uses the assay to analyze collections of human sera. Transfection efficiency and expression levels are normalized through the use of a positive human serum pool as a control. Unlike most serological assays in which a titer reflects the dilution at which a defined end point is reached, the unit of M2 antibody is defined for a single (1:40) dilution of serum. This allows large numbers of sera to be analyzed

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Correspondence: Suzanne Epstein, PhD, OCTGT, CBER, FDA, 1401 Rockville Pk, HFM-730, Rockville, MD 20852-1448 (suzanne.epstein@fda.hhs.gov).

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within a single run, but does not measure titers or other antibody properties revealed by dilution series. Results for healthy donors of different ages showed that antibodies to M2 were found in a higher percentage of, and at higher levels, in adults aged ≥ 40 years, compared with younger donors. If anti-M2 antibodies were present, they usually recognized both seasonal and swine-origin M2.

For influenza virus, there are no human "preimmune" sera to establish a threshold of positivity, because most humans have been exposed to an influenza virus at some time. Even cord blood could contain maternal antibodies to influenza virus proteins. The investigators faced this problem by identifying human sera with similar binding to transfected cells expressing seasonal M2 and to 293 T untransfected control cells ($\leq 3\%$ difference) and considering these specimens negative. The choice of a 3-unit threshold is arbitrary, and most of the conclusions in the article would not be altered by choosing a slightly different cutoff.

Only one of the significant observations would be changed by using a different threshold: the comparison of kinetics for M2 and HA antibody responses in the course of infection with the 2009 pandemic virus. Do antibodies to M2 really increase earlier in the course of infection than hemagglutination inhibiting (HI) antibodies? By days 6–10 after symptom onset, antibody responses have begun to rise in a minority of donors. Two very different assays are being used, with thresholds defined in different ways and one plotted on a \log_2 scale. M2 titers of ≥ 3 U and HI titers of ≥ 20 were considered positive. Although an HI titer of 10 is marginal and does not offer much protection, it still seems to differ from background. In the dot plot, the response to HA appears to be rising by days 6–10 above the values from days 1–5, just as does the response to M2. If the threshold had been 10 U for anti-M2 and an HI titer of 10, the results would not support a kinetic difference.

Nonetheless, the investigators made good use of the hinted difference. They

went beyond making measurements and counting positives to consider what the results might mean biologically. They hypothesized there was significance to the possible difference in kinetics and reasoned that an early rise of anti-M2 antibodies in some donors might mean those individuals were already primed to M2. Testing of paired serum samples supported this idea; individuals who started out with higher activity showed greater increases by the time of the second sample than those who started out negative. The same was not true for HI antibodies, at least for this instance. An alternative explanation for the difference in M2 responses between donor subgroups might be that the subgroups differ in HLA types. It would be interesting to know whether antibodies to the HA stem appear with kinetics suggesting priming.

Induction of antibodies to M2 is apparently difficult to achieve in very young children, even upon infection with the pandemic virus (Table 2). In contrast, a single infection leads to robust HI responses in this age group. It seems that boosting is required to induce much antibody to M2. This is also the case in mice with 3 infections needed for strong responses to M2 [13].

The investigators suggest the possibility that pandemic and seasonal strains of influenza A viruses differ in ability to induce anti-M2 antibodies. This idea is interesting but remains speculative. It would be difficult to go beyond anecdote with human sera, since there are few pandemics and the age of donors would be an unavoidable confounding variable. For example, with a large set of serum specimens collected in 2008 (before the pandemic), unless all the donors are young, they could have experienced infection with the pandemic virus of 1968 or the virus that reemerged in 1977. For older donors, there would probably be no records of whether they were infected during earlier pandemics. Therefore, identifying purely seasonal responses would be a challenge. This particular question about pandemic versus seasonal viruses could be addressed in animal models, where a range of structural forms could

be compared readily, and the authors mention unpublished studies of that kind. In animals, one could ask what features of a pandemic virus, not necessarily the M2 itself, lead to the difference in responsiveness.

The data demonstrate the success of the M2-FCA in evaluating M2-specific human antibody responses, and show that it can be useful in future clinical studies. Such studies could ask important questions: Do the modest levels of antibody to M2 induced by natural infection contribute to protection against subsequent infections? Or does a meaningful contribution to protection require a response intensified by M2 vaccines? The observed negative correlation of anti-M2 antibodies with influenza incidence by age group is not informative about this question. It likely just reflects, at the population level, a negative correlation with previous influenza experience in general. To assess a role in protection, anti-M2 antibody levels must be compared to infection outcomes on an individual basis, using linked samples. Thus, studies of surveillance cohorts or human challenge studies are necessary. It would be difficult to address this question even with the improved M2-FCA assay. Large cohort sizes would be needed to distinguish protection by anti-M2 antibodies from other contributions such as CD8⁺ T cells [15] and antibodies to neuraminidase [16, 17].

Researchers who work only in animal models or in vitro molecular systems may think the progress in the Zhong et al article is modest. However, many with public health and epidemiology interests will see it as a major step. Given the difficulty of assembling suitable human samples and reliably detecting the signal above the noise, it would have been an accomplishment just to measure the responses systematically. The authors have gone beyond that, using their insights from the data, to pose and to begin addressing several questions important for infectious disease studies and vaccine development. The rapid, simple, and sensitive M2-FCA will add value to

future studies of human influenza surveillance and vaccination.

Notes

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