Epitope specificity of anti-HA2 antibodies induced in humans during influenza infection

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Background The conserved, fusion-active HA2 glycopolypeptide (HA2) subunit of influenza A hemagglutinin comprises four distinct antigenic sites. Monoclonal antibodies (MAbs) recognizing three of these sites are broadly cross-reactive and protective.

Objectives This study aimed to establish whether antibodies specific to these three antigenic sites were elicited during a natural influenza infection or by vaccination of humans.

Methods Forty-five paired acute and convalescent sera from individuals with a confirmed influenza A (subtype H3) infection were examined for the presence of HA2-specific antibodies. The fraction of antibodies specific to three particular antigenic sites (designated IIF4, FC12, and CF2 here) was investigated using competitive enzyme immunoassay.

Results Increased levels of antibodies specific to an ectodomain of HA2 (EHA2: N-terminal residues 23–185 of HA2) were detected in 73% of tested convalescent sera (33/45), while an increased level of antibodies specific to the HA2 fusion peptide (N-terminal residues 1–38) was induced in just 15/45 individuals (33%). Competitive assays confirmed that antibodies specific to the IIF4 epitope (within HA2 residues 125–175) prevailed in 86% (13/15) over those specific to the other two epitopes during infection. However, only a negligible increase in HA2-specific antibodies was detectable following vaccination with a current subunit vaccine.

Conclusions We observed that the antigenic site localized within N-terminal HA2 residues 125–175 was more immunogenic than that within residues 1–38 (HA2 fusion protein), although both are weak natural immunogens. We suggest that new anti-influenza vaccines should include HA2 (or specific epitopes localized within this glycopolypeptide) to enhance their cross-protective efficacy.

Keywords Anti-HA2 antibodies, epitope specificity, HA2 epitopes, human convalescent serum, influenza A infection, virus-specific antibodies.

Introduction Influenza A viruses are known to cause acute respiratory disease in humans, spreading in the form of repeated epidemics or pandemics. However, the course of infection with newly emerged influenza viruses, or the threat posed by them, cannot be predicted. Because of the high antigenic variability of influenza A viruses, the efficacy of current vaccines against influenza is limited and vaccines need to be updated yearly. For this reason, a new approach to vaccine preparation has been developed,1–6 which is based on epitopes shared by influenza A viruses of different hemagglutinin (HA) subtypes able to induce cross-protective antiviral immunity. Much attention has been focused on the virus membrane protein, M2,7–10 while several other recent studies have looked at the cross-protection induced by HA2 glycopolypeptide (HA2) – the antigenically conserved part of HA.11–16 Protective HA2-specific antibodies are noted to recognize both sequential and conformational epitopes.11,15,17–20

The antibody response induced by the HA glycoprotein during influenza infection is directed against the variable immunodominant HA1 glycopolypeptide (HA1) with the receptor-binding site responsible for virus attachment to the cell surface. Virus-neutralizing (VN) antibodies recognizing epitopes close to this receptor site effectively block virus attachment and thus play an important role in antiviral defense. These antibodies represent only a fraction of the whole spectrum of anti-HA antibodies induced during influenza infection, and their specificity is narrow, because
of the high variability of the HA1. Indeed, VN antibodies do not effectively neutralize the infectivity of drifted epidemic strains even within a subtype. However, antibodies specific to other parts of HA, including those which are specific to the relatively conserved, fusion-active HA2, are also induced during influenza A infection.

HA2 glycopolypeptide constitutes part of the stem of the HA homotrimer and plays an important role in virus entry. It is responsible for the fusion of viral and endosomal membranes, enabling the release of ribonucleoprotein into the cytosol and its transport into the nucleus. Anti-HA2 antibodies do not prevent attachment of the virus to the cell surface; nevertheless, they do contribute to a milder course of influenza infection. It has been reported that the fraction of antibodies specific to the HA2 increases significantly after repeated infection of mice with antigenically different viral strains of the same subtype. HA2-specific antibodies have also been detected in convalescent sera from humans with a confirmed influenza infection.

Because of the broad cross-reactivity of HA2-specific antibodies and intra- or inter-subtype sequence homology of HA2 epitopes, HA2 is the subject of several studies focused on designing a vaccine with a broader spectrum of efficacy against influenza. Antibodies specific to three of four antigenic sites located on the fusion-active HA2 subunit actually inhibit that fusion activity and protect mice against lethal influenza infection. Furthermore, antibodies recognizing conformational epitopes on the stem of the HA trimer and antibodies specific to the fusion peptide have a similar effect in vitro and in vivo.

To determine the immunogenicity of particular independent antigenic sites on HA2, we investigated the quality and specificity of antibodies induced in humans by HA2 during both natural influenza infection and vaccination.

We examined forty-five paired acute and convalescent sera derived from patients of differing ages all of whom were confirmed to have an influenza infection (A/Fujian/411/2002(H3N2)-like virus) during the epidemic season 2003/2004. Titers of specific antibodies were determined using the hemagglutination-inhibition (HI) test, while titers of virus-specific antibodies and antibodies specific to the influenza virus HA2 were evaluated by ELISA. Epitope specificity was assessed in convalescent sera with sufficiently increased levels of antibodies induced by HA2.

To determine and quantitate epitope-specific anti-HA2 antibodies, inter-subtype cross-reactive monoclonal antibodies (MAbs) recognizing three different antigenic sites on HA2 were used. Levels of virus- and HA2-specific antibodies induced in naturally infected human patients were compared to levels found in individuals vaccinated before the influenza season.

Material and methods

Human sera
Paired acute and convalescent human sera derived from patients of differing ages, confirmed as having an influenza A (H3N2) infection during the 2003/2004 season, were obtained from the Public Health Authority of the Slovak Republic. Acute serum was obtained from blood samples taken from patients at the point when clinical symptoms were presented, while convalescent serum was obtained 3 weeks later. A second, separate set of serum samples was obtained from volunteers vaccinated with one dose of recommended subunit influenza vaccine before the 2003/2004 epidemic season. Post-vaccination sera were obtained 3 weeks after vaccines were administered.

Antigens
Preparation and purification of the EHA2 construct comprising HA2 N-terminal residues 23–185 of the influenza A/Aichi/2/1968 (H3N2) virus was carried out as described previously.

Another antigen, a fusion peptide, represented a 38-amino acid region of the HA2 N-terminus of influenza A/Memphis/2/1985 (H3N2) strain, held under the accession number ABD61777 (IIGFGAIAGFIEGNGMVD GWYGRHRQIQSSEGTQGAADL 38). This sequence corresponded to that of H3N2 strains circulating during the 1985 influenza season. Fusion peptide (purity, 94.8%; molecular weight, 4059-44) was synthesized and supplied by ProImmune (Oxford, UK).

Viruses
Influenza A/Fujian/411/2002 (H3N2)-like virus, A/Wyoming/3/2003 (H3N2), a representative of influenza A viruses circulating during the 2003/2004 epidemic season, was used as the antigen in ELISA binding tests. Virus was propagated in fertilized chicken eggs and purified by differential gradient centrifugation in sucrose (10–50% w/w).

Monoclonal antibodies
Monoclonal antibodies specific to the HA2 of influenza virus A/Dunedin/4/73 (H3N2) were prepared as described previously. Hybridomas originating from splenocytes of BALB/c mice immunized with HA2 produced specific antibodies that were cultured in DMEM with 20% bovine fetal calf serum. These specific antibodies were purified from the culture medium by affinity chromatography on protein A-Sepharose, using standard methodology.

Hemagglutination-inhibition test
Inhibition of agglutination of turkey erythrocytes was performed using a standard method described elsewhere. Before testing, human sera were treated with a
receptor-destroying enzyme (RDE) from *Vibrio cholerae* according to instructions supplied (DENKA). Briefly, sera were incubated overnight with RDE solution (1:3, v/v) at 37°C and then heated at 56°C for 30 minutes in a water bath to inactivate non-specific inhibitors.

**ELISA binding test (ELISA)**

Levels of IgG antibodies in human sera specific to the HA2 polypeptide and also those specific to the whole virus (referred to as “virus-specific antibodies”) were detected by ELISA using appropriate antigens from the following: (i) purified influenza virus A/Wyoming/3/2003 (H3N2) – 300 ng/100 µl/well; (ii) purified EHA2 (aa 23–185 HA2) – 30 ng/100 µl/well; or (iii) synthetic fusion peptide – 500 ng/100 µl/well. Antigens were diluted in phosphate-buffered saline (PBS) and adsorbed to microtitration plates overnight at 4°C. After saturation of plates with blocking buffer (1% nonfat dry milk in PBS), twofold dilutions of human sera were added (100 µl/well). After incubating at room temperature for 90 min, antibodies bound to the solid phase were detected using Rabbit anti-Human IgG/HRP conjugate (Dako). Antigen–antibody reactions were visualized by adding ortho-phenylenediamine and 0.03% H₂O₂ in McIlvaine buffer, pH 5, and optical density was measured at a wavelength of 492 nm.

**Competitive enzyme immunoassay (competitive EIA)**

To characterize the epitope specificity of anti-HA2 antibodies present in human sera, competitive EIA was performed using antigenic peptides (EHA2 or fusion peptide) in solid phase. Prior to performing competitive assays, the amount of antigen was optimized to ensure a suitable sensitivity detection system (EHA2, 30 ng/100 µl; fusion peptide, 500 ng/100 µl). Monoclonal antibodies recognizing particular antigenic sites (MAb IIF4, FC12 or CF2) were used as competitors at a concentration of 1000 ng/100 µl.

Binding of antibodies in human sera was detected in the presence of a particular competitor MAb and compared to the control (lacking the competitor). Changes in binding of human antibodies to HA2 or to the fusion peptide in the presence or absence of the competitor were evaluated. Where antibodies specific to the epitope recognized by the MAb were present in human sera, we observed a decrease in binding of human antibodies to the antigen in solid phase (HA2 or fusion peptide) as they were outcompeted by the MAb.

**Mathematical evaluation of results**

ELISA serum titers were estimated as the reciprocal of the serum dilution at the point where the regression line drawn through three experimental points of the titration curve (closest to the cut-off value) intercepted the cutoff line. A cutoff value 0.5 was chosen arbitrarily. The same evaluation was adopted for competitive EIAs. The fraction of anti-HA2 epitope-specific antibodies in human paired sera was calculated based on the titration of acute and convalescent sera diluted in the presence or absence of the competitor MAb. The fraction of antibodies specific to a given epitope was calculated as:

\[
\text{Fraction} = \frac{\text{titer without competitor} - \text{titer in the presence of competitor}}{\text{titer without competitor}}.
\]

**Results**

**Antibody titer increase during influenza infection in relation to patient’s age**

Antibodies recognizing regions located close to the receptor-binding site on viral HA influencing red blood cell agglutination represent a fraction of the spectrum of virus-specific antibodies induced during an influenza infection. All the patients participating in this study responded to an influenza infection by an increased level of virus-specific antibodies. On average, a 26-fold increase was observed (Figure 1). Similarly, the level of HI antibodies was noted to increase in the sera of all patients who overcame the infection, with the exception of one patient whose acute serum already displayed a high HI antibody titer (not documented). There was a trend for less effective induction of both virus-specific and HI antibodies in older patients (Figure 1), although we acknowledge that a higher number of serum samples would be required for statistical evaluation to support this proposition.

**Induction of HA2-specific antibodies during influenza infection**

As HA2 is a conserved antigen, we supposed that the level of anti-HA2 antibodies would increase significantly during repeated exposure to epidemic strains of influenza, and particularly to those of the same HA subtype. To investigate this, we compared the titers of anti-HA2 antibodies in convalescent sera with those of acute sera in 45 patients. We found increased levels of anti-HA2 antibodies after virus infection in the sera of 33 of these 45 individuals (Figure 2A). On average, a 2.2-fold increase in anti-HA2 titer was detected, although sporadic incidences of greater than fivefold increases were observed. A slightly higher increase in anti-HA2 titer (on average fourfold) was observed in six patients in the over 40 age-group, but there was no significant difference in the levels of titer increase observed in patients of various age-groups (Figure 2B). No correlation between the increase in anti-HA2 and virus-specific antibody titers was found (evaluated by Mann–Whitney rank test).
Spectrum of antibodies specific to the particular epitopes of HA2

Competitive EIA was used to evaluate the epitope specificity of anti-HA2 antibodies induced in humans during influenza infection. We have previously identified four independent antigenic sites (I to IV) on the HA2 molecule. Monoclonal antibodies recognizing sites I, II, and IV protect mice against a lethal dose of influenza virus, while MAb specific to site III does not. In the study described here, we used protective HA2-specific MAb recognizing antigenic sites I (MAb CF2), II (MAb IIF4), and IV (MAb FC12) as competitors in the EIA test. Site I is a part of the HA2 fusion peptide located at residues 23–38, while sites II and IV are localized within residues 125–175. These MAb competed with antibodies of corresponding specificity present in human sera for binding to the HA2 antigen (i.e., EHA2 or synthetic fusion peptide).

Of 45 paired acute and convalescent sera, only 15 convalescent samples exhibited a sufficiently increased level of anti-HA2 antibodies to be suitable for further specificity analysis (Figure 3A). The fraction of antibodies specific to individual antigenic sites could not be evaluated in acute sera because of the generally low level of anti-HA2 antibodies.

We quantified the fraction of antibodies specific to particular HA2 antigenic sites in convalescent serum samples, revealing that in 13 of 15 cases, the level of antibodies specific to the epitope recognized by MAb IIF4 was higher than that of antibodies specific to the epitope recognized by MAb FC12 (Figure 3B). To determine the presence of antibodies specific to the CF2 epitope in human sera, a synthetic fusion peptide was employed as the antigen (HA2 residues 1–38). A positive reaction with the fusion peptide in ELISA, that is, greater than cutoff (background +3sd), was demonstrated in 15 convalescent sera, although only
one patient’s serum exhibited a detectable increase (2-6-fold) in these antibodies (Figure 4). Antibodies specific to the CF2 epitope present in this convalescent serum represented about 50% of all fusion peptide-reactive antibodies.

**Hemagglutination-inhibiting and HA2-specific antibody responses elicited in vaccinated individuals**

The induction of HA2-specific antibodies by vaccination was analyzed in paired sera of eight human volunteers. All individuals were vaccinated prior to the epidemic season 2003/2004 with one dose of the subunit vaccine recommended for use during that season (“Influvac”). Acute and convalescent sera described earlier originated from the same influenza season. The main criterion for the efficacy of anti-influenza vaccine is the induction of HI antibodies. We therefore compared the levels of HI antibodies induced by vaccination with Influvac \((n = 8)\) with the titers of antibodies induced during a natural infection \((n = 45)\).

As illustrated in Figure 5, both vaccination and a natural influenza infection induced increased levels of HI antibodies (3- and 14-fold, respectively). In contrast, only a negligible increase was observed in anti-HA2 antibody levels following vaccination compared to the approximately twofold increase observed after a natural infection.

**Discussion**

HA2 glycopolypeptide-specific antibodies have been shown to contribute to protection against influenza infection. They reduce replication of the virus both *in vitro* \(^{34,35,43}\) and *in vivo* \(^{11,16,36}\) and accelerate recovery from the disease. However, effective protection requires induction of enough specific antibodies with a sufficiently high effective binding affinity. HA2 glycopolypeptide-specific antibodies are regularly elicited in mice during influenza infection, and the
proportion of these antibodies increases significantly after repeated infection with antigenically different virus strains of the same HA subtype. The aim of this study was to investigate the level of HA2-specific anti-influenza antibodies induced in humans during a natural influenza infection and to identify their epitope specificity.

The fraction of anti-HA2 antibodies induced during influenza infection was evaluated in patients of different age-groups. Humans are typically exposed to influenza viruses several times during their lives, so that HA2-specific antibodies were already present in the acute human sera we examined. We anticipated an increase in HA2-specific antibodies particularly in the sera of adult patients repeatedly exposed to influenza infections. However, such an increase was actually only detected in serum samples from some patients. In the majority of patients, a negligible increase or no change of serum anti-HA2 antibody titers was recorded, although a slightly higher titer increase was observed in sera derived from older patients (individuals of over 40 years old).

The HA2 subunit of influenza A HA comprises several epitopes that are conserved within various HA subtypes. We thus set out to evaluate the immunogenic potential of three selected HA2 epitopes in influenza-infected humans. More antibodies were specific to antigenic site II located in HA2 residues 125–175, recognized by MAb IIF4, than to antigenic site IV (an independent antigenic site also located within residues 125–175), recognized by MAb FC12. Antibodies specific to antigenic site I, recognized by MAb CF2, were induced very poorly during influenza infection, with only 33% of convalescent sera containing detectable antibodies specific to this site. An increase in CF2-like antibodies in convalescent vs. acute serum was only observed in one patient. Our data suggest that these particular antigenic sites recognized by protective MAbs are actually relatively weak natural immunogens. Even after vaccination, a negligible increase in titer of HA2-specific antibodies was observed.

The current vaccines effectively protect against epidemic influenza strains, which are antigenically identical or very similar to the vaccination strain. They induce VN antibodies specific to the immunodominant HA1 part of HA. In post-vaccination human sera, only a very slight increase in anti-HA2 antibodies was detected. Thus, enhancement of the immunogenicity of selected HA2 antigenic sites or epitopes leading to a stronger anti-HA2 antibody response should be considered when designing new influenza vaccines.

The ability of the HA2 N-terminus (constituting the fusion peptide) to induce cross-protection against lethal influenza infection in mice has already been demonstrated. Vaccines based on conserved epitopes of influenza virus proteins could moderate the severity of human disease in the period between a new pandemic virus emerging and the availability of an actual anti-influenza vaccine. It is during this critical period that immunity against a new pandemic virus is not yet present in the majority of human population, enabling the infection to spread rapidly and often with devastating impact. Monoclonal antibodies to the three epitopes whose immunogenic potential was examined here reduced influenza virus replication both \textit{in vitro} and \textit{in vivo}. We believe that more effective induction of antibodies targeted to these selected HA2 epitopes could contribute to improved prevention of influenza infections in humans or could at least help to reduce the severity of disease caused by newly emerged influenza viruses.

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