Antibody-Dependent Cell-Mediated Cytotoxicity to Hemagglutinin of Influenza A Viruses After Influenza Vaccination in Humans

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**Background.** Detection of neutralizing antibodies (nAbs) to influenza A virus hemagglutinin (HA) antigens by conventional serological assays is currently the main immune correlate of protection for influenza vaccines. However, current pre-pandemic avian influenza vaccines are poorly immunogenic in inducing nAbs despite considerable protection conferred. Recent studies show that Ab-dependent cell-mediated cytotoxicity (ADCC) to HA antigens are readily detectable in the sera of healthy individuals and patients with influenza infection.

**Methods.** Virus neutralization and ADCC activities of serum samples from individuals who received either seasonal or a stock-piled H5N1 avian influenza vaccine were evaluated by hemagglutination inhibition assay, microneutralization assay, and an improved ADCC natural killer (NK) cell activation assay.

**Results.** Immunization with inactivated seasonal influenza vaccine led to strong expansion of both nAbs and ADCC-mediating antibodies (adccAbs) to H3 antigen of the vaccine virus in 24 postvaccination human sera. In sharp contrast, 18 individuals vaccinated with the adjuvanted H5N1 avian influenza vaccine mounted H5-specific antibodies with strong ADCC activities despite moderate virus neutralization capacity. Strength of HA-specific ADCC activities is largely associated with the titers of HA-binding antibodies and not with the fine antigenic specificity of anti-HA nAbs.

**Conclusions.** Detection of both nAbs and adccAbs may better reflect protective capacity of HA-specific antibodies induced by avian influenza vaccines.

**Keywords.** antibody; antibody-dependent cell-mediated cytotoxicity; hemagglutinin; influenza A virus; vaccines.

Hemagglutinin (HA) is the major glycoprotein expressed on the surface of influenza A viruses. A total of 18 antigenically different HA subtypes have been identified so far, with each sharing 40%–60% amino acid sequence identity [1]. Seasonal H1 and H3 subtypes of influenza A viruses have been circulating among human populations for decades and cause annual influenza epidemics. Recently, emerging avian influenza A viruses, including H5, H7, and H9 subtypes, have caused serious infections in humans and pose a new threat for public health.

Vaccination is an efficient approach to prevent human influenza illness. The current seasonal trivalent inactivated influenza vaccines (TIVs) are highly immunogenic in inducing neutralizing antibodies (nAbs) to the HA antigens [2]. Two types of HA-specific nAbs have been identified so far: conventional nAbs (cnAbs) and broadly nAbs (bnAbs). Conventional nAbs primarily recognize antigenic sites located within the HA globular head [3]. Whereas the globular head undergoes constant antigenic drift, cnAbs are often strain-specific [4]. Nevertheless, detection of cnAbs by conventional serological assays such as hemagglutination inhibition (HI) assay and/or microneutralization (MN) assay is currently the main way of assessing protective capacity after influenza virus infection or vaccination. In addition to strain-specific cnAbs, bnAbs to HA antigens have been identified [5]. These bnAbs primarily target amino acid sequences within the membrane proximal HA stem region that are conserved among diverse HA subtypes [5]. Levels of bnAbs are extremely low or undetectable in human sera after seasonal influenza vaccination [6].

In contrast to seasonal TIV, pre-pandemic avian influenza vaccines containing H5, H7, or H9 subtypes are poorly immunogenic in inducing nAbs [7, 8]. Multiple dosing or coadministration with an adjuvant is generally required to elicit detectable levels of nAbs after immunization [9]. However, it has been shown in animal models that avian influenza vaccines can provide considerable protection despite no or low levels of nAbs induced [10]. This raises an important question of whether detection of cnAbs alone can fully reflect the protective capacity of anti-avian HA Abs after vaccination with current avian influenza vaccines.

In a recent study, Jegaskanda et al [11] reported that pre-existing HA-specific Abs in normal human sera (NHS) possessed cross-reactive Ab-dependent cell-mediated cytotoxicity.
from 24 healthy adults (median age, 32.5 years; range, 21–48) pre- (day 0) and postvaccination (day 20–48) [15]. The panels of paired human sera were tested in the present study.

Hemagglutination Inhibition Assay

Details of 6 HA-specific chimeric mAbs were described previously [15].

Chimeric Monoclonal Antibodies

The HI assay was performed according to the standard procedure using 0.5% turkey red blood cells as described previously [16].

Materials and Methods

Human Serum Samples

One panel of single NHS, sampled from 72 healthy adults (median age, 40 years; range, 20–65) between 1999 and 2006, and 2 panels of paired human sera were tested in the present study. The first panel of paired sera consists of 24 paired sera sampled from 24 healthy adults (median age, 32.5 years; range, 21–48) pre- (day 0) and postvaccination (day 20–21) with 1 dose of 2011–2012 seasonal TIV. The sera were acquired through a contract and received as anonymous samples. Thus, a review by the CDC Institutional Review Board was exempted. The second panel of paired sera were collected from 18 healthy adult volunteers (median age, 41.3 years; range, 30–62) who participated in a clinical trial of an avian H5N1 vaccine under informed consent. The paired sera were sampled pre- (day 0) and postvaccination (day 21–60) after 2 doses of 3.75 µg per dose of AS03-adjuvanted inactivated avian H5N1 vaccine, derived from A/Indonesia/05/2005 virus. Use of the sera in the present study was approved by the CDC National Center for Immunization and Respiratory Diseases human subjects review.

Chimeric Monoclonal Antibodies

Details of 6 HA-specific chimeric mAbs were described previously [15].

Hemagglutination Inhibition Assay

The HI assay was performed according to the standard procedure using 0.5% turkey red blood cells as described previously [16].

Microneutralization Assay

Virus neutralization titers of human sera were determined by a standard MN assay as described previously [16].

Antibody-Dependent Cell-Mediated Cytotoxicity Natural Killer Cell Activation Assay

Antibody-dependent cell-mediated cytotoxicity natural killer (NK) cell activation assay was improved from a flow cytometry-based ADCC method described previously [17]. Ninety-six-well nickel-coated plates (Thermo Scientific) were coated with 200 ng/well of full-length, trimeric, recombinant HA antigens with Histidine Tag (Influenza Reagent Resource) at 4°C overnight. The plates were then washed 5 times with 200 µL/well of sterile 10 mM phosphate-buffered saline (PBS) (pH 7.2). Human serum samples were serially diluted with PBS and added into each well at 100 µL/well. The start dilution was 1:40. The plates were incubated for 1 hour at 37°C and then washed 5 times. Human NK cell lines expressing either high-affinity (158 V/V) or low-affinity (158 F/F) FcγRIIIa receptor and the parental NK-92 control cells were used as effector cells as described previously [18]. Natural killer cells were mixed with appropriately diluted (usually 1:25) phycoerythrin-conjugated mouse anti-human CD107a (BD Pharmingen) in the presence of 1:1500 diluted protein transport inhibitor containing monensin (BD Bioscience). Natural killer cells (5 × 10^5) in 100 µL of the above mixture were then added into each well of the plate and incubated for 4 hours at 37°C. The cells were washed twice and fixed with 250 µL/well 4% paraformaldehyde (Sigma-Aldrich). Data acquisition was performed on an LSR II flow cytometer (Becton Dickenson). The results were expressed as end-point titers, eg, the highest serum dilution that achieved the 3% of the arbitrary threshold. Each serum sample was tested in duplicate. The final titer was the geometric mean titer (GMT) of the duplicate titers. Evaluation of human NK cell lines as effector cells and the arbitrary threshold of the assay are described in detail in the Supplementary Material.

Enzyme-Linked Immunosorbent Assay

Total influenza HA-specific immunoglobulin (Ig)G Abs in human sera were determined by an enzyme-linked immunosorbent assay (ELISA) method described previously using the same recombinant HA antigens as described above as coating antigens [19].

Results

Assessment of Both Neutralizing Antibodies (Abs) and Ab-Dependent Cell-Mediated Cytotoxicity Abs to Hemagglutinin Antigens of Influenza A Viruses in Human Sera

We developed an improved ADCC NK cell activation assay utilizing human NK cell lines as effector cells (Supplementary Figures 1 and 2). To examine the relation between HA-specific nAbs and adccAbs in human sera, we first measured VN and ADCC activities to HA antigen of a then representative seasonal A/New Caledonia/20/1999 H1N1 virus at the time frame when
a panel of 72 NHS were collected between 1999 and 2006. As expected, sera with a “protective” level of pre-existing nAbs to the seasonal H1N1 virus were common (33.33%) among the 72 sera tested (Table 1). Unexpectedly, a substantially higher proportion of the NHS panel (69.44%) had the H1-specific ADCC titers above the arbitrary 1:160 positive threshold of HA-specific ADCC. Note that the ADCC titers of the 24 sera with pre-existing HI Abs to the H1N1 virus were 4.2-fold higher than the rest of the 48 sera without detectable levels of HI Abs (GMT: 1031 vs 245).

We then measured VN and ADCC activities to the H3 antigen of 2011–2012 seasonal TIV H3N2 vaccine virus in 24 paired sera collected from a cohort of the TIV-immunized healthy adults. As expected from the prescreening of this serum panel, relatively low levels of both nAbs and adccAbs (GMT: 36 and 94, respectively) were detected, with approximately 45% of the 24 prevaccination sera having titers greater than 40 by MN (Table 2). Vaccination with the seasonal TIV led to considerable expansion of both nAbs and adccAbs to the H3 antigen (GMT: 370 and 446, respectively) in the 24 post-vaccination sera, and a high percentage of the 24 prevaccination sera had levels of H3-specific VN and ADCC activities above their respective thresholds of 40 and 160 (100% and 83.83%, respectively).

Finally, we assessed nAbs and adccAbs in 18 paired sera collected from healthy adults who volunteered to receive an AS03-adjuvanted prepandemic H5N1 avian influenza vaccine containing A/Indonesia/05/2005 H5N1 virus antigens. As shown in Table 2, none of the 18 prevaccination sera contained detectable levels of nAbs to the H5N1 vaccine virus. Unexpectedly, almost all of the 18 prevaccination sera (94.44%) had detectable levels of adccAbs to the H5 antigen (GMT: 436). At present, the reasons for a high baseline ADCC titers to the H5 antigen in this serum panel are not known. High ADCC Abs titer to H5N1 and H7N9 avian influenza A viruses were also observed in healthy adults and children in a recent independent study [20].

Table 2. Neutralizing and ADCC-Mediating Antibodies Induced After Seasonal vs Avian Influenza Vaccination

| HA Specificity | Serum | VN GMT (95% CI) | No. Titers ≥40 (%) | ADCC GMT (95% CI) | No. Titers ≥160 (%) |
|----------------|-------|----------------|------------------|------------------|------------------|
| A/Perth/16/2009 | Pre-    | 36 (21–62)      | 11/24 (45.83)   | 94 (66–133)      | 10/24 (41.8)    |
| (H3N2)         | Post-   | 370 (249–549)   | 24/24 (100)     | 446 (248–803)    | 20/24 (83.83)   |
| A/Indonesia/05/2005 | Pre-    | 5 (5–5)         | 0 (0.00)        | 436 (260–729)    | 17 (94.44)      |
| (H5N1)         | Post-   | 62 (32–119)     | 13 (72.22)      | 2785 (1953–3914) | 18 (100)        |

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CI, confidence interval; GMT, geometric mean titer; HA, hemagglutinin; MN, microneutralization; NK, natural killer; VN, virus neutralization.

a Paired serum samples were collected from 24 adult volunteers on pre- (day 0) and post-vaccination (day 20–21) with 1 dose of 2011–2012 inactivated trivalent influenza vaccine containing A/Perth/16/2009 (H3N2) vaccine component, respectively. The sera were tested with MN assay to determine titers of VN antibodies against A/Perth/16/2009 (H3N2) virus and with ADCC NK cell activation assay to determine the H3-specific ADCC activity, respectively.

b Paired serum samples were collected from 18 healthy adult volunteers on pre- (day 0) and post-vaccination (day 21–60) with 2 doses of AS03-adjuvanted H5N1 vaccine (A/Indonesia/05/2005), respectively. The sera were tested with MN assay for VN antibodies against A/Indonesia/05/2005 (H5N1) virus and with ADCC NK cell activation assay for the H5 HA-specific ADCC activity, respectively.
titers, the 13 sera with HI Abs and the 9 sera without HI Abs showed a similar level of ADCC activity to the H1 antigen (903 vs 806) (Figure 1B). The difference was statistically not significant ($P = .5525$).

**Antibody (Ab)-Dependent Cell-Mediated Cytotoxicity-Mediating Capability of Chimeric Monoclonal Abs With a Wide Range of Virus Neutralization Capability**

The data thus far reveal that, at the polyclonal level, HI Abs and HA-specific nAbs were indistinguishable in their capability to induce ADCC in vitro, provided that equivalent amounts of HA-binding Abs were present in the sera. However, this does not rule out the possibility that VN and ADCC are mediated by 2 separate subsets of HA-specific Abs in the polyclonal human sera. Therefore, we used a panel of 6 HA-specific chimeric mAbs to dissect this possibility. All of the 6 mAbs have identical Fc fragments derived from human IgG1 and differ in the Fab portions that recognizes diverse antigenic sites on the globular head of the H1 antigen [15]. As shown in Table 3, 3 of the 6 mAbs (069-A09, 145-D11, and 146-C07) possessed high VN capability (MN titers: 12–24 ng/mL). Two of the mAbs (065-D01 and 065-C05) showed intermediate (16–32-fold lower) VN capability (MN titers: 195–391 ng/mL). Monoclonal Ab 145-C09 had the lowest VN capability among the 6 mAbs tested (MN titer: 3125, 130- to 260-fold lower). However, independent of the differences in the fine antigenic specificity and VN capability, all of the 6 mAbs showed similar strength of ADCC activity (ADCC titers: 12–50 ng/mL).

**Correlation Between Antibody (Ab)-Dependent Cell-Mediated Cytotoxicity-Mediating, Virus Neutralization and Hemagglutinin Antigens-Binding Ads**

Finally, we analyzed the potential correlation between adccAbs, nAb and HA-binding Abs to the seasonal H1N1 virus from the 72 NHS tested above. As shown in Figure 2A, overall, H1-specific ADCC titers are positively correlated with the amount of HA-binding Abs (Spearman coefficient: 0.75). Similar levels of correlation were observed when HA ELISA titers and HI titers from the same serum panel were analyzed (Spearman coefficient: 0.68) (Figure 2B). Note that the titers of HA-specific adccAbs were unable to reach high enough levels among the total HA-binding Ads to achieve a stronger correlation in the serum groups analyzed (Supplementary Table 1).

**DISCUSSION**

In the present study, we improved the robustness of the influenza-associated ADCC assays by incorporating human NK cell lines as effector cells (Supplementary Figure 1). This circumvents a major limitation associated with the usage of fresh human peripheral blood mononuclear cells effectors in this type of analysis [17, 20]: eg, (1) limited sources of fresh human blood donors for routine analysis; and (2) potential interassay variations associated with different blood donor sources with heterogeneous NK cell activation status and uncharacterized phenotypes of FcγRIIIa receptors. In addition, a target-free
surrogate assay for the conventional ADCC assays using influenza virus-infected targets, as proposed originally by Jegaskan-da et al [17], eliminates the potentially inevitable batch-to-batch variations of virus infectivity to target cells.

We then used the improved ADCC NK cell activation assay to evaluate the potential of measuring HA-specific ADCC activities as a possible new addition to the current immune correlates of protection after influenza vaccination. Our results suggest that in vitro measurement of HA-specific ADCC activities may complement conventional serological methods in assessment of the full spectrum of in vitro functionality of anti-HA Abs induced by avian influenza vaccines.

Thus far, the data from us and others have provided sufficient evidence that nnAbs to influenza HA antigens possess the capability to induce ADCC in vitro [12, 21] and in the present work. The question remains whether this subset of anti-HA Abs is biologically relevant in protection against human influenza illness. Several lines of evidence obtained in animal models support this possibility. First, vaccination with either seasonal or avian influenza vaccines often conferred a certain degree of cross-protection against lethal challenges with heterologous viruses in the absence of nAbs [8, 22, 23]. Second, titers of HA-specific nnAbs were correlated with protection observed under such circumstances [24, 25]. Third, passive transfer of HA-specific mAbs or polyclonal, HA-monospecific immune sera without detectable levels of HA-related VN capability led to complete resolution of influenza infection and/or improved viral clearance in the lung of the recipient mice [26–28]. It has become clear that although nnAbs cannot prevent influenza infection, they may reduce severity of clinical influenza illness considerably [22, 29]. Mitigation of laboratory-confirmed human influenza by current seasonal influenza vaccines, including aversion of influenza-associated hospitalization or death, has been well documented in the literature [30, 31]. The exact immune mechanism(s) that correlate with this protective effect are not fully understood at present. It is conceivable that HA-specific nnAbs, alone or together with other humoral immune components such as neuraminidase-inhibition Abs and anti-M2 Abs, may contribute to the observed protection via FcR-dependent mechanisms such as ADCC. Prospective clinical trials may help establish the biological relevance of HA-specific ADCC activities in protection against human influenza illness.

It has been long recognized that HA-specific nAbs, classically measured by HI assay, were positively correlated with the probability of protection among naturally infected or vaccinated individuals [32]. An HI titer of 1:40 is generally considered as an immune correlate corresponding to a 50% reduction in the risk of contracting seasonal influenza in adults. According to this threshold, approximately one third of the 72 NHS tested had pre-existing protective levels of nAbs to the then circulating seasonal H1N1 virus (Table 1). Vaccination with a seasonal TIV boosted nAbs to the H3N2 vaccine component considerably in the 24 healthy adults. Protective levels of H3-specific nAbs were detected in all of the 24 postvaccination sera (Table 2). At present, it is not clear whether both VN and ADCC are required in order for HA-specific nAbs to clear influenza A viruses efficiently in vivo. Earlier studies have shown that immune protection mediated by HA-specific nAbs was independent of Fc fragment-associated functional activities [33, 34].

We noted that 24 of the 72 NHS showed strong activities in both VN and ADCC to the seasonal H1 antigen examined (Table 1). Moreover, the titers of adccAbs in the sera were 4.2-fold higher than those 48 sera without nAbs. At first glance, this seemed to indicate that the subset of nAbs might possess stronger capability to trigger ADCC activities than the nnAb subset. Detailed analyses revealed no evidence to support this assumption. First, 2 subgroups of sera with equivalent titers of H1-binding Abs showed similar strength of ADCC activities to the H1 antigen, independent of nAbs (Figure 1). Second, a panel of mAbs with substantially different VN capability, yet

Figure 2. Correlation between neutralizing, antibody-dependent cell-mediated cytotoxicity (ADCC)-mediating and anti-hemagglutinin (HA)-binding antibodies. A total of 72 normal human sera were tested for titers of neutralizing, ADCC mediating, and HA binding to the H1 specificity of A/New Caledonia/20/1999 H1N1 virus. Correlation between neutralizing, ADCC-mediating, and HA-binding antibodies was analyzed by Spearman correlation analysis using commercial Prism 5 software. All of the titers were expressed in log2 scale.
identical affinity to FcγRIIIa receptor on NK cells, displayed similar strength of ADCC activities (Table 3). These observations demonstrate that both nAbs and nnAbs can trigger equally strong ADCC activities in vitro. In contexts where the main Abs induced are neutralizing, as with the anti-H3 response after seasonal TIV vaccination (Table 2), the titers of adccAbs will mirror the MN titers.

We observed that relatively low levels of nAbs to the H5N1 vaccine virus were detected in the 18 postvaccination sera after vaccination with 2 low doses of H5N1 avian vaccine (Table 2). This result is not surprising, in light of well-documented observations in the literature that inactivated avian H5N1 vaccines were generally poorly immunogenic in inducing nAbs [7, 35]. However, we noted that the titers of anti-H5 adccAbs expanded substantially despite weak induction of nAbs after the H5N1 vaccination. This implies that a large proportion of H5-specific Abs induced were nonneutralizing yet capable of triggering ADCC activities in vitro. Although not tested in this study, this may hold true for other HA subtypes of avian influenza vaccines as well. In fact, it was observed recently in the mouse model that after vaccination with either inactivated or recombinant H7 vaccines, the titers of H7-binding Abs were approximately equivalent to those specific for seasonal H1 or H3 antigens tested in parallel, although the levels of the H7-specific nAbs in the sera were substantially lower [36]. Similar results were also obtained in humans who were vaccinated with H7 influenza vaccines [37]. In addition to HA, other influenza antigens, such as NA and M2, are also capable of triggering ADCC activity that may contribute to cross-protection against influenza. In a recent study, Terajima et al [20] reported a high level of ADCC titers to avian viruses in healthy adults and older children. In this study, whole influenza virus-infected cells were used as targets of which ADCC activity to all surface antigens, including HA, NA, and M2, were detected. Nonetheless, it is worth noting that high ADCC Ab titers to avian influenza A viruses were detected in human sera by both ADCC assay formats ([20] and Table 2).

We found that multiple factors may affect strength of in vitro ADCC activities. The strength of HA-specific ADCC activity is largely associated with the titers of HA-binding Abs in the human sera tested (Figure 2 and Supplementary Table 1) and, to lesser extent, the affinity of FcγRIIIa receptor on NK cells (Supplementary Figure 1B) and IgG subclasses of the HA-specific Abs (Supplementary Table 2). Diverse antigenic sites on the HA globular head did not appear to have a major impact on the strength of HA-associated ADCC activities, because a panel of 6 mAbs with identical Fc fragments derived from a human IgG1 showed similar strengths of ADCC activities, independent of the obvious differences in their VN capability (Table 3). This observation is different from the result of a recent study showing that whereas 5 anti-HA stalk human mAbs induced superior ADCC activities, 3 anti-HA head mAbs tested in parallel failed to do so [38]. At present, we do not know the reasons for this discrepancy, because similar ADCC NK cell activation protocols were used by both research groups. One remote possibility is that the anti-HA head mAbs tested by both groups may happen to belong to 2 different classes of anti-HA nAbs [38]. Whereas our anti-HA head chimeric mAbs might be selected from those that require FcγR effector mechanism to induce ADCC, the 3 anti-HA head mAbs tested by Dillilo et al did not [38]. Nevertheless, it appears that both anti-HA head and anti-HA stalk Abs are able to induce ADCC activities in vitro.

CONCLUSIONS

We wish to point out that HA-specific ADCC activities measured in vitro may not reflect the ADCC activities in vivo after natural influenza infection, which is most likely a well-balanced process modulated by multiple factors under normal circumstances. However, immunopathology associated with high titers of influenza antigen-specific, low-avidity, nnAbs may occur under certain circumstances in nature [39, 40]. It is conceivable that FcR-dependent mechanisms such as ADCC may be involved in the detrimental process. In this regard, monitoring ADCC activities associated with nonneutralizing anti-HA Abs may provide new insights into the immune mechanisms of Ab-enhanced influenza virus respiratory diseases.

Supplementary Data

Supplementary material is available online at Open Forum Infectious Diseases online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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