Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Influenza virus-specific antibody dependent cellular cytoxicity induced by vaccination or natural infection

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ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form 18 November 2016
Accepted 24 November 2016
Available online xxxx

Keywords:
Influenza virus
Antibodies
Natural killer cells
Antibody dependent cellular cytotoxicity

ABSTRACT

Influenza viruses are responsible for substantial morbidity and mortality during seasonal epidemics. Vaccination is the most effective method to prevent infection, however due to antigenic drift of the viral surface protein hemagglutinin (HA), annual influenza virus vaccination is required. In addition to seasonal viruses, certain (avian) influenza A viruses of other subtypes, like H5N1 or H7N9, cause sporadic zoonotic infections. Therefore, the availability of game-changing novel vaccines that induce "universal" immune responses to a wide variety of influenza A virus subtypes is highly desirable. The quest for universal influenza vaccines has fueled the interest in broadly-reactive antibodies specific for the stalk of hemagglutinin (HA) and biological activities of antibodies other than direct virus neutralization, like antibody-dependent cellular cytotoxicity (ADCC). In the present study, we investigated the ADCC response upon influenza virus vaccination and infection in humans using a robust ADCC assay that is based on the use of recombinant HA and a continuous NK cell line that expresses FcγRIII (CD16). This assay offers advantages over existing methods, like ease to perform and possibilities to standardize. We showed that HA-specific ADCC mediating antibodies are induced by vaccination with adjuvanted trivalent seasonal and monovalent H1N1pdm09 inactivated vaccines, and by infection with H1N1pdm09 virus. In addition, the use of chimeric influenza HA with a H1 stem but antigenically irrelevant head domain derived from an avian virus allowed detection of H1-stalk-specific ADCC mediating antibodies. This assay will facilitate the assessment of ADCC mediating serum antibodies after (universal) influenza vaccination or infection and may define ADCC activity as a correlate of (cross-) protection in the future.

1. Introduction

Influenza viruses belong to the family of *Orthomyxoviridae* and are responsible for substantial morbidity and mortality during seasonal epidemics. Vaccination is the most effective method to prevent infection, however antigenic drift of the viral surface protein hemagglutinin (HA) - the major target for virus neutralizing antibodies – necessitates regular updates of the vaccine composition and annual influenza virus vaccination to protect high risk patients from seasonal strains. In addition, introduction of novel subtypes of influenza A virus into the human population (antigenic shift) can lead to pandemic outbreaks, as has occurred three times in the previous century. The first influenza pandemic of the 21st century was caused by a novel influenza A(H1N1pdm09) reassortant virus containing gene segments from avian, human and swine influenza virus origin [1,2].

Natural influenza virus infection engages both the humoral and cellular adaptive immune system, eliciting virus-specific antibody and T-cell responses respectively. In particular cytotoxic T lymphocytes contribute to clearance of infection, but the main correlate of protection from infection with an antigenically matching influenza virus is the presence of neutralizing antibodies directed to HA. Standard methods for measuring levels of serum antibodies to influenza virus are hemagglutination inhibition (HI) and virus neu-

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http://dx.doi.org/10.1016/j.vaccine.2016.11.082
0264-410X/

tralization (VN) assays. HI titers have become the accepted correlate of protection and are widely used to assess vaccine efficacy. An HI titer of $\geqslant$1:40 has been shown to correlate with 50% protection against influenza virus infection in susceptible populations [3]. The HI assay does have limitations though: HI assay results vary depending on the virus strain and the use of animal red blood cells is difficult to standardize, making comparison of results across studies difficult. In addition, antibodies specific to HA-stalk cannot be detected in this assay and human exposure to animal influenza viruses does not consistently lead to detection of HI antibodies.

The HA glycoprotein consists of two domains: the immunodominant but variable globular head domain and the more conserved stalk domain. VN antibodies to HA are typically directed to the head domain and prevent binding of virus to the receptor of target cells [4]. Most HI mediating antibodies are strictly strain specific and fail to recognize drift variants or viruses of other subtypes. While most HA-specific antibodies after influenza vaccination or infection are directed against the head domain, antibodies to the more conserved HA-stalk are also induced. These HA-stalk-specific antibodies can neutralize influenza virus by preventing pH dependent conformational changes of HA in the endosome or display other biological activities that contribute to protective immunity [5].

Because the HA-stalk is relatively conserved, antibodies to this region display a high degree of cross-reactivity. In addition to direct neutralizing activity, these antibodies mediate antibody-dependent phagocytosis (ADP) [6,7], antibody-dependent complement fixation (ADC) [8] and antibody-dependent cellular cytotoxicity (ADCC) [9,10], all of which may contribute to protective immunity. The induction of HA-stalk-specific antibodies has raised considerable interest as a venue to develop universal vaccines [11–14], which in turn fueled renewed interest in alternative modes of action of antibodies, in particular ADCC. In this process, recognition of viral antigens on the surface of infected cells by antibodies can result in specific killing of these cells by NK cells or neutrophils, which are activated by interaction between the Fc portion of the antibodies and the Fcγ-receptorIIIα (FcγRIIIα, CD16) on these cells. It has been shown in mice that the optimal protective effect of HA-stalk-specific antibodies (in addition to direct neutralizing activity) is indeed dependent on the presence of Fcγ-receptors [10,15–19]. In healthy human subjects, ADCC mediating antibodies without overt neutralizing activity, have also been detected [20,21], as well as in experimentally infected non-human primates [22].

To assess the potential of seasonal and candidate universal influenza vaccines to induce ADCC mediating antibodies, the availability of accurate and standardizable assays to determine levels of ADCC mediating serum antibodies is highly desirable. In this study, we detected functional HA-specific ADCC mediating antibodies in serum samples from children and adults pre- and post-vaccination or following infection with H1N1pdm09 virus. Subjects were either vaccinated with an adjuvanted monovalent or trivalent influenza vaccine, or experienced infection with H1N1pdm09 virus. In the subjects that received trivalent vaccine, ADCC mediating antibodies to influenza A/H3N2 and B virus were also detected. Finally, using chimeric influenza HA proteins, we were able to demonstrate the presence of HA-stalk-specific ADCC mediating antibodies after vaccination and natural infection with H1N1pdm09 virus.

2. Results

2.1. Detection of ADCC mediating antibodies

For use in the ADCC assay, purified NYMC-X181 virus (H1N1pdm09 vaccine strain based on A/Cal/07/09) or purified full-length recombinant HA protein was used as coating antigen. As source of NK cells, PBMC or NK92.05-CD16 cells were used. The presence of ADCC mediating antibodies was detected by analyzing NK cells for the degranulation marker CD107a after incubation with antibody bound to viral antigen. When PBMC were used, activated NK cells were identified as LIVE/DEAD$^-$CD3$^-$CD20$^-$CD14$^-$-CD56$^+$CD107a$^+$ cells (Fig. 1A). In the case of NK92.05-CD16 cells, activated NK cells were identified as being LIVE/DEAD$^-$CD56$^+$-CD107a$^+$ (Fig. 1B).

Sera obtained from 5 co-workers positive for H1N1pdm09-specific HI antibodies (EMC1-5, Table 1), a pool of sera obtained from 78 seronegative children, and two intravenous immunoglobulins (IVIG) preparations were analyzed for the presence of ADCC mediating antibodies under the respective experimental conditions. All samples were tested at 3 different dilutions (1:40/1:160/1:640). The use of whole virus resulted in higher percentages of CD107a$^+$ NK92.05-CD16 cells compared to the use of recombinant HA (potentially reflecting non-HA-specific ADCC mediating antibodies). However backgrounds values were also higher when purified virus was used (negative control between 10% and 20% with purified virus vs 0–5% with recombinant HA). Despite differences in background CD107a$^+$ percentages, results obtained with either antigen preparation correlated significantly ($R = 0.8579$, Fig. 1C). Results obtained with NK92.05-CD16 as source of NK cells correlated well with those obtained with PBMC ($R = 0.9351$, Fig. 1D), however with PBMC much lower percentages of CD107a$^+$ NK cells were observed compared to NK92.05-CD16 cells.

Thus, NK92.05-CD16 and recombinant HA perform optimally in the ADCC assay, gave results comparable to those obtained with more traditional reagents, and provide the opportunity to standardize the assay.

2.2. Determination of ADCC endpoint titers and correlation to HI

In an initial attempt to determine the endpoint serum dilution at which CD107a$^+$ cells could still be detected, linearity of the assay proved to be relatively poor (Fig. 1E, dotted lines). Blocking the plates with 5% BSA after coating with viral antigen improved linearity considerably (Fig. 1E, black lines). Subtracting the values obtained using uncoated control plates also reduced background levels sometimes observed with the negative serum pool (Fig. 1E, grey lines).

Of the five serum samples EMC1-5, two IVIG preparations and the negative serum the endpoint H1N1pdm09-specific ADCC titers were determined by 4-fold serially diluting the samples 1:10–1:163,840. An arbitrary cut-off value for presence of ADCC mediating antibodies was set at 5% CD107a$^+$ NK92.05-CD16 cells (Fig. 2A). At 1:10 dilution and occasionally at a dilution of 1:40 lower percentages of CD107a$^+$ cells were observed than at higher dilutions, indicative of a prozone effect. Serum sample EMC1 had the highest endpoint titer (1:10240), IVIG1, EMC2, EMC3 and EMC5 had a titer of 1:2560, whereas IVIG2 and EMC4 had an endpoint titer of 1:640 (Fig. 2B). ADCC endpoint titers correlated significantly with HI titers in these serum samples ($R = 0.8456$, Fig. 2B).

2.3. ADCC mediating antibodies in H1N1pdm09 virus infected adults

Consecutive serum samples from individuals diagnosed with RT-PCR confirmed H1N1pdm09 virus infection, were obtained (CF1-22, Table 1) at day of clinical onset and up to three different time-points after disease (between 1 and 43 days post onset [dpo]). These samples were assessed for ADCC activity at a dilution of 1:160 on plates coated with 400 ng recombinant HA, in the linear part of the dilution-response curve. H1pdm09-specific ADCC mediating antibodies were already detected in 0 dpo serum samples

Please cite this article in press as: de Vries RD et al. Influenza virus-specific antibody dependent cellular cytoxicity induced by vaccination or natural infection. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.11.082