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Research Paper

What Lies Beneath: Antibody Dependent Natural Killer Cell Activation by Antibodies to Internal Influenza Virus Proteins

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ABSTRACT

The conserved internal influenza proteins nucleoprotein (NP) and matrix 1 (M1) are well characterised for T cell immunity, but whether they also elicit functional antibodies capable of activating natural killer (NK) cells has not been explored. We studied NP and M1-specific ADCC activity using biochemical, NK cell activation and killing assays with plasma from healthy and influenza-infected subjects. Healthy adults had antibodies to M1 and NP capable of binding dimeric FcγRIIIa and activating NK cells. Natural symptomatic and experimental influenza infections resulted in a rise in antibody dependent NK cell activation post-infection to the hemagglutinin of the infecting strain, but changes in NK cell activation to M1 and NP were variable. Although antibody dependent killing of target cells infected with vaccinia viruses expressing internal influenza proteins was not detected, opsonising antibodies to NP and M1 likely contribute to an antiviral microenvironment by stimulating innate immune cells to secrete cytokines early in infection. We conclude that effector cell activating antibodies to conserved internal influenza proteins are common in healthy and influenza-infected adults. Given the significance of such antibodies in animal models of heterologous influenza infection, the definition of their importance and mechanism of action in human immunity to influenza is essential.

1. Introduction

Seasonal influenza epidemics result in 30 to 50 million cases of severe illness and 250 to 500 thousand deaths each year mainly in the very young, the elderly and the chronically ill. To prevent influenza infection a trivalent or quadrivalent vaccine, containing inactivated split virion preparations from two influenza A viruses (H1N1 and H3N2) and one or two influenza B viruses, is widely administered especially to high-risk groups (World Health Organization, 2015). Influenza vaccination primarily induces neutralising antibodies (NAbs) against the envelope protein hemagglutinin (HA) (Atmar et al., 2007; Martin Mdel et al., 2010). NAbs bind to HA inhibiting viral attachment to cell surface sialic acid and prevent viral fusion with host cells (Krause et al., 2011; Wang et al., 2010). However the effectiveness of antibody-mediated neutralisation is limited by the continual antigenic drift of the HA protein (Sandbulte et al., 2011; Shil et al., 2011). The accumulation of mutations in HA and re-assortment events between antigenically distinct viruses (antigenic shift) can result in the emergence of influenza variants not recognized by NAbs (Fielding et al., 2011; Hardelid et al., 2011; Yang et al., 2012). To minimize the impact of antigenic change the influenza vaccine is updated annually. Furthermore seasonal vaccination is strain-specific and is only weakly protective against infection with divergent influenza viruses. Global influenza pandemics can occur when there is minimal immunity to novel influenza viruses capable of infecting humans (Peiris et al., 2009).

In addition to neutralisation, antibodies (Abs) mediate a number of other effector functions through their Fc region. These functions include complement activation (O'Brien et al., 2011; Ohta et al., 2011), Ab-dependent phagocytosis (ADP) (Huber et al., 2001) and Ab-dependent

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cellular cytotoxicity (ADCC) (Greenberg et al., 1978; Hashimoto et al., 1983a, 1983b; Vella et al., 1980). Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that play an important role in the elimination of virally infected and transformed cells. ADCC is mediated when the NK cell FcγRIIIa (CD16a) receptor interacts with the Fc region of IgG Abs bound to their viral antigens (Ags) on the surface of virally infected cells. IgG-Fc region binding to and crosslinking of FcγRIIIa leads to NK cell activation, which includes degranulation releasing perforins and granzymes, as well as the secretion of anti-viral cytokines such as IFNγ and TNF. It has been shown that ADCC Abs enhance protection against several clinically important viral pathogens (Laoprasopwattana et al., 2007; Vogt et al., 2011; Xiao et al., 2010). Our group has previously demonstrated that healthy influenza-exposed adults have Abs to HAs from non-circulating strains of influenza in the absence of NAbs. We have also shown that HA-specific Abs lead to NK cell activation and influenza clearance in vitro (Jegaskanda et al., 2013a, 2013b, 2013c, 2014a). There has been a recent interest in ADCC Abs targeting more conserved influenza surface Ags such as the extracellular domain of matrix 2 protein (eM2) (El Bakkouri et al., 2011; Kim et al., 2014; Lee et al., 2014) and the stalk region of HA (DiLillo et al., 2014; Ellebedy et al., 2014; Margine et al., 2013a, 2013b).

Influenza proteins inside the viral envelope are highly conserved across different strains and subtypes of influenza. Two internal proteins that have been extensively studied in the context of influenza infection are nucleoprotein (NP) and matrix protein 1 (M1). NP binds and transports the RNA genome of influenza and M1 is a structural protein that coats the inside of the influenza virion. Both NP and M1 contain well-characterized epitopes for $CD4^+$ and $CD8^+$ T-cell responses to influenza virus infection. Ongoing studies aim to create a heterosubtypic influenza vaccine by specifically inducing T-cell responses to conserved NP and M1 epitopes (Chen et al., 2014; Hessel et al., 2014; Lambe et al., 2013; Lillie et al., 2012). An additional role for Abs against internal influenza proteins is starting to emerge. In animal models, NP-specific IgG and vaccination with whole NP have successfully provided protection against heterologous influenza challenge (Epstein et al., 2005; Lamere et al., 2011a; Ulmer et al., 1993; Wraith et al., 1987). Passive transfer of non-neutralising NP Abs can also fully protect against influenza challenge in mice, but the mechanism of protection has yet to be elucidated (Carragher et al., 2008; LaMere et al., 2011b). NP can also be found on the surface of influenza-infected cells in vitro, along with barely detectable levels of M1 (Bodewes et al., 2013; Virelizier et al., 1977; Yewdell et al., 1981). It is conceivable that non-neutralising Abs to M1 and NP may be involved in clearance of infected cells through Fc mediated effector functions. Improving our understanding of the humoral response to internal influenza proteins may contribute to the development of a universally protective vaccination strategy. Herein we have demonstrated M1 and NP Abs are a common feature of influenza-exposed or infected human adults and these Abs have the ability to trigger activation of NK cells.

2. Materials and Methods

2.1. Donors and Plasma Samples

We studied healthy adults, subjects with clinical influenza virus infection and subjects with experimental influenza virus infection for NK cell activating Abs to internal influenza proteins. Fourteen healthy donors were recruited to donate plasma samples from September 2013 to December 2014. The median age of the donors is 29.5, with a range of 22–52. All of the donors had the trivalent influenza vaccine one to 10 times since 2005. Plasma samples were also taken from four influenza-naïve pigtail macaques (*Macaca nemestrina*) to serve as influenza Ab negative samples. Nine of the healthy donors recruited above and one of the influenza-naïve macaques were used in the recombinant soluble FcγRIIIa ELISA described below. Plasma samples from two of the healthy donors and one macaque were also tested in an NK cell activation assay with purified primary NK cells. Macaque studies were approved by the Commonwealth Scientific and Industrial Research Organizational animal health ethics committee. The WHO Collaborating Centre for Reference and Research on Influenza provided six sera samples from three subjects collected in 2013 with likely A/California/07/2009 (H1N1 pdm09) influenza virus infection. One serum sample was taken at the patient's initial doctor's visit with an influenza-like illness and a second sample was collected approximately two weeks later at a follow-up appointment. Influenza H1N1 pdm09 virus infection was confirmed by a complement fixation test (CFT) and hemagglutination inhibition (HI) assay (Table 1). Plasma samples from 11 volunteers challenged with A/Wisconsin/67/2005 (H3N2) influenza were provided from a previously published study conducted on behalf of the University of Oxford. Initially all participants, aged 18–45, had undetectable serum Abs to the challenge A/Wisconsin/67/2005 (H3N2) strain (HI ≤ 10). The volunteers were infected with A/Wisconsin/67/2005 (H3N2) intranasally while under quarantine. They were monitored for viral shedding, disease symptoms and a HI assay was performed 36 days post-challenge (Table 2) (Lillie et al., 2012). Healthy blood donors provided PBMCs as a source of NK cells in some assays. The relevant human ethics committees approved all studies.

2.2. Intravenous Immunoglobulin (IVIG)

We studied 18 IVIG preparations collected over 7 years from 2004 to 2010 as a representation of pooled IgG from thousands of healthy HIV negative donors (Sandoglobulin, CSL Behring, Australia). The 18 IVIG preparations were made by cold ethanol fractionation and were 96% IgG. Stock IVIG preparations are at a concentration of 60-200 mg/ml and prior to use all preparations were diluted to a concentration of 10 mg/ml in Fetal Calf Serum (FCS) as previously described (Jegaskanda et al., 2014a).

2.3. Cell Lines and Viruses

The NK cell line GFP-CD16 (176V) NK-92 (Gong et al., 1994) was used to perform some of the NK cell activation assays. The parental cells of GFP-CD16 (176V) NK-92 are NK-92 cells from the ATCC (CRL-2407). GFP-CD16 (176V) NK-92 cells have been transduced with a retrovirus to express the high affinity variant of CD16 (V176) in the pBMN-IRES-EGFP vector. On these cells surface expression of CD16 (V176) correlates with GFP expression. GFP-CD16 (176V) NK-92 cells were kindly provided by Dr. Kerry Campbell from the Institute for Cancer Research in Philadelphia, PA.

A549 (ATCC) and CEM.NKr-CCR5 cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were used as targets for cytotoxicity assays. Recombinant vaccinia viruses (rVV) were generated by homologous recombination of vaccinia infected cells with non-conjugative plasmids expressing influenza genes into the vaccinia 7.5 k or TK genes as previously described (Mackett et al., 1984). rVVs containing individual HA, NP or M1 genes from influenza A/Puerto Rico/8/1934 (H1N1) were kindly provided by Drs. Jonathan Yewdell and Jack Bennink (NIH, Bethesda, MD) and expanded by Dr. Weisan Chen

Table 1
Time of pre- and post-seroconversion samples, CFT and HI titre for three naturally influenza-infected patient sera samples received from the WHO Collaborating Centre for Reference and Research on Influenza.

Patient	Time point	CFT result	HI titre H1N1 A/California/07/2009
1	Day 0	<10	<10
	Day 17	>120	80
2	Day 0	10	<10
	Day 16	>320	20
3	Day 0	10	<10
	Day 12	>320	80