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The multifaceted effect of PB1-F2 specific antibodies on influenza A virus infection

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ABSTRACT

PB1-F2 is a small influenza A virus (IAV) protein encoded by an alternative reading frame of the PB1 gene. During IAV infection, antibodies to PB1-F2 proteins are induced. To determine their function and contribution to virus infection, three distinct approaches were employed: passive transfer of anti-PB1-F2 MAbs and polyclonal antibodies, active immunization with PB1-F2 peptides and DNA vaccination with plasmids expressing various parts of PB1-F2. Mostly N-terminal specific antibodies were detected in polyclonal sera raised to complete PB1-F2. Passive and active immunization revealed that antibodies recognizing the N-terminal part of the PB1-F2 molecule have no remarkable effect on the course of IAV infection. Interestingly antibodies against the C-terminal region of PB1-F2, obtained by immunization with KLH-PB1-F2 C-terminal peptide or DNA immunization with pC-ter.PB1-F2 plasmid, partially protected mice against virus infection. To our knowledge, this is the first report demonstrating the biological relevance of humoral immunity against PB1-F2 protein *in vivo*.

Introduction

Influenza continues to be a major public health problem. The World Health Organization (WHO) estimates that in a typical year, 10–20% of the world's population is infected with influenza, resulting in 3–5 million severe illnesses and up to half a million deaths. During pandemics, the losses are even greater. Pandemic global outbreaks have caused severe illness with high mortality rates; the 1918 Spanish outbreak in particular killed at least 20 million people worldwide. Currently, the most efficient strategy for control of influenza is yearly vaccination (Morens et al., 2010; Stohr, 2003; Taubenberger and Morens, 2006).

The flu is caused by the influenza virus which is a segmented, enveloped, and negative strand RNA virus. The genome of influenza virus consists of eight separate RNA segments that code for viral proteins: two envelope glycoproteins-hemagglutinin (HA) and neuraminidase (NA), matrix protein (M1), an ion channel protein (M2), nucleoprotein (NP), three proteins of polymerase complex (PB1, PB2 and PA), non-structural proteins NS1 and nuclear export protein (NEP/NS2), until recently considered to be non-structural protein (Muramoto et al., 2013; Shi et al., 2012). While searching for alternative reading frame peptides encoded by influenza A viruses (IAV) that are recognized by CD8+T cells, a PB1-F2 protein encoded by the (+1) ORF in PB1 gene was discovered (Chen et al., 2001). PB1-F2 is also a nonstructural protein. A third PB1-related protein translated from PB1, N40, has recently been identified. In addition to its mode of translation, PB1-F2 has several unique features. These include its absence from some IAV isolates (Zell et al., 2007), variable expression in the individual infected cells, rapid degradation, mitochondrial localization, formation of a nonselective ion channel (Henkel et al., 2010; Chen et al., 2001; Yamada et al., 2004), and apoptotic or pro-apoptotic properties. Further studies using mouse models support a role for PB1-F2 in pathogenicity and lethality (Alymova et al., 2011; de Wit et al., 2008; McAuley et al., 2010b) probably by disrupting a function of alveolar macrophages (Coleman, 2007). IAVs knocked out for the expression of PB1-F2 were not attenuated in the replication in tissue culture, but their pathogenicity and lethality for mice was considerably reduced. Also, PB1-F2-knockout viruses were cleared from lungs more rapidly and induced earlier immune response to the infection (Zamarin et al., 2006) implying that PB1-F2 played a role in the suppression of immune response responsible for viral clearance. PB1-F2 enhances inflammation during the primary viral infection of mice and increases both the frequency and severity of secondary bacterial pneumonia (McAuley et al., 2007).

During IAV infections, Abs are generated to both structural (*HA, NA, NP, NS2, M1, M2*,) and non-structural protein (*NS1*). Only Abs to HA can efficiently neutralize virus infectivity. Antibodies to PB1-F2 protein are induced in response to influenza A virus infection (Krejnusova et al., 2009). We have shown that PB1-F2-specific Abs

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could be detected via immunoprecipitation or immunofluorescence assays both in immune mouse and human convalescent sera. It has been reported that humans generate anti-PB1-F2 antibodies, as measured using a phage library expressing PB1-F2 sequences (Khurana et al., 2009). It is not known whether these antibodies can modulate the functional outcomes of PB1-F2 expression and course of disease. In the present study, we have used a number of assays for analysis of anti-PB1-F2 antibody responses, focusing on their effect on virus infection. We find that PB1-F2 specific antibodies directed against the C-terminal part of the molecule reasonably protect mice against IAV infection. On the other hand PB1-F2 specific antibodies which are directed predominantly to the N-terminal part of the molecule do not remarkably affect course of infection. Based on this analysis and the data presented in this study, it is clear that the biological relevance of anti-PB1-F2 specific antibodies in IAV infections is complex.

Results and discussion

"*In silico*" analysis predicts that PB1-F2 specific antibodies are most likely induced to the N-terminal part of PB1-F2 molecule.

Initially we applied "*in silico*" analysis for prediction of epitopes (B cell epitopes) recognized by PB1-F2 specific antibodies in immune sera. The complex prediction of beta-turns (Fig. 1A.1), accessibility (Fig. 1A.2), flexibility (Fig. 1A.3), hydrophilicity (Fig. 1A.4),antigenic propensity (Fig. 1A.6), was performed (December 2012http://tools.immuneepitope.org/tools/bcell/iedb_input). Although widely employed immunogenicity prediction algorithm (Kolaskar and Tongaonkar, 1990) (Fig. 1A.5) has predicted antibody binding sites even in the C-terminus, novel BepiPred method using a combination of a hidden Markov model and a propensity scale method determined a major antibody binding site in the N-terminus (Fig. 1A). Five out of six analyses predict antibody binding sites in N-terminal part of the PB1-F2, one in both N-terminal a C-terminal parts of the molecule and one mainly in the C-terminal part.

ELISA with peptides corresponding to various regions of PB1-F2 and immunofluorescence confirmed that N-terminal part of molecule is the major target for specific antibodies.

To validate the outcome of "*in silico*" analysis, PB1-F2 specific serum obtained by immunization of mice with purified PB1-F2-MBP protein was performed. As shown in Fig. 1B, typical serum contained antibodies recognizing in ELISA predominantly N-terminal peptide (3–13aa) and less efficiently also C-terminal peptide (65–87aa). According to "*in silico*" analysis, specific antibody binding sites should also be localized in region 14–41. Regrettably, no peptide(s) corresponding to this region was available. Accordingly, results of peptide ELISA correspond properly with "*in silico*"analysis. Interestingly, PB1-F2 specific MAb AG55 (Fig. 1C) bind only to N-terminal peptide (3–13). We are aware that direct binding of short peptides to the surface of plate partially or completely abolishes their conformation and mobility. Therefore PB1-F2 specific antibodies (recognizing conformational epitopes) could not be detected in such ELISA setting. To exclude direct binding, N-terminally biotinylated peptides derived from PB1-F2 ORF (7–16aa or 62–71aa) were attached to streptavidin pre-coated plate. Streptavidin pre-coated or direct coating of N terminally biotinylated peptides derived from PB1-F2 ORF (7–16aa or 62–71aa) was compared in its ability to interact with IMS anti PB1-F2-MBP (supplementary Fig. 1). Streptavidin pre-coating of wells and capture of PB1-F2 ORF derived peptides (7–16aa, 62–71aa) via biotin even worsened reactivity of all the screened anti PB1-F2-MBP sera. Employing of irrelevant IMS raised after MHV68 infection and irrelevant peptide derived from HA2 ORF (170–178aa) confirmed high specificity and relevance of used ELISA assay. In the experiments that followed we replaced peptide ELISA with indirect immunofluorescence of MDCK cells transiently transfected with pPB1-F2, pNter.PB1-F2, pCter.PB1-F2 and pPB1-F2 Stop 3aa, respectively. Similarly as in peptide ELISA PB1-F2 specific antibodies were detected in the immune sera raised against PB1-F2-MBP. Conformation is well preserved under these conditions. In immunofluorescence experiments the positive staining was detected only in transfected MDCK cells expressing either full length (pPB1-F2) or N-terminal part of PB1-F2 (pNter.PB1-F2) (Fig. 1D upper row). Interestingly, the staining pattern of MDCK cells expressing full length of PB1-F2 was apparently different from staining pattern corresponding to N-terminal part of PB1-F2. We assume that in MDCK cells full length PB1-F2 is localized in mitochondria, however N-terminal part PB1-F2 missing C-terminal mitochondrial localization signal is spread through cytosol and nucleus. Similarly nuclear localization of the PB1-F2 is typical for avian strain derived PB1-F2 (Chen et al., 2010; Kosik et al., 2013). We were unable to detect specific staining of MDCK cells expressing C-terminal part of PB1-F2. Although weak a clear signal was detected in ELISA for C-terminal peptide no staining of cells was seen in cells transfected by pCter.PB1-F2 following incubation with PB1-F2 specific serum obtained by immunization of mice with purified full length PB1-F2-MBP. We believe that this discrepancy can be explained by the higher sensitivity of ELISA as compared with indirect immunofluorescence of transfected cells. To confirm expression ability and transfection efficiency of the pPB1-F2, pNter.PB1-F2, pCter.PB1-F2 samples from the same transfection experiment were stained with PB1-F2 N terminally specific MAb (AG55) or PB1-F2-C terminally specific IMS anti PB1-F2 C_{73-87} –KLH respectively. As expected AG55 positive staining was only observed in the case of full length or N terminal part of the PB1-F2 (Fig. 1D middle row), while positive staining of transfected cells with IMS anti PB1-F2-C_{73-87} –KLH was only observed in the case of full length or C terminal part of the PB1-F2 (Fig. 1D below row). Neither IMSs nor MAb stained cells transfected by pPB1-F2 Stop3aa. To ensure no detectable expression of the PB1-F2 protein after transfection by pPB1-F2 Stop3aa western blot analysis was performed. It was not possible to detect PB1-F2 in cells transfected with pPB1-F2 Stop3aa or cells transfected with insert less plasmid pTriEx4 (Merck) neither with MAb AG55 nor IMS anti PB1-F2-MBP. Strong expression of the PB1-F2 protein was detected both MAb AG55 or IMS anti PB1-F2-MBP in the case of transfection with pPB1-F2 (supplementary Fig. 2). Based on the above presented results we suggest that PB1-F2 specific antibodies bind predominantly to the N-terminal part of the molecule.

Passive immunization with N-terminally specific MAbs has no remarkable effect on the course of infection.

To examine the effect of PB1-F2 specific antibody on virus infection, the mixture of two MAbs AG55 and LB66 was first passively transferred to a group of mice by intravenous rout and the mice were subsequently (within 2 h) challenged by PR8 (1LD50). For positive control, the group of mice were passively immunized with HA specific MAb H17L2, highly efficient in virus neutralization. In negative control the mice received HHV2 specific MAb 499. All MAbs used for passive immunization were of the same isotype. Survival rates and body weight changes were monitored daily. Virus in the lungs was determined by RCA on day 2, 6 and 10 days post infection. Two days post infection the similar titer of virus was detected in lungs of mice passively immunized with the mixture of N terminally PB1-F2 specific MAbs as in mice immunized with a negative control MAb or PBS respectively. As expected in the lungs of mice immunized H17L2 MAb there was almost no virus detected 2 days post infection. Six days post infection the virus in lungs was clearly detected only in mice immunized with negative control MAb and PBS. Ten days post infection, no virus was detected in the lungs of any groups of mice (Fig. 1A). Although weight loss was most remarkable in the