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Virus-neutralizing antibody response of mice to consecutive infection with human and avian influenza A viruses

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Summary. – In this work we simulated in a mouse model a naturally occurring situation of humans, who overcame an infection with epidemic strains of influenza A, and were subsequently exposed to avian influenza A viruses (IAV). The antibody response to avian IAV in mice previously infected with human IAV was analyzed. We used two avian IAV (A/Duck/Czechoslovakia/1956 (H4N6) and the attenuated virus rA/Viet Nam/1203-2004 (H5N1)) as well as two human IAV isolates (virus A/Mississippi/1/1985 (H3N2) of medium virulence and A/Puerto Rico/8/1934 (H1N1) of high virulence). Two repeated doses of IAV of H4 or of H5 virus elicited virus-specific neutralizing antibodies in mice. Exposure of animals previously infected with human IAV (of H3 or H1 subtype) to IAV of H4 subtype led to the production of antibodies neutralizing H4 virus in a level comparable with the level of antibodies against the human IAV used for primary infection. In contrast, no measurable levels of virus-neutralizing (VN) antibodies specific to H5 virus were detected in mice infected with H5 virus following a previous infection with human IAV. In both cases the secondary infection with avian IAV led to a significant increase of the titer of VN antibodies specific to the corresponding human virus used for primary infection. Moreover, cross-reactive HA2-specific antibodies were also induced by sequential infection. By virtue of these results we suggest that the differences in the ability of avian IAV to induce specific antibodies inhibiting virus replication after previous infection of mice with human viruses can have an impact on the interspecies transmission and spread of avian IAV in the human population.

Keywords: avian influenza A viruses; sequential influenza A infection; antibody response; virus-neutralizing antibodies; HA2-specific antibodies; original antigenic sin

Introduction

Severe human infections caused by avian influenza A viruses (IAV) of H5N1 subtype were first reported in the year 1997 (De Jong *et al*., 1997; Subarrao *et al*., 1998; Subbarao and Katz, 2000). Since then, sporadic human infections by avian IAV of H5N1 and other HA subtypes have occurred with a fatality rate of close to 60% (Chen *et al*., 2014; Freidl *et al*., 2014; WHO report, 2014). Thus avian IAV, mainly of H5 and H7 subtypes, as well as originally swine, later human pandemic influenza A(H1N1) pdm09 virus with its avian ancestors, still pose a pandemic threat to humans (Hatta and Kawaoka, 2002; Subarrao *et al*., 2006; Garten *et al*., 2009). The interspecies transmission of IAV is a complex process resulting from many interactions between the virus and the host. Thus, both viral and host factors determine the disease severity, the course of infection and its outcome (Cheung *et al*., 2002; Seo *et al*., 2002, 2004; Webster, 2006; Wong and Yuen, 2006; Sandrock and Kelly, 2007; Chandrasekaran *et al*., 2008; Blazejewska *et al*., 2011; Boivin *et al*., 2011; Gabriel *et al*., 2011; Schmolke *et al*., 2011; Horby *et al*., 2012; Imai and Kawaoka, 2012; Kaplan and Webby, 2013; Gabriel *et*

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Abbreviations: HA = hemagglutinin**;** IAV = influenza A virus**;** MAb = monoclonal antibody; OAS = original antigenic sin; VN = virus-neutralizing

al., 2013). There are however only limited studies on the interaction of avian IAV with the human immune system. Several studies on subclinical infections in humans based on serological analysis of individuals who had been in contact with infected poultry (Chang SY *et al*., 2013; Liu *et al*., 2013) have been reported, but it is difficult to predict the outcome of the exposure of humans to non-adapted avian viruses. The analysis of the reaction of immune system to these inter-species influenza infections could therefore get us closer to understanding the ability of non-adapted IAV to spread among humans and to cause severe disease. The fact that some influenza A (H7N9) viruses isolated from humans displayed features suggesting partial adaptation to mammals (Hui and Hayden, 2014; Wang *et al*., 2014) could be important from the epidemiological point of view. It could help to elucidate how the immune response of humans exposed to avian viruses can influence the IAV virus spread in the human population. The main role in this process is played by virus-neutralizing antibodies which are of narrow strain-specificity (Kostolansky *et al*., 2000). In the case of zoonotic infections, it is exclusively the heterosubtypic immune response that can be effective in the reduction of virus replication. Such heterosubtypic antibody response is targeted at the shared, conserved parts of influenza virus antigens, including HA (Gocnik *et al*., 2008; Varečková *et al*., 2008; Stropkovska *et al*., 2009; Sui *et al*., 2009; Bommakanti *et al*., 2010; Prabhu *et al*., 2010; Wang *et al*. 2010; Janulikova *et al*., 2012; Du *et al*., 2013; Stanekova *et al*., 2013).

In this study we focused on the interaction of the virus and the host immune system and analyzed the antibody response of mice to the avian IAV. By the sequential double infection of mice with human and avian viruses we simulated the situation in nature, when individuals previously infected with human viruses, or vaccinated with influenza vaccine, come into the contact with avian IAV. The main goal was to examine how a previous infection of mice with human viruses influences the induction of antibodies inhibiting the replication of avian viruses.

Material and Methods

Viruses. Human viruses A/Mississippi/1/1985 (H3N2) – "A/Miss" and A/Puerto Rico/8/1934 (H1N1) -"A/PR8" were adapted to mice according to Fislova *et al*. (2009). Avian virus, low pathogenic (LPAI) A/Duck/Czechoslovakia/1956 (H4N6) – "A/Duck" did not replicate in mice even after multiple passaging in mouse lungs, though it was fully replicating *in vitro* in MDCK cells as well as in fertilized chicken eggs. Therefore, a non-adapted A/Duck was used in all experiments. On exposure of mice to this virus, the presence of viral mRNA as well as the synthesis of viral proteins were proven in mouse lungs. Another model virus, the recombinant of highly pathogenic (HPAI) H5N1 A/Viet Nam/1203-2004 (H5N1) "rA/Vietnam" virus, containing HA and NA from A/Viet Nam/1203-2004 of avian H5N1 virus, internal proteins (PB2, PA, NP, NS, M) from A/PR8/1934 H1N1virus and PB1 from A/Texas/1977 (H3N2) virus was attenuated by deleting the multi-basic cleavage site between HA1 and HA2 in HA molecule and was adapted to mice as described before (Fislová *et al*., 2009). All virus stocks used in this work (except rA/Vietnam), originated from the collection of viruses at the Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Virus rA/Vietnam was kindly provided by Dr. Ferko and Dr. Romanova (GHB, Vienna, Austria). Viruses were propagated in 10-day-old fertilized chicken eggs for 48 hr at 35°C. Infectious allantoic fluid was aliquoted and stored at -80°C. The virus titer was evaluated by a standard micro-hemagglutination assay using guinea pig erythrocytes. Purified viruses were prepared from the infectious allantoic fluid by differential centrifugation in sucrose gradients (Russ *et al*., 1976).

EHA2. EHA2 was expressed in *Escherichia coli* BL21 transformed with pLM-1 plasmid encoding the region of aa 23–185 of HA2 gp of recombinant X-31 virus (H3N2). The latter originated from A/Aichi/2/1968 (H3N2) virus, kindly provided by Drs D.C. Wiley and J. Chen, Harvard University, Boston, USA. EHA2 was purified as previously described (Janulíková *et al*., 2012).

Plaque assay. Virus was titrated by a standard plaque assay method (Varečková *et al*., 2003).

Animal experiments. Six-week-old female BALB/c mice (Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used for infection. Mice were infected intranasally under a light narcosis with the appropriate dose of influenza virus (30–50 PFU/mouse or as indicated) in a volume 40 µl/mouse.

Preparation of mouse-adapted viruses. Adaptation of human influenza A viruses A/Miss (H3N2) and A/PR8 (H1N1) to mice has been described before (Fislová *et al*., 2009). Avian influenza virus A/Duck (H4N6) and rA/Vietnam (H5N1) were adapted to mice as follows: Two 6-week old BALB/c mice were infected intranasally under a light anesthesia with 40 µl of allantoic fluid containing virus. Their lungs were harvested 2 days after the infection. Lungs were homogenized in 1 ml of PBS, pH 7.2. Cell debris was sedimented and supernatant (40 µl) was used for further infection. After 6 passages, the viruses were propagated in 10-day old chicken embryos for 48 or 36 hr. The infectious dose of lethal viruses for mice was estimated by the titration of mouse-adapted virus on mice and the median lethal dose (LD50) was determined according to Reed and Muench (1938).

Ethical statement. All animal experiments were performed in accordance with the European Union standards and the fundamental ethical principles including animal welfare requirements were respected. Experiments with avian influenza viruses were conducted under BSL-3 containment, including work with animals.

Sera collection. Blood was obtained from the facial vein of mice before and 14 days after each infection and serum was prepared by standardly used protocol.

Preparation of detector peroxidase-labeled monoclonal antibody (MAb): NP-specific MAb 107L was conjugated to horse-

radish peroxidase according to the protocol described in work by Tkáčová and Varečková (1996).

Virus-neutralization micro-assay. Two-fold dilutions of mouse sera in phosphate buffered saline (PBS, pH 7.2) were mixed with 8 $TCID_{50}$ of virus in a volume ratio of 1:1. After incubation at 25°C for 60 min, the mixture of virus with the appropriate dilutions of serum (100 µl) was added to a confluent monolayer of MDCK cells cultured in a 96-well plate. Infectious virus was adsorbed onto the cells for 45 min at 25°C. Cell monolayers were washed with PBS and 100 µl of serum-free ULTRA-MDCK medium containing 4 µg/ml of TPCK-trypsin was added per well and incubated at 37°C in a humid atmosphere of 5% CO_2. After 18 hr of infection, cell monolayers were washed three times with PBS and fixed with cold methanol at +4°C for 15 min. Replicated virus was detected by HRP-labeled monoclonal antibody 107L-Px specific for influenza A nucleoprotein as previously described (Varečková *et al*., 2002). The reaction was stopped after 1 hr of incubation at 37°C and visualized with the substrate solution (100 µl/well) of 3-amino-9-ethylcarbazol containing hydrogen peroxide (0.03%). After an approximately 30 min. incubation at 25°C, the plates were washed and evaluated microscopically. The distinctly red-colored cells were considered positive for infection. The titer of virus-neutralization antibodies was defined as the reciprocal value of the highest serum dilution, at which differentiated red intracellular staining was still visible.

ELISA binding test. Antigen (purified virus 300 ng/100 µl/well or purified EHA2 30 ng/100 µl/well) diluted in PBS, pH 7.2 was adsorbed to the 96-well microtitration plate overnight at 4°C. Plates were then washed 3 times with PBS, pH 7.2 and virus was treated with the McIlvain solution, pH 5 for 30 min. at 25°C. Subsequently the plates were washed and saturated with 0.5% ovalbumin for 1 hour at 25°C. Serial 2-fold serum dilutions (100 µl/well) were then added and incubated for 90 min at 25°C. After 3 washes (0.02% Tween20 in PBS), a goat anti-mouse IgG conjugated with horseradish peroxidase (GAM-Ig-Px, Bio-Rad) diluted 3000-times in 0.5% ovalbumin was added. Following 90 min incubation at 25°C and 3 washes, the reaction was visualized by the addition of peroxidase substrate solution OPD (o-phenylendiamine) containing 0.03% H_2O_2 and absorbancy at 492 nm was measured.

Results

We focused on specific antibody response induced after the infection of mice with human or/and avian influenza A viruses. The aim of this work was to analyze the level of biologically active antibodies specific to avian IAV following previous exposure to human IAV. Mouse-adapted human viruses of H1 and H3 subtypes and avian viruses of H4 and H5 subtypes were used for infection.

We used two human IAV strains differing in virulence and pathogenicity, A/PR8 of H1 subtype of high virulence and A/Miss of H3 subtype of medium virulence, and two avian IAV strains non-lethal for mice, non-replicating virus A/Duck of H4 subtype and replicating virus A/Vietnam of H5 subtype. In all experiments, mice were primarily infected with a sublethal dose of human virus of H1 or H3 subtype (0.1 LD_{50}). Two weeks after the primary infection, two subsequent identical doses of infectious avian virus (A/Duck or A/Vietnam) were intranasally applied to mice in a 14-day interval. The levels of VN antibodies specific to avian IAV as well as to human viruses were analyzed.

Analysis of the virus-neutralizing antibody response of mice infected with human IAV and subsequently exposed to avian IAV A/Duck (H4N6)

We showed that no VN antibodies specific to A/Duck were induced after a single dose of H4 virus, but a significant VN titer was observed after the second and third infective dose of this virus (Fig. 1). A previous infection of mice with human viruses of H1 or H3 subtype did not significantly influence the level of A/Duck-specific VN antibodies, regardless of the HA subtype of the human IAV used for primary infection. The VN titers ranged between 256 and 1024.

The VN antibodies specific to the human virus used for primary infection (H1 or H3 subtype), were already induced after a single dose of these viruses and their titers reached the values of 1200 and 1600 for H3 and H1 viruses, respectively. Surprisingly, the titer of VN-antibodies specific to the primary human virus increased after the subsequent repeated exposure of mice to the avian virus of H4 subtype and ranged from 2000 to 3200 (Fig. 1).

Antigen-specificity of antibodies elicited after infection with non-replicating avian virus of H4 subtype in mice which had been previously exposed to well-replicating mouse-adapted human IAV of H3 subtype was analyzed by ELISA-binding test. The first group (n = 3) represented mice infected with three repeated doses (6.4 PFU/mouse) of A/Duck (H4N6) virus. The second group represented mice (n = 3) infected with one sub-lethal dose of A/Miss (H3N2) virus and then with an identical dose of avian A/Duck (H4N6) virus. The other two groups of mice were infected according to the same scheme, but with 10-times higher doses of virus i.e. 64 PFU/mouse. The levels of antibodies specific to both viruses used for infection (A/Duck and A/Miss) were evaluated by ELISA-binding test (Fig. 2). We showed that primary infection of mice with A/Miss virus also elicited antibodies which cross-reacted with A/Duck virus and vice versa, regardless of the infectious dose of avian virus.

The cross-reactivity of antibodies induced by the double infection described above could be attributed to the immune stimulation by the highly conserved internal antigens. In these sera, however, a subpopulation of antibodies reactive with HA2 of H3 subtype was also present, and the level of these antibodies increased markedly following infection with heterologous virus of H4 subtype (Fig. 2).

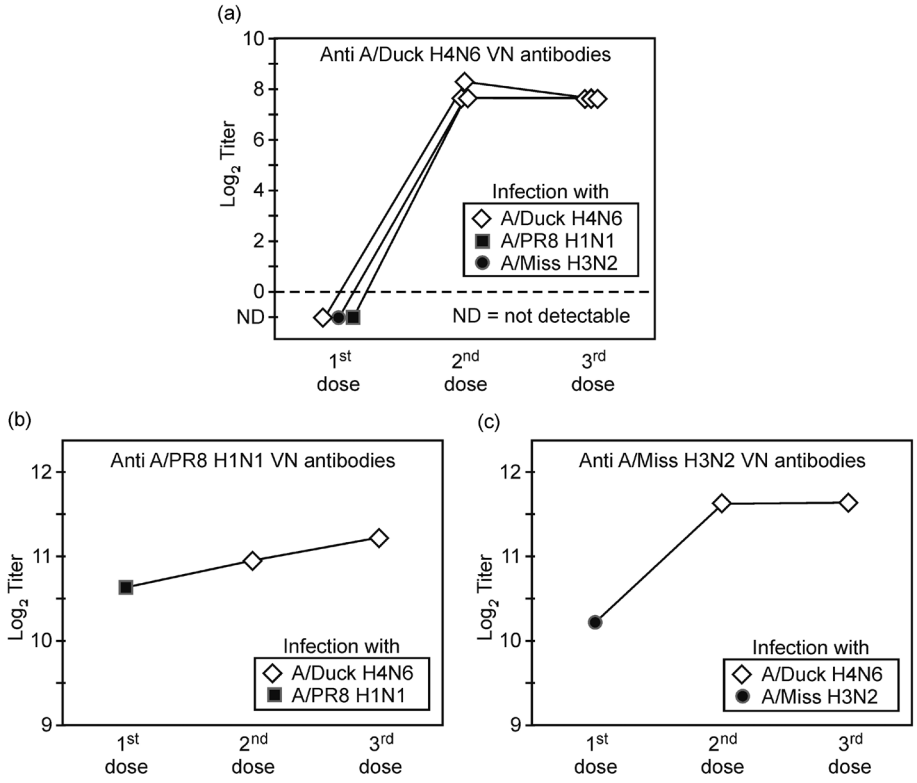

Fig. 1

Titers of virus-neutralizing antibodies specific to virus A/Duck (H4 subtype, avian) (a), A/Miss (H3 subtype, human) (b) or A/PR8 (H1 subtype, human) (c) in sera of BALB/c mice infected with repeated doses of A/Duck virus, or infected with one sublethal dose of human IAV (H1 or H3) and subsequently with two identical doses of avian (H4) virus

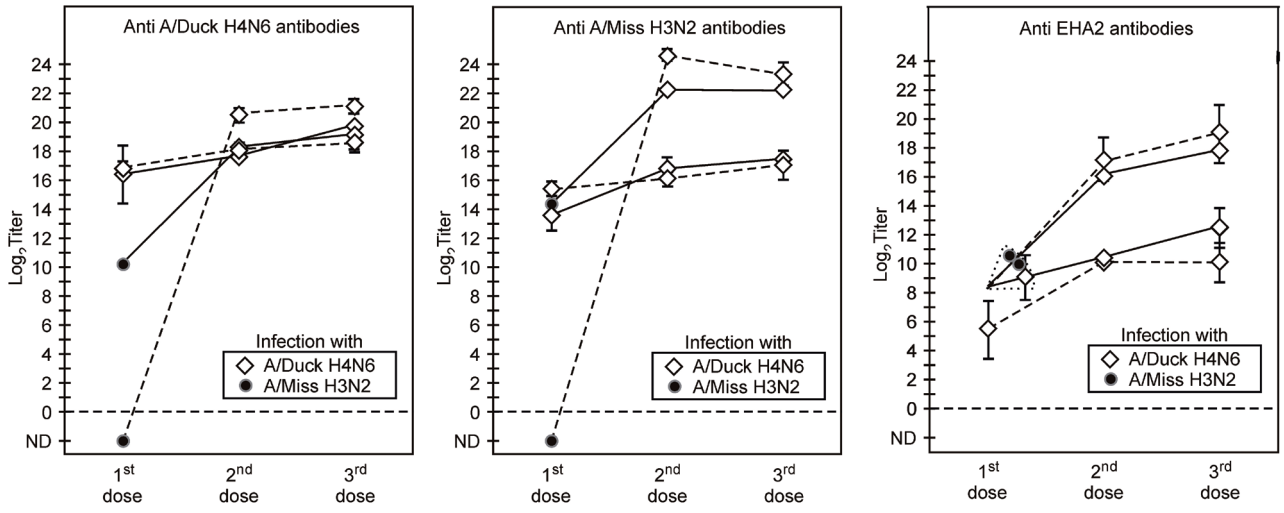

Fig. 2

Titers of virus-specific antibodies induced in sera of mice infected with A/Duck virus or with sublethal dose of A/Miss virus followed by the infection with two doses of A/Duck virus

Titers were estimated by ELISA binding test with purified influenza viruses A/Duck (H4 subtype-avian), A/Miss (H3 subtype-human) and with purified EHA2 of IAV of H3 subtype. The two diferent virus doses are plotted with continuous line (64.0 PFU) or dashed line (6.4 PFU). ND = not detectable.

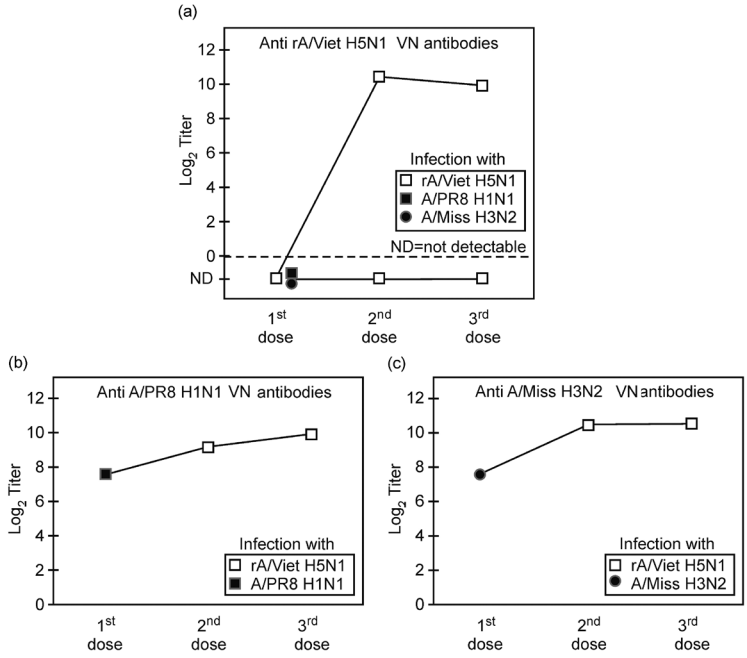

Fig. 3
Titers of virus-neutralizing antibodies specific to virus rA/Vietnam (H5 subtype, avian) (a), A/PR8 (H1 subtype, human) (b) or A/Miss (H3 subtype, human) (c) in sera of BALB/c mice after repeated infection with rA/Vietnam virus, or after infection with one sublethal dose of human IAV (H1 or H3) and subsequently with two doses of avian (H5) virus

Analysis of virus-neutralizing antibody response of mice infected with human IAV and subsequently with avian rA/Vietnam (H5N1) virus

The above scheme of infection was also used for the analysis of antibody response to the H5 virus induced after repeated infection of naive mice with rA/Vietnam (H5N1), or after infection of mice previously exposed to sub-lethal dose of human virus of H1 or H3 subtype and then infected with H5 avian virus. No H5-specific VN antibodies were detected in the sera of mice infected with one dose of H5 virus, but after the second infective dose of this virus, a detectable level of VN antibodies was elicited (Fig. 3). Surprisingly, no H5-specific VN antibodies were detected in the sera of mice infected with H5 virus after their previous infection with human IAV (H1 or H3 subtype). In contrast, the VN antibodies specific to H1 or H3 viruses used for primary infection were elicited in mice already after a single dose of the human virus and their titers increased after the subsequent infection of mice with H5 virus, similarly as it was shown for the H4 virus (see above).

Discussion

In this study we aimed to analyze the level of biologically active avian IAV-specific antibodies after previous exposure of animals to human IAV. Following intranasal infection with an avian non-adapted IAV A/Duck, no infectious virus was detected in mouse lungs. Analysis of biologically active antibodies, however, revealed that antibodies reducing virus replication of H4 virus were produced. The infection of mice with a sublethal dose of human IAV (H3 or H1) followed by infection with non-replicating avian H4 virus resulted in a significant antibody response specific to H4 as well as to H3 or H1 viruses. Strangely, when the infection with human IAV was followed by the infection with avian IAV of H5 subtype, no VN antibodies specific to this avian IAV were detected. This means that individuals could be protected against the human viruses, but their immune system was not able to react by producing VN antibodies specific to the corresponding new avian virus of H5 subtype. This could be related to the ability of H5 viruses to induce a strong cytokine response after infection (Cheung *et al.*, 2002; Wong and Yeng, 2006; Xie *et al.*, 2014) or it could be the impact of the original antigenic sin (OAS). Kim *et al.* (2009) showed that sequential infection of mice with live viruses caused much more profound original antigenic sin than their infection following immunization with whole inactivated IAV vaccine.

OAS is a phenomenon in which prior exposure to an antigen results in a suboptimal immune response to a related new antigen and a very strong immune response to the prior

antigen. We observed this phenomenon in our study also. We showed that the titer of antibodies neutralizing human IAV induced in sera of mice infected with a sublethal dose of human viruses of H3 or H1 subtype increased significantly after subsequent exposure of mice to avian viruses of H4 or H5 subtypes. The production of H4-specific VN antibodies after such double infection was not markedly influenced by the previous exposure of mice to human virus. No H5-specific VN antibodies, however, were elicited after previous infection of mice with human IAV. On the other hand, a population of antibodies reducing the replication of H5 virus, but not clearing it, was induced after the repeated infection with this mouse-adapted avian IAV (results not shown). These results correspond to the observation of Gray *et al.* (2014), who showed that many infections of humans with avian IAV (of subtypes H4, H5, H6, H9 or H12) were subclinical with generally none or only very low titers of VN antibodies having been detected. This phenomenon might influence the process of adaptation of avian viruses to mammalian hosts, including humans.

In our work we showed that a population of cross-reactive antibodies is induced after a sequential infection of mice with human and avian IAV. These antibodies also include HA2-specific antibodies. HA2 gp is a relatively conserved protein, and some HA2 epitopes are even shared among HA subtypes (Kostolanský *et al.*, 2002; Varečková *et al.*, 2008). HA2- specific antibodies recognizing HA stem were shown to be protective (Gocník *et al.*, 2007, 2008; Prabhu *et al.*, 2009; Sui *et al.*, 2009; Wang *et al.*, 2010; Staneková *et al.*, 2011; Janulíková *et al.*, 2012), though they do not mediate virus neutralization by blocking the receptor-binding site on HA. We therefore suggest that anti-stem HA antibodies could help individuals exposed to dangerous avian IAV to overcome the infection in a subclinical manner.

Based on our results, we can conclude that the contact of humans with avian IAV without proved clinical symptoms of disease might be sufficient for the induction of biologically active antibody response. Consequently, these antibodies could represent the immune pressure on the partially adapted viruses of avian origin and fuel the process of their adaptation to mammals or elimination of viruses with limited replication from the human population before their adaptation to the new host.

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