Antibodies induced by the HA2 glycopolyepptide of influenza virus haemagglutinin improve recovery from influenza A virus infection


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The haemagglutinin (HA) of influenza A virus consists of two glycopolyepptides designated HA1 and HA2. Antibodies recognizing HA1 inhibit virus haemagglutination, neutralize virus infectivity and provide good protection against infection, but do not cross-react with the HA of other subtypes. Little is known regarding the biological activities of antibodies against HA2. To study the role of antibodies directed against HA2 during influenza virus infection, two vaccinia virus recombinants (rVVs) were used expressing chimeric molecules of HA, in which HA1 and HA2 were derived from different HA subtypes. The KG-11 recombinant expressed HA1 from A/PR/8/34 (H1N1) virus and HA2 from A/NT/60 (H3N2) virus, whilst KG-12 recombinant expressed HA1 from A/NT/60 virus and HA2 from A/PR/8/34 virus. Immunization of BALB/c mice with rVV expressing HA2 of the HA subtype homologous to the challenge virus [A/PR/8/34 (H1N1) or A/Mississippi/1/85 (H3N2)] did not prevent virus infection, but nevertheless resulted in an increase in mice survival and faster elimination of virus from the lungs. Passive immunization with antibodies purified from mice immunized with rVVs confirmed that antibodies against HA2 were responsible for the described effect on virus infection. Based on the facts that HA2 is a rather conserved part of the HA and that antibodies against HA2, as shown here, may moderate virus infection, future vaccine design should deal with the problem of how to increase the HA2 antibody response.

INTRODUCTION

Influenza A viruses cause an acute respiratory disease. The best way of prevention remains vaccination (Luke & Subbarao, 2006). The main criterion of the effectiveness of influenza vaccines is their ability to stimulate the production of virus-neutralizing antibodies, which are considered to be the most important in defence against influenza virus infection (Palladino et al., 1995; Gerhard, 2001; Tamura et al., 2005). The vaccine strains are recommended by the WHO (2006) based on monitoring of epidemic strains during the 'flu season. Vaccines have to be updated yearly, as their protective potential becomes less effective due to unpredictable and frequent changes in their virus surface glycoproteins (Cox et al., 2003; Lipatov et al., 2004; Stephenson et al., 2004). Therefore, new strategies to extend the protective effectiveness of vaccines against influenza virus infection are continually being looked for.

The current strategy for the development of a 'universal' vaccine with a protective effect against influenza A viruses of various subtypes is based on the conserved antigens shared by influenza viruses of different subtypes, which have the potential to stimulate a protective immune response. The most promising studies have focused on the M2 protein (Neirynck et al., 1999; Mozdzanowska et al., 2003).

The influenza virus HA2 glycopolyepptide (gp) represents a relatively conserved part of the haemagglutinin (HA) molecule (Nobusawa et al., 1991; Gerhard et al., 2006). It is able to induce a specific antibody response during natural infection of humans (Styk et al., 1979) and mice (Kostolánsky et al., 2002). Antibodies induced by HA2 are highly cross-reactive within a given subtype and even among various subtypes of influenza A virus (Becht et al., 1984; Russ et al., 1987; Sánchez-Fauquier et al., 1987; Varečková et al., 2002). In contrast to HA1-specific antibodies, anti-HA2 antibodies do not prevent the haemagglutination mediated by the virus and do not neutralize infectivity of the virus (Becht et al., 1984; Russ et al., 1987; Sánchez-Fauquier et al., 1987). However, some inhibit the fusion activity of influenza virus HA (Varečková et al., 2003a) and reduce virus replication in vitro as well as in vivo (Varečková et al., 2003b; Gocnik et al., 2007).

A supplementary figure showing an example of Western blot titration of sera from mice immunized with rVV KG-11 and KG-12 and challenged with 0.7 LD₅₀ A/PR8 (H1N1) virus is available with the online version of this paper.
After infection, a higher level of antibodies specific to the HA2 is induced than after immunization with purified influenza virus (Fislova´ et al., 2005). Therefore, to study the protective effect of HA2-specific antibodies, we used immunization with HA2 expressed by recombinant vaccinia virus (rVV) (Moss, 1996) to stimulate the complex immune response, similarly to natural infection (Bennink et al., 1984).

To evaluate the contribution of specific antibodies to protection against influenza A virus infection, mice were immunized with rVV expressing influenza HA composed of HA1 and HA2 of different subtypes (H1 and H3) (Gould et al., 1987). The level of specific antibody response and the course of infection in mice immunized with rVV expressing chimeric HA were compared with those that received the wild-type vaccinia virus (wt-VV). To confirm the contribution of HA2-specific antibodies to protection, immunoglobulins induced by rVV expressing HA2 of the subtype homologous to the challenge virus were passively transferred to mice and the course of subsequent influenza infection was evaluated.

**METHODS**

**Viruses and viral antigens.** Influenza A viral strains A/PR/8/34 (H1N1) (A/PR8) and A/Mississippi/1/85 (H3N2) (A/Miss) originated from the collection of viruses at The Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Virus A/NT/60/68 (H3N2) (A/NT60) was obtained from the Sir William Dunn School of Pathology, Oxford University, UK.

**Mouse-adapted influenza viruses.** A/PR8 (H1N1) and A/Miss (H3N2) were obtained after six to nine lung-to-lung passages in inbred BALB/c mice (Kostolansky et al., 2002). The final lung homogenate in 1 ml PBS (pH 7.2) was used for propagation in embryonated hen’s eggs (200 ml per egg). Infectious allantoic fluid was stored in small aliquots at –70 °C.

**Purified viruses.** A/PR8, A/Miss and A/NT60 were prepared from infectious allantoic fluid by sucrose gradient centrifugation as described previously (Russ et al., 1974).

**Vaccinia viruses.** wt-VV CR-19 and rVVs KG-11 and KG-12 expressing the chimeric HA composed of HA1 and HA2 of different subtypes (Fig. 1), prepared as described previously (Gould et al., 1987), were a kind gift by Dr Yewdell (NIH, Bethesda, MA, USA). VVs were cultured on human osteosarcoma 143 TK− cells in Dulbecco’s modified Eagle’s medium (DMEM) with 5 % fetal bovine serum. After 3 days of incubation at 37 °C in a humid atmosphere with 5 % CO2, cells were harvested. The cell sediment was resuspended in 10 mM Tris/HCl (pH 9). Virus was released from the cells after disruption by repeating cycles of freezing and thawing and subsequent sonication. The titre of infectious VVs (expressed in p.f.u. ml−1) was determined by plaque titration on a 143 TK− cell monolayer.

**Immunization and infection of experimental animals.** Six-week-old female BALB/c mice were immunized intraperitoneally with two doses (1 × 106 p.f.u. per mouse each in 0.5 ml) of one of the three VVs with a 2-week interval. Fourteen days after the second dose, mice were infected intranasally in a light ether narcosis with a given infectious dose of mouse-adapted influenza virus A/PR8 or A/Miss. The survival rates were followed until day 14 post-infection (p.i.). Results were evaluated statistically using Fisher’s exact test. Differences were considered to be significant for P<0.05. The titres of infectious virus and the presence of specific viral RNA (vRNA) were determined in the lungs of two mice per group on days 2, 4, 7, 9 and 14 p.i. Macroscopic damage in the lungs (the presence of haemorrhagic and necrotic lesions) caused by virus infection was scored as the percentage of damaged lung tissue.

In all animal experiments presented in this paper, animals were treated according to European Union standards and the fundamental ethical principles including animal welfare requirements were respected.

**Sera collection.** Blood was collected (repeatedly from the same individuals) from the sinus orbitals of three mice in each experimental group 14 days after the first and second immunization and after infection. Sera from three individuals of each group and a particular draw were pooled in equal aliquots.

**Determination of the titre of infectious virus in lungs of mice following infection.** Mouse lungs were homogenized in 1 ml PBS. The titre of the infectious virus in lung suspension was estimated by a modified rapid culture assay (Tkacova´ et al., 1997). Briefly, a confluent Madin–Darby canine kidney (MDCK) cell monolayer grown in a 96-well microtitre plate was infected with twofold dilutions (in PBS, pH 7.2) of lung homogenate (100 µl per well). After 45 min of adsorption at room temperature, the inoculum was removed, 100 µl well per well of serum-free DMEM containing 0.5 µg TPCK trypsin (Sigma) ml−1 was added and cells were incubated overnight at 37 °C in a humid atmosphere with 5 % CO2. Cells were fixed with cold methanol and replicated virus was detected using antinucleoprotein (NP) monoclonal antibody (mAb) 107L (Vareckova´ et al., 1995) and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) as described previously (Gocnik et al., 2007). After addition of the substrate 3-amino-9-ethylcarbazole together with 0.03 % H2O2, infected cells were scored under the light microscope as having distinct, intracellular red staining. The titre of the infectious virus was determined as the reciprocal value of the highest dilution of the tested sample in which infected cells were still detected.

**Detection of vRNA in the lungs of infected mice.** Total RNA was isolated from 200 µl lung homogenate using an RNA Instapur System (Eurogentec) according to the manufacturer’s instructions. The final RNA precipitate was resuspended in 20 µl RNase/DNase-free H2O supplemented with 2 U RNase inhibitor (Fermentas) µl−1. The 20 µl reaction mixture for reverse transcription (60 min at 42 °C) contained 5 µl total RNA, 1 mM each dNTP, 1 × RT buffer (Fermentas), 0.2 µg random heptamer (Invitrogen), 200 U Moloney

![Fig. 1. Chimeric molecules of influenza A virus HA expressed by rVV KG-11 and KG-12 (Gould et al., 1987).](http://vir.sgmjournals.org)
murine leukemia virus reverse transcriptase (Fermentas) and H2O up to the final volume. Amplification of cDNA was carried out using oligonucleotide primers specific for influenza A NP. Primers specific for NP were designed to cover the homology in RNA sequences of H1 and H3 subtypes of influenza A viruses: 5'-GTGAGGATGCACAGCTGGTCTAAC-3' (forward) and 5'-TACCCCTCTTTTGCAAGTGTTAC-3' (reverse) and produced a 599 bp fragment. The PCR mixture of 20 μl contained 2 μl undiluted cDNA, 1 × PCR Master Mix (Fermentas), 10 pmol each primer and H2O up to the final volume. The PCR was performed as described previously (Vareckova et al., 2006). The detection limit of the RT-PCR was 1.12 pg specific cDNA.

Western blotting. Proteins of purified influenza A virus (2 mg ml−1) were separated by 12% SDS-PAGE under reducing conditions. Proteins were electrophoretically onto nitrocellulose membrane (Hybond-C Extra; Amersham Biosciences) in 10 mM Tris/glycine buffer. The membrane was blocked overnight at 4 °C in 10 mM Tris/HCl (pH 7.5) containing 5% glycine and 3% non-fat dried milk. After saturation, the membrane was washed twice with PBS (pH 7.2) and cut into strips. Nitrocellulose strips with the blotted proteins were incubated individually with twofold dilutions of the immune serum in PBS containing 3% non-fat dried milk for 2 h at room temperature and washed with 0.01% Triton X-100 in PBS. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (in 3% non-fat dried milk for 1 h at room temperature and washed with 0.01% Triton X-100 in PBS. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (in 3% non-fat dried milk for 1 h at room temperature) by adding the substrate diaminobenzidine tetrahydrochloride with 0.03% H2O2. After colour development, the reaction was stopped by washing the strips with H2O. The titres of antibodies in the sera were evaluated as the reciprocal value of the highest dilution of serum in which a band of appropriate molecular mass was still detected. To identify the position of the HA1 and HA2 gpl on the nitrocellulose membrane, molecular mass standards were used and immunochromatographic detection was carried out using mAb IF4 specific for HA2 (Russ et al., 1987) or mAb 107L specific for the NP of influenza A virus (Vareckova et al., 1995).

Passive transfer of antibodies elicited by rVV immunization of mice. The IgG fraction from sera of mice immunized with two consecutive identical doses of rVV KG-11, rVV KG-12 or wt-VV CR-19 (as described above) was purified by affinity chromatography on protein A-Sepharose. Six-week-old female BALB/c mice were immunized intravenously with 150 μg purified IgG per mouse and challenged with influenza virus A/Miss (Russ et al., 1987) or mAb 107L specific for the NP of influenza A virus. The survival rates were followed for 14 days p.i. The titre of the infectious virus and specific vRNA was determined in mouse lungs on days 2, 4, 7, 9 and 14 p.i.

RESULTS

Immunization of mice using rVV

To induce a specific anti-HA antibody response (Bennink et al., 1984), BALB/c mice were immunized with two consecutive doses of one of the two rVV (Fig. 1): rVV KG-11, expressing chimeric HA composed of HA1 of influenza virus A/PR8 (H1N1) and HA2 of A/NT60 (H3N2), or rVV KG-12, expressing chimeric HA composed of HA1 of influenza virus A/NT60 and HA2 of A/PR8. The control group of mice received wt-VV CR-19, expressing no influenza A virus-specific protein. After immunization, mice were challenged with influenza virus A/PR8 (H1N1) or A/Miss (H3N2), which are of the same HA subtype as the HA1 and HA2 expressed by the rVV. The effect of the H1/H2-specific antibody response on the course of influenza infection was evaluated.

Survival of BALB/c mice infected with influenza A virus following immunization with rVV expressing chimeric HA

Mice immunized with two identical doses of one of the rVV (see Fig. 1) were infected intranasally with A/PR8 (H1N1). The effect of immunization on infection with three different infectious doses (0.7, 1 and 3 LD50) was compared. Of the mice in the control group infected with 0.7 LD50 and treated with wt-VV (n=14), 64.3% survived the infection; those that succumbed to infection died within 7–11 days p.i. (Fig. 2a). Immunization with rVV KG-11 or KG-12 (n=14 per group) significantly improved the survival of mice infected with influenza A/PR8 virus to 100% (P=0.024). Significant protection was also observed in mice infected with 1 LD50 of A/PR8 virus: 95.2% (P=0.0012) for KG-11 (n=21) and 90% (P=0.0060) for KG-12 (n=20) (Fig. 2b). The protective effect of immunization with rVV KG-12, expressing HA2 of the HA subtype identical to the challenge virus, disappeared when the infectious dose of challenge virus A/PR8 was increased to 3 LD50 (Fig. 2c) (n=20), whilst the significant protection induced by immunization with KG-11 (expressing HA1 identical to the challenge virus) remained at 94% (P=0.0001; n=17). Thus, HA2 (expressed by rVV) induced a specific immune response with the protective potential observable at doses of the challenge virus of less than approx. 3 LD50.

To determine whether immunization with rVV also conferred protection to mice against infection with an influenza A virus of the H3 subtype, immunized mice (n=8 per group) were infected with the virus A/Miss (H3N2). This virus was used because mouse-adapted virus A/NT60 (H3N2), the HA1 and HA2 of which were expressed by rVV, is not lethal for mice. For sensitive detection of the protective effect of immunization with rVV expressing HA2 of the corresponding subtype, we used an infectious dose of challenge virus of 0.7 LD50. In the control group immunized with wt-VV, 37.5% of mice died between 10 and 14 days p.i. (survival 62.5%), whilst all mice (P=0.099) immunized with rVV KG-11 or KG-12 survived (Fig. 2d).

Thus, immunization with rVV expressing HA1 and HA2 of the different HA subtypes (H1 and H3) protected mice against lethal infection with an infectious dose close to 1 LD50 of influenza A viruses of both H1 and H3 subtypes, regardless of the composition of the chimeric HA molecule. However, this does not mean that HA2 gp is as effective an inducer of protective immunity as HA1 gp: using a low infectious dose of virus in these experiments (0.7 LD50) allowed sensitive detection of the protection induced by HA2 gp, but restricted the evaluation of differences between the ability of HA1 and HA2 gp to stimulate
At a higher infectious dose of challenge virus (≥3 LD₅₀), the protection induced by HA2 diminished, whilst the protection induced by HA1 identical to the challenge virus remained unchanged (survival not less than 94%) and was significant.

**Kinetics of influenza virus infection of mice previously immunized with rVV expressing chimeric HA**

The levels of infectious virus and vRNA in the lungs were determined on days 2, 4, 7, 9 and 14 p.i. using a rapid culture assay and RT-PCR in the groups of mice infected with the lowest infectious dose of virus (0.7 LD₅₀) where the maximal protection induced by HA2 was achieved. The virus titre in the lungs of mice from the control group, which received wt-VV and were challenged with virus A/PR8 (H1N1), reached a maximum on day 4 p.i. (17 log₂ units) and decreased up to day 9 p.i., with no infectious virus or vRNA detectable on day 14 p.i. (Fig. 3a). The maximum level of infectious virus in the lungs of mice immunized with rVV KG-12, expressing HA2 of the subtype homologous to the challenge virus, was significantly lower and the titre decreased more rapidly (no infectious virus and no vRNA were detected on day 9 p.i.) than in the control group. However, when mice were immunized with rVV KG-11, expressing HA2 of the heterologous HA subtype but HA1 of the same subtype as the challenge virus, no infectious virus or vRNA was present in the lungs of mice throughout the period of examination.

Haemorrhagic and necrotic lesions caused by infection in the mouse lungs peaked on day 7 p.i. (80–100% damage) in the control group and remained until day 14 p.i., when a mild improvement was observed (results not shown). The maximal damage to lungs was lower and delayed (50–70% on day 9 p.i.) in mice that received rVV KG-12 (expressing HA2 identical to the challenge virus A/PR8). The extent of the damage decreased significantly on day 14 p.i. (20–30%). No visible macroscopic changes in the lung tissue were observed in the group of mice immunized with rVV KG-11 (expressing HA1 identical to the challenge virus A/PR8). This was in accordance with the lack of detection of infectious virus and vRNA in the lungs.

The course of virus replication differed when influenza virus A/Miss (Fig. 3b) was used for challenge of immunized mice. In the control group, the maximal titre (about 16 log₂ units) was reached on day 2 p.i. and clearance of infectious virus and vRNA from the lungs was achieved on day 14 p.i. Induction of an immune response specific to HA2 of the HA subtype homologous to that of the infecting virus (the group of mice treated with rVV KG-11) led to more rapid clearance of virus from the lungs in comparison with the control group. In this group, virus and vRNA in the lungs became undetectable on day 9 p.i. Immunization with rVV KG-12, expressing HA1 of the HA subtype homologous to the infecting virus, did not prevent replication of the virus. However, it significantly lowered the virus titre on day 2 p.i. (to about 9 log₂ units) in
comparison with the control group or with the group immunized with homologous HA2 (KG-11): the maximum was delayed by 2 days (reached on day 4, about 13 log₂ units). Infectious virus and vRNA were completely cleared from the lungs by day 9 p.i.

Lower levels of infectious virus in the lungs of immunized mice also resulted in decreased damage to the lung tissue following infection with A/Miss. The high degree of damage (80–90%) in the control group reached a maximum on day 7 p.i. and remained unchanged until day 14 p.i. (data not shown). In the group of mice immunized with KG-11, maximal damage was reached on day 7 p.i. and then improved up to day 14, by which time it had decreased to 10%. In contrast, in the group of mice immunized with rVV KG-12, the impairment of lung tissue was delayed for 2 days (maximum of about 65% on day 9 p.i.) and was approximately 40–50% on day 14 p.i. Thus, immunization of mice with rVV KG-11 or KG-12 increased the rate of clearance of virus from the lungs of infected mice and helped to reduce damage to lung tissue.

**Analysis of the antibody response induced by immunization and infection**

We correlated the course of infection in mice immunized with rVV or wt-VV with the level of specific anti-HA antibodies determined by Western blotting. Sera from three mice per group were collected on day 14 after each immunization step and following infection. The levels of specific anti-HA1 and anti-HA2 antibodies induced by immunization with the rVVs and wt-VV were compared before and after infection with 0.7 LD₅₀ influenza A virus A/PR8 or A/Miss. Antibodies specific for the challenge virus were detected using purified viruses of the homologous HA subtypes and intersubtype-specific antibodies were detected using viruses of the heterologous HA subtype to the challenge virus.

Specific anti-HA1 and anti-HA2 antibodies that reacted with the virus of HA subtype identical to the subtype of the corresponding HA gp expressed by rVV could already be detected after the first immunization and their level increased after the second immunization. No detectable specific anti-HA antibodies were induced after immunization with wt-VV (Figs 4 and 5; a representative Western blot titration is available as supplementary data in JGV Online). Similar results were also obtained by ELISA (data not shown). Subsequent infection of mice with influenza A virus increased the antibody response preferentially against the HA chain of the challenge virus, which was of homologous HA subtype to that expressed by the rVV.

It should be noted that in all sera the titres of the anti-HA1 antibodies detected by Western blotting, in contrast to ELISA (data not shown), were lower than the titres of the anti-HA2 antibodies. This indicated that the majority of antibodies induced by the immunodominant epitopes on HA1 recognized conformational epitopes, unlike the antibodies induced by epitopes on HA2.

After infection with virus A/PR8, levels of anti-HA antibodies in mice immunized with rVV KG-11 did not increase. The explanation for this is that the stimulation of the immune response by HA1 identical to the challenge virus (A/PR8) elicited an antibody response that fully protected mice against infection. In the case of infection of mice with A/Miss after immunization with rVV KG-12, which expressed HA1 that was of the same HA subtype as the challenge virus (A/NT60) but was not identical to it, mice were protected against lethal infection, although virus replication occurred to a limited degree.
After infection with either of the viruses (A/PR8 or A/Miss) and previous stimulation with wt-VV, relatively low titres of anti-HA2 antibodies (1600–6400) were observed. However, infection with either challenge virus following immunization with rVV expressing HA2 of the HA subtype homologous to the challenge virus resulted in a 32-fold higher titre in comparison with the control mice immunized with wt-VV (Figs 4 and 5).

Thus, immunization with rVV expressing HA2 of a subtype homologous to the challenge virus led to improved survival of mice, earlier elimination of virus from the lungs and stimulation of higher levels of HA2-specific antibodies than in the control. Although it is known that rVV expressing influenza virus HA stimulate both T- and B-cell immunity (Bennink et al., 1984), in our subsequent experiments, we studied whether antibodies alone induced by HA2 expressed by rVV contributed to the observed protection.

**Passively transferred antibodies elicited by rVV moderate influenza virus infection of mice**

To verify the role of antibodies induced by chimeric molecules of HA expressed by rVV in the protection of mice against lethal infection (2 LD<sub>50</sub>), purified immunoglobulins from the sera of mice immunized with rVV were applied intravenously to mice prior to infection with A/Miss. In the control group, receiving IgG from mice immunized with wt-VV, none of the mice survived (n=12) (Fig. 6a). When mice (n=11) were passively immunized...
with IgG induced by rVV KG-11, which was specific to HA2 of the HA subtype homologous to the challenge virus (H3), 36.4% (P=0.0373) of them survived the challenge. In the group of mice passively immunized with IgG induced by rVV KG-12 (n=11), specific to HA1 of the same subtype (H3) but not identical to the challenge virus, 45.5% of individuals survived (P=0.0137). The reason why antibodies induced by HA1 of the subtype homologous to the challenge virus were not able to prevent infection can be explained by the antigenic difference between HA1 of A/NT60 expressed by rVV KG-12 and the challenge virus A/Miss. Passive transfer of IgG specific to HA1 or HA2 of the HA subtype homologous to the challenge virus also led to earlier clearance (by 2 days) of infectious virus and vRNA from the lungs in comparison with the control group, which received IgG induced by wt-VV (Fig. 6b). It should be noted that the amount of specific antibody passively transferred to mice prior to infection with influenza virus was comparable to the quantity of antibody in the sera of mice induced after immunization with rVV.

Therefore, we concluded that antibodies elicited by rVVs expressing HA2 of the HA subtype homologous to the challenge virus contributed to the observed protection against influenza virus infection.

**DISCUSSION**

In our studies, we focused on the contribution of HA2-specific antibodies to protection against influenza virus...
We showed that immunization with rVV expressing HA2 of the same HA subtype as the challenge virus (and HA1 of a different subtype) protected mice against lethal infection with a low dose (0.7 LD<sub>50</sub>) of challenge virus and led to earlier clearance (at least 2 days) of infectious virus and vRNA from the lungs of immunized mice than in the control group. The milder course of infection following immunization was also apparent by the lower rate of lung damage. The rate of protection was similar for infection with influenza A viruses of either H1 (A/PR8) or H3 (A/Miss) subtype.

When mice were stimulated with HA2 of the heterologous HA subtype to the infecting virus but with homologous HA1, the kinetics of influenza virus infection differed following challenge with A/PR8 and A/Miss. As could be expected, rVV KG-11, expressing HA1 identical to that of the challenge virus, stimulated highly specific neutralizing antibodies, which completely prevented infection with A/PR8. As a result, we did not detect any infectious virus or vRNA in the lungs of mice immunized with rVV KG-11, and the titres of anti-HA1 as well as anti-HA2 antibodies were not boosted by the infection (Fig. 4). In contrast, following infection with influenza virus of H3 subtype A/Miss, previous immunization with rVV KG-12 expressing HA1 of the same HA subtype (H3) but of an antigenically different strain (A/NT60) did not induce virus-neutralizing antibodies effective in the prevention of infection, but induced antibodies sufficient to protect mice against lethal infection. In this case, the titre of anti-HA1 antibodies increased significantly following infection, indicating that virus replication had occurred. The course of infection in the group of immunized mice was milder than in the control group (wt-VV) (Fig. 3b). In addition, cross-reactive antibodies specific to HA2 (induced by immunization with HA2 of the heterologous subtype), which could also help to manage the course of infection (Fig. 5), were detected in sera from this group of mice.

Immunization with rVV induces both T- and B-cell immunity specific to epitopes on HA (Gould et al., 1987; Bennink et al., 1984). It has been already shown that CD8<sup>+</sup> cells recognizing epitopes on HA2 gp contribute to the specific immune response following influenza virus infection (Gould et al., 1987; Saikh et al., 1995). Here, the role of antibodies in moderation of influenza virus infection was confirmed by passive transfer experiments. We showed that antibodies induced by rVV KG-11 (expressing HA1 of the HA subtype homologous to the challenge virus) or KG-12 (expressing HA2 of the HA subtype homologous to the challenge virus) applied to mice prior to infection with A/Miss resulted in increased survival of mice compared with the control (wt-VV). The protective potential of anti-HA2 antibodies was dependent on the infectious dose. HA2 is a weaker inducer of protective immunity than HA1, as the protective effect of immunization with HA2 diminished at a higher infectious dose (> 3 LD<sub>50</sub>). However, this does not reduce the significance of the observed protective effect of anti-HA2 antibodies, as under physiological conditions the infectious dose should not exceed the lethal dose used in our experimental system. The mechanism of promoting the infection by HA2-specific antibodies differs from that of HA1-specific antibodies. Whilst virus-neutralizing HA1-specific antibodies block the binding of virus to target cells, it is thought that HA2-specific antibodies are effective in the second stage of the replication cycle: fusion of the viral and endosomal membranes (Gerhard, 2001). Another possible mechanism for the reduction of virus replication mediated by HA2-specific antibodies is antibody-dependent cell-mediated cytotoxicity.

**Fig. 6.** Survival of mice infected with 2 LD<sub>50</sub> of influenza virus A/Miss (H3N2) (a) and kinetics of virus infection (b) following passive transfer of purified immunoglobulins from the sera of mice immunized with wt-VV (■), or with rVV KG-11 (○) or rVV KG-12 (▲) expressing the chimeric influenza HA composed of HA1 and HA2 of various subtypes. The significance of survival was evaluated using Fisher’s exact test. The kinetics of virus infection was followed by assessment of the titre of infectious virus in mouse lungs using a rapid culture method on MDCK cells and by the detection of vRNA in lungs of mice by RT-PCR using primers specific for influenza A NP, as described in the legend to Fig. 3.

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When mice were stimulated with HA2 of the heterologous HA subtype to the infecting virus but with homologous HA1, the kinetics of influenza virus infection differed following challenge with A/PR8 and A/Miss.

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The role of HA2-specific antibodies in reducing the severity of influenza A virus infection described here is in accordance with our previous observations that intravenously applied mAbs specific to HA2 (Gocnık et al., 2007) improve the course of infection with influenza A viruses of a given subtype. To achieve a sufficiently high level of HA2-specific antibodies, the immunization requires repeated doses of HA2 expressed by rVV, which has an enhancing effect similar to repeated natural infection with different influenza strains of the same subtype (Kostolanský et al., 2002).

In conclusion, the results of this study indicate that antibodies specific to HA2 are able to lighten the course of influenza infection and to accelerate the recovery process. Taking into account the fact that HA2-specific antibodies are cross-reactive within the HA subtype and some of them also among subtypes, future vaccines should be designed to enhance the anti-HA2 antibody response.

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