DIFFERENCES IN ANTIBODY RESPONSES OF MICE TO INTRANASAL OR INTRAPERITONEAL IMMUNIZATION WITH INFLUENZA A VIRUS AND VACCINATION WITH SUBUNIT INFLUENZA VACCINE

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Summary. – Two antigenically related but different influenza A virus strains of H3N2 subtype, A/Dunedin/4/73 (H3N2) (Dunedin) and A/Mississippi/1/85 (H3N2) (Mississippi), were used for intranasal (i.n.) and intraperitoneal (i.p.) immunization of mice and respective antibody responses were compared. In ELISA, using purified influenza A virus as antigen, the highest titer of antiviral antibodies was observed after a repeated i.n. infection, in which the Dunedin strain was followed by the Mississippi strain and vice versa. Similarly, in virus neutralization (VN) test, the highest titer of VN antibodies was found after a repeated i.n. infection. The subunit vaccine INFLUVAC, when administered intramuscularly (i.m.), induced only a poor antibody response as assayed by ELISA. Moreover, the INFLUVAC vaccination elicited a 100-fold lower titer of VN antibodies than the i.n. infection and an approx a 10-fold lower titer than the i.p. immunization. A repeated INFLUVAC vaccination did not lead to a significant increase of VN antibody titer. Also the antibody response to HA2gp – a conserved part of influenza hemagglutinin (HA) that might contribute to the induction of specific antiviral antibodies – was followed. Similarly to the VN antibody response, the highest HA2 antibody titer was induced after a repeated i.n. infection, whereas the lowest HA2 antibody titer was observed after a single or repeated INFLUVAC vaccination. Overall, the HA2 antibody titers remarkably well corresponded to the VN potential of the examined sera.

Key words: antibodies; ELISA; influenza A virus; vaccine; INFLUVAC; virus neutralization

Introduction

Influenza represents a global epidemiological problem, which affects hundreds of million people yearly. The most frequent cause are spontaneous genetic mutations leading to the antigenic changes accumulated predominantly in viral HA, the major influenza virus antigen involved in specific immune recognition by the host. The decisive role in immune defense mechanisms against influenza plays the antibody response, particularly neutralizing antibodies specific to HA (Wiley *et al*., 1981; Gerhard *et al*., 1981; Caton *et al*., 1982; Wiley and Skehel, 1987). The antiviral T-cell mediated immunity (CD4+, CD8+) can confer to the protective immunity too; the T-cell specificity may be directed against the peptides derived from any viral protein, but nucleoprotein is the most important (Bennink and Yewdell, 1988; Jameson *et al*., 1998; Hilleman, 2002). Therefore the spread of an influenza virus strain is influenced by current immune status of population. A specific immunity to actual strain(s) can be elicited by vaccination. Currently, there are either inactivated vaccines or live cold-adapted vaccines. Inactivated vaccines are trivalent, containing HA and neuraminidase (NA) of current influenza A virus strains of

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Abbreviations: A/Sydney = A/Sydney/5/97 (H3N2), A/Beijing = A/Beijing/262/95 (H1N1); Dunedin = A/Dunedin/4/73 (H3N2); HA = hemagglutinin; HAI = hemagglutination-inhibition; i.m. = intramuscularly; i.n. = intranasally; i.p. = intraperitoneally; Mississippi = A/Mississippi/1/85 (H3N2); NA = neuraminidase; RCA = rapid culture assay; VN = virus neutralization, virus-neutralizing

subtypes H3N2 and H1N1 and influenza B virus. Cold-adapted live influenza vaccines are constructed as virus reassortants composed of the HA and NA genes from currently circulating strains together with the genes originating from an attenuated, cold-adapted donor strain (Kawaoka, 2001). Cold-adapted live vaccines are recommended especially to unprimed individuals and children as they stimulate mucosal and cell-mediated immunity, whereas inactivated vaccines are more suitable for adults and elderly persons. The efficiency of both live and inactivated vaccines for children over 3 years and adults is considered to be virtually identical (Gendon, 2004). It has been reported that the efficacy of inactivated influenza vaccines ranges at 70–90% in healthy adults and at 30–70% in elderly persons (measured by induction of serum hemagglutination-inhibition (HAI) antibodies (Bardiya and Bae, 2005)).

The limited efficiency of current influenza vaccines and narrow specificity to antigenically matched strains are the major problems to be solved. Some attempts to improve the vaccines' immunogenicity included the use of immuno-potentiators (to increase B- and T-dependent immunity) or virosomes (to increase CD8+ CTL-mediated immunity) (Bardiya and Bae, 2005). Other authors have focused their interest on the use of a virus antigen other than HA in the role of possible target of newly designed vaccine: the M2 protein that has been described to contain an immunogenic epitope actively conferring immune protection (Zou *et al*., 2005) and was proposed as a base for a bivalent (human and avian) influenza vaccine (Liu *et al*., 2005). The common principle of these attempts or considerations or ideas is to "enrich" the vaccine with a virus antigen, which is immunogenic, able to elicit protective response and is antigenically conserved to ensure cross-reactivity with different influenza virus strains either within subtype or even between subtypes. From this point of view, besides the mentioned M2 protein, another candidate might appear the HA2gp (light chain) of viral HA. As it is known, HA2gp is a part of HA which is antigenically conserved within an individual subtype and even among different subtypes (Both *et al*., 1983; Sánchez-Fauquier *et al*., 1987; Russ *et al*., 1987; Nobusawa *et al*., 1991; Varečková *et al.*, 2005). HA2gp is responsible for the fusion of viral and endosomal membranes during the early phase of cell infection (Maeda and Ohnishi, 1980; Huang *et al*., 1981; Skehel and Wiley, 2000). Kostolanský *et al*. (2002) have described HA2gp as a strong immunogen for antibody response in mice after natural i.n. infection. Finally, Varečková *et al*. (2003a) have observed a fusion-inhibition activity of monoclonal antibodies (MAbs) to HA2 *in vitro* and described a reduction of virus replication caused by some of these MAbs (Varečková *et al.,* 2003b).

The aim of this work was to compare the immunogenicity of intact, mouse-adapted influenza A virus administered to mice i.n. or i.p. with that of INFLUVAC subunit influenza vaccine administered i.m. Antibodies to all viral antigens ("antiviral antibodies") were determined by ELISA, while VN antibodies (VN antibodies), basically directed to HA, were assayed by VN test. A particular attention was paid to the quantification of HA2 antibodies in the sera.

Materials and Methods

Virus strains. A/Dunedin/4/73 (H3N2) (Dunedin), A/Mississippi/1/85 (H3N2) (Mississippi), A/Sydney/5/97 (H3N2), and A/Beijing/262/95 (H1N1) (obtained from the National Institute for Medical Research, London, UK) were propagated in fertilized chicken eggs and purified from allantoic fluid by sucrose gradient centrifugation (Russ *et al*., 1974). Mouse-adapted influenza A virus strains Dunedin and Mississippi (Kostolanský *et al*., 2002) were used for i.n. immunization.

Recombinant HA2 (aa 23–185) from the A/Aichi/2/68 (H3N2) virus strain expressed in *Escherichia coli* was prepared according to Chen *et al*. (1995) (kindly gifted by D.C. Wiley and J. Chen).

INFLUVAC subunit influenza vaccine (Solvay Duphar B.V., the Netherlands) contained 15 μg HA and 15 μg NA per dose of each of the influenza virus strains A/Sydney/5/97 (H3N2), A/Beijing/262/95 (H1N1), and B/Beijing/184/93-like.

Intranasal immunization of mice. Female 5-week-old BALB/c mice (2 animals in a group) were given i.n. 0.002 LD_{50} of virus (infectious allantoic fluid diluted in PBS pH 7.2) in 40 μl under light ether anesthesis. In the case of repeated immunization, mice received the second dose 21 days after the first one. The mice were bled 14 days after the last virus dose.

Intraperitoneal and intramuscular immunization of mice. Mice of the same strain, sex and age as above (2 animals in a group) were given i.p. 15 μg of purified virus per mouse without adjuvant or i.m. 1/10 of the recommended human dose of the INFLUVAC vaccine (containing 1.5 μg of HA/NA preparation of each virus strain). In the case of repeated immunization, mice were given the same amount of the antigen in the same route 21 days after the first one. The mice were bled 14 days after the last dose.

ELISA of serum antibodies was performed according to Kostolanský *et al*. (2002). Sera from two animals were examined by ELISA in triplicate. A purified homologous virus strain adsorbed onto the wells was used as detecting antigen. The twofold negative control value was chosen as the cut-off level (the limit of positivity). The titer (ELISA titer) was estimated as the reciprocal of the dilution of serum at the intercept of the regression line with the cut-off level. The first positive value point and the two closest neighbor points were chosen to compute the regression line. Standard deviation (SD) of the titer was computed according to the formula: SD (titer) = (average SD of the A values of the points used for computing the regression line)/(slope of the regression line).

Virus-neutralization (VN) test in vitro was a modification of the rapid culture assay (RCA) described by Tkáčová *et al*. (1997). Briefly, confluent MDCK cell monolayer grown in 96-well microplates were overlaid with 100 μl per well of a mixture of 4 $TCID_{50}$ (determinated by RCA) of particular virus strain (Dunedin or Mississippi or Sydney) with twofold dilutions of tested serum, preincubated for 1 hr at room temperature. After 45 mins of adsorption at room tem-

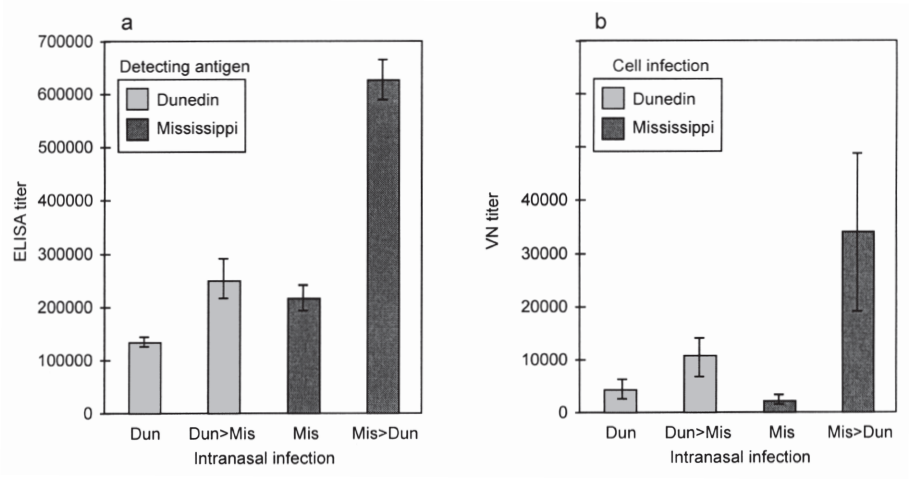

Fig. 1

ELISA (a) and VN (b) titers of antibodies in sera of mice following single i.n. infection with influenza A virus strains Dunedin or Mississippi, or successive infection with Dunedin-Mississippi or Mississippi-Dunedin

Dun = Dunedin; Mis = Mississippi.

perature the cells were washed with PBS and incubated overnight with a serum-free ultra-MDCK culture medium containing 0.5 μg/ml TPCK-treated trypsin (Sigma) in humid 5% CO_2 atmosphere. The cells were then washed, fixed with cold methanol, washed again, and incubated with the detector horseradish peroxidase-conjugated MAb 107L (Tkáčová and Varečková, 1996) diluted in 5% nonfat dried milk for 1 hr at 37°C. The binding of the detector antibody was visualized after adding aminoethyl carbazole with 0.03% H_2O_2. The reaction was scored microscopically. Sera from two animals were examined by VN test in triplicate. The titer (VN titer) was estimated as the reciprocal value of the highest dilution of serum neutralizing the virus infectivity and the SD was calculated in a standard way.

Results

Antibody response to intranasal infection with purified virus

Mice were infected i.n. with the virus strains Dunedin or Mississippi. Although these virus strains belong to the same H3 subtype they are sufficiently evolutionary distant to enable a successive infection of mice. The antibodies (antiviral antibodies) induced by single or repeated i.n. infection were assayed by ELISA. After the single infection, the antibodies were determined using a homologous (immunizing) virus. After the repeated infection (Dunedin-Mississippi or Mississippi-Dunedin) the antibodies bound to each of the two viruses were determined.

ELISA titers (Fig. 1a) showed that both virus strains, Dunedin and Mississippi, in the single infection, represent comparable inducers of antibody response. The repeated infection increased the antibody titer 2–3 times.

The VN titers (Fig. 1b) for the single infection with the virus strains Dunedin or Mississippi were similar, 2,000–4,000. However, the repeated infection increased the VN titer much more strongly, about 20 times. The VN titers after the repeated infection were higher when assayed with the priming virus (results not shown). Apparently, the original antigenic sin phenomenon involves VN antibodies (Francis *et al.*, 1953).

Antibody response to intraperitoneal immunization with intact virus

With the aim to compare the antibody response to natural i.n. infection and that to i.p. immunization we administered an intact purified virus i.p. to mice in a single or two doses. The ELISA titers (Fig. 2a) showed that (i) the antibody response to the single i.p. immunization was lower than that to the i.n. infection (Fig. 1a), and (ii) the repeated i.p. immunizaton enhanced antibody response similarly to the repeated i.n. infection.

The VN titers (Fig. 2b) after the single i.p. immunization were generally very low. Moreover, the repeated i.p. immunization had no effect in contrast to the i.n. infection (Fig. 1b). It follows that the i.n. immunization was highly effective concerning the VN antibody response, while the i.p. immunization with intact virus was ineffective in this respect.

Antibody response to intramuscular vaccination with INFLUVAC vaccine

In these experiments the antibody response of mice to i.m. vaccination with subunit influenza vaccine INFLUVAC was followed. The vaccine contained HA and NA of influenza

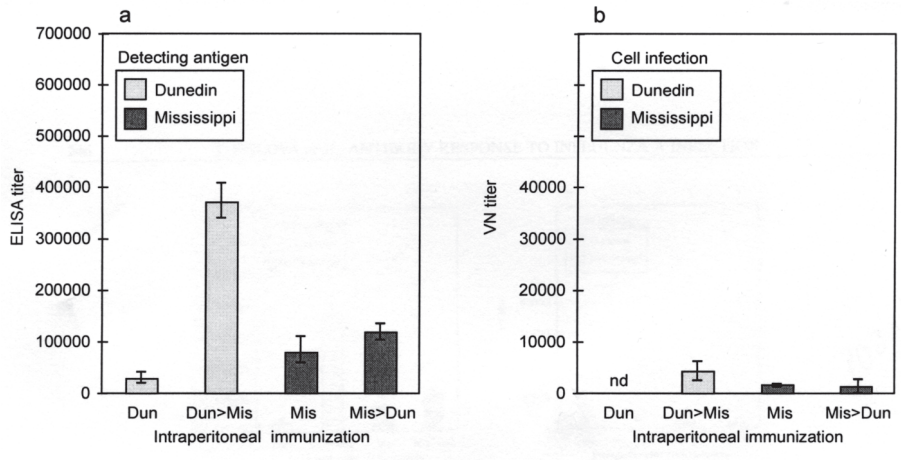

Fig. 2

ELISA (a) and (b) VN titers of antibodies in sera of mice following single i.p. immunization with influenza A virus strains Dunedin or Mississippi, or successive i.p. immunization with Dunedin-Mississippi or Mississippi-Dunedin

nd = not done. For other abbreviations see Fig. 1.

virus strains A/Sydney/5/97 (H3N2), A/Beijing/262/95 (H1N1), and B/Beijing/184/93-like. Each animal was given 1/10 of the dose recommended by the manufacturer for humans, i.e. 1.5 μg of HA and NA of each virus strain without adjuvant per mouse in one or two doses. Antibodies specific to particular virus strain were assayed using A/Sydney/5/97 (H3N2) and A/Beijing/262/95 (H1N1) as antigens. Antibodies specific to B/Beijing/184/93-like were not followed.

The ELISA titers of antibodies indicated that the vaccine induced a significant A/Sydney/5/97 (H3N2)-specific as well as A/Beijing/262/95 (H1N1)-specific antiviral antibody response (Fig. 3a). This was, however, lower than that induced by i.p. immunization (Fig. 2a), despite its repeating. However, it must be taken into account, that the vaccine did not contain internal virus proteins.

Nevertheless, the most important parameter – titers of VN antibodies – was not satisfactory (Fig. 3b). The single immunization resulted in a very low VN titer for A/Sydney/5/97 (H3N2). Neither a repeated immunization led to an apparent increase of VN titer.

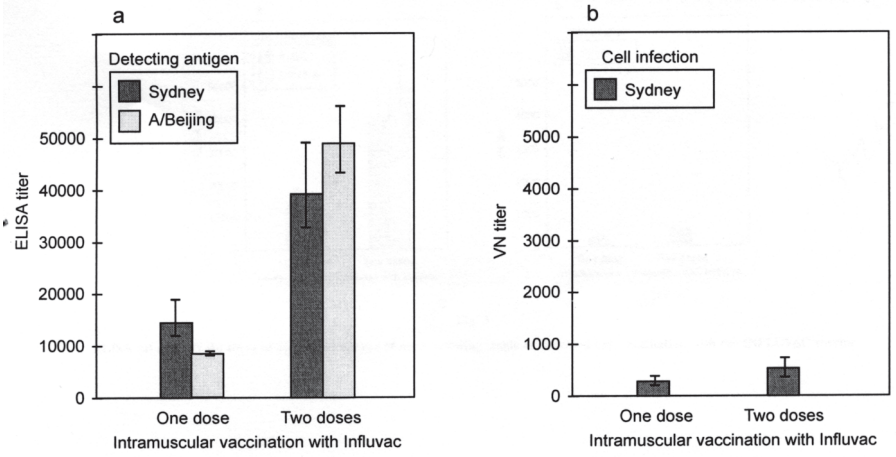

Fig. 3

ELISA (a) and VN (b) titers of antibodies in sera of mice following single or repeated i.m. vaccination with the INFLUVAC vaccine

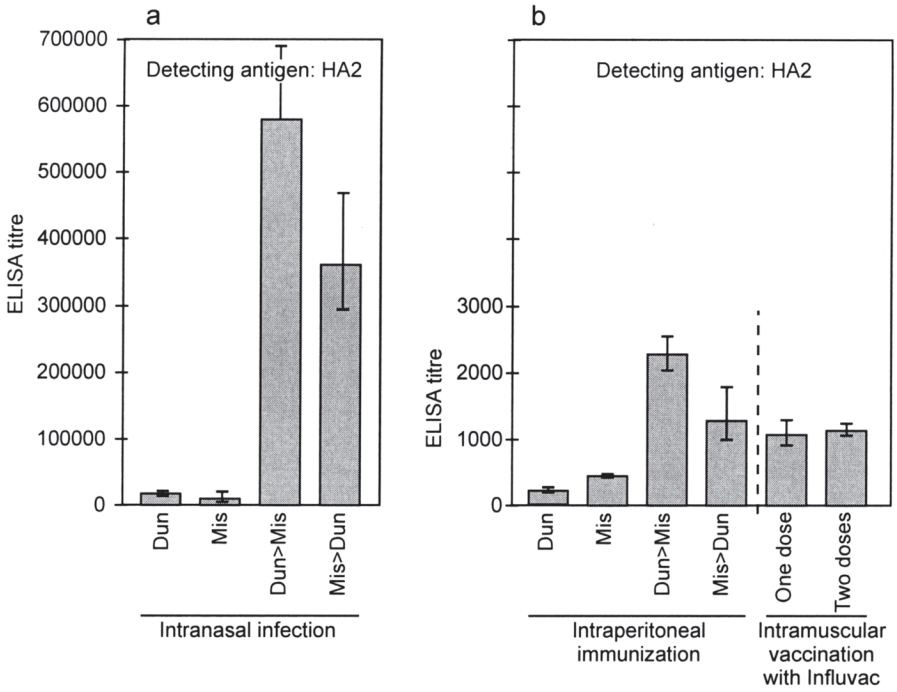

Fig. 4

ELISA titers of HA2 antibodies in sera of mice following three different immunization procedures

For the abbreviations see Fig. 1.

Antibody response to immunization with HA2

All the mouse sera described above were assayed also for HA2-specific antibodies by ELISA. As all these sera contained antibodies specific to viral antigens belonging to the H3 subtype, a purified HA2 derived from the X-31 virus (H3 subtype) was used as antigen in the assay. The titers of HA2 antibodies after each type of immunization described above were low when a single dose of antigen was applied (Fig. 4). The repeated immunization led to increased titers of HA2 antibodies in the case of i.n. and i.p. but not i.m immunization. The most marked increase, about 100-fold was observed after i.n. immunization. A less pronounced increase, about 10-fold was noted after i.p. immunization. In contrast, a non-significant raise was observed with the INFLUVAC vaccine.

In conclusion, a significant increase in the titer of HA2 antibodies was achieved after the repeated i.n. infection, when a complete replication cycle(s) occurred and viral antigens were processed and presented to the immune system.

Discussion

The aim of this work was to evaluate the immunogenicity and effectiveness of the subunit influenza vaccine INFLUVAC applied i.m. in comparison to that of intact influenza virus applied i.n. or i.p. This evaluation was based on quantification of antibodies in mouse sera by ELISA and VN test.

Before drawing any conclusions from this experimental approach it should be stressed that ELISA and the VN test do not assay the same kind of antibodies; whereas ELISA using intact virus as detector antigen assays the antibodies to all viral antigens regardless of their biological activity ("antiviral antibodies"), VN test assays only the antibodies neutralizing the virus used in the test ("VN antibodies"). These antibodies are basically directed to viral HA and represent only a part of antiviral antibodies.

Titration of antiviral antibodies in mouse sera by ELISA showed that the most effective immune response was achieved by i.n. infection. Previous observations have shown that the infectious dose eliciting a significant antibody response may vary from about 1 LD_{50} to 0.0003 LD_{50} (Kostolanský *et al.*, 2002) The dose of 0.002 LD_{50} used in this study was chosen as sufficient for induction of a specific immune response by replicating virus. To verify the effect of a repeated natural infection, we used two virus strains of the same HA subtype but antigenically different to allow the secondary infection. The titers as assayed by both ELISA and VN test raised significantly after the second i.n. virus

dose, suggesting the importance (existence) of a boosting effect on antibody response also in the case of natural infection. In fact, repeated seasonal influenza infections with new epidemic strains of the same subtype lead to a preferential increase of antibody response to the antigenically conserved virus antigens. The results of this study indicate that a repeated infection with an antigenically different virus strain induces predominantly those antibodies, which are directed against the priming virus strain in accord with the principle of the original antigenic sin (OAS) phenomenon (Francis *et al*., 1953). It should be stressed that the OAS phenomenon was clearly manifested also on the VN antibodies described in this study.

The level of these antibodies after the i.p. application of single dose of intact virus was lower than that after natural infection. Two i.p. doses led to significantly higher ELISA titers as compared to single i.p. dose, but still not as high as those obtained with two i.n. doses. This suggests that the i.p. administration of virus was not as efficient as the natural infection. The replication of virus ensures a complete immune processing of viral antigens and, consequently, a complex immune response including mucosal immunity (specific secretory IgA) and induction of cytotoxic T lymphocytes. Hypothetically, we cannot exclude the possibility of a limited local virus replication following the i.p. administration of purified intact virus. However, even though this kind of virus replication would have taken place, the i.p. infection was not sufficient to induce a stronger antibody response than the i.n. infection.

The i.m. administration of the INFLUVAC vaccine elicited a significant specific antibody response, though lower than that resulting from the application of intact virus. The dose administered to mice was adjusted to the 1/10 of human dose, i.e. 1.5 μg of each virus strain HA. Such an amount of antigen roughly corresponded to the amount of HA in the dose of virus strains used for the i.p. immunization (in total 15 μg of viral proteins). A similar dose of inactivated complete virus strain PR/8 (up to 6 μg), used by Zhu *et al.* (2005), elicited an immune response with a comparable antibody titer. Barackman *et al.* (1999) have elicited a significant antibody response in mice by i.n. application of only 1 μg of viral HA, however, in combination with an adjuvant. Our findings showed that the repeated application of INFLUVAC vaccine caused only a slight increase in specific antibody level. It should be taken into account that the INFLUVAC vaccine contained only isolated HA and NA and therefore the post-vaccination sera did not contain antibodies to other (internal) viral proteins.

The prevention of influenza infection is mediated by VN antibodies (O'Neill *et al*., 2000; Gerhard, 2001), basically specific to HA (Lamb and Krug, 1996; Govorkova and Smirnov, 1997; Edwards and Dimmock, 2001; Hilleman, 2002). Therefore, the level of VN antibodies in post-vaccination sera is the main parameter determining the efficiency of particular vaccination. From this point of view our results suggest that the most effective immunization against influenza is i.n., the natural way of infection, especially a repeated one. This kind of immunization elicited not only the relatively (proportionally) highest VN response, but also the VN titers reached the highest values in comparison to other immunization approaches used in this study. This observation might be relevant for the use of a cold-adapted live human influenza vaccine. A yearly seasonal vaccination with actual cold-adapted virus strain may lead to the effect of a repeated exposure to virus with a consequence of high VN response. Since the VN antibody titer determines the efficiency of particular influenza vaccine, the INFLUVAC vaccine can be considered much less effective, even after a repeated i.m. administration. This concerns the absolute value of the titer of VN antibodies as well as its proportion to the titer of antiviral antibodies. However, one must keep in mind that mice immunized with INFLUVAC were immunologically naive, without previous exposure to influenza virus as is usual in humans. This fact could contribute to low antiviral and VN titer values in mouse anti-INFLUVAC sera.

A low efficiency of induction of VN antibodies by a subunit vaccine has been reported by Stephenson *et al.* (2003). In that study, a whole-virus vaccine produced a better immune response than a subunit vaccine. On the other hand, also the route of vaccine administration may influence its effectiveness. Barackman *et al.* (1999) have found i.n. inoculation of an inactivated subunit influenza vaccine to induce a better antibody response than i.m. administration of the same vaccine.

Bardiya and Bae (2005) have reported the efficacy of an inactivated influenza vaccine, measured by induction of serum HAI-inhibition antibodies, to be limited, ranging at 70–90% in healthy adults and at 30–70% in elderly persons. According to Cox *et al.* (2004), an inactivated vaccine induced protection in 60–90% of individuals. Moreover, a commercial inactivated trivalent vaccine poorly induced mucosal IgA antibodies and cell-mediated immunity. A prerequisite for the induction of mucosal and cell-mediated immunity is the use of a live influenza vaccine.

In this study we documented that a single but better a repeated i.n. immunization with live virus induced a strong antibody response to HA2gp. Recent data (Varečková *et al*., 2003a,b) suggest that cross-reactive HA2 antibodies may contribute to the VN response induced by virus. For the future it remains to improve the effectiveness of subunit influenza vaccines or live attenuated influenza vaccines with emphasis on the potentiation of antibody response to those viral antigens which are conserved and able to induce a protective response.

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