CROSS-PROTECTION OF MICE IMMUNIZED WITH DIFFERENT INFLUENZA A (H2) STRAINS AND CHALLENGED WITH VIRUSES OF THE SAME HA SUBTYPE

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Summary. – Cross-protection of mice immunized with inactivated preparations of human and avian influenza A (H2) viruses was determined after lethal infection with mouse-adapted (MA) variants of human A/Jap x Bell/57 (H2N1) and avian A/NJers/78 (H2N3) viruses. The MA variants differed from the original strains by acquired virulence for mice and changes in the HA antigenicity. These studies indicated that mice vaccinated with human influenza A (H2) viruses were satisfactorily protected against challenge with A/Jap x Bell/57-MA variant; the survival rate was in the range of 61% – 88.9%. Immunization of mice with the same viral preparations provided lower levels of protection against challenge with A/NJers/78-MA variant. Vaccination of mice with the avian influenza A (H2) viruses induced better protection than with human strains against challenge with both MA variants. Challenge with A/NJers/78-MA variant revealed that 76.2% – 95.2% of animals were protected when vaccinated with avian influenza virus strains isolated before 1980, and that the protection reached only 52.4% – 60.0% in animals vaccinated with strains isolated in 1980 – 1985. The present study revealed that cross-protection experiments in a mouse model could provide necessary information for the development of appropriate influenza A(H2) virus vaccines with a potential for these viruses to reappear in a human population.

Key words: human and avian influenza A viruses; H2 subtype, cross-protection; mouse-adapted variants; lethal infection

Introduction

A complete protection against influenza virus infection is remarkably difficult to achieve due to continuous antigenic changes of viral glycoproteins. Rapid evolution of influenza A virus genes in humans depends on periodic introduction of genes from avian virus pools (Gorman *et al.*, 1990, 1991). Numerous influenza A virus subtypes and their variants antigenically related to human epidemic strains have been conserved in the avian population (Hinshaw *et al.*, 1982).

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Abbreviations: HA = haemagglutinin, haemagglutination; HAI = haemagglutination-inhibition; i.m. = intramuscularly; i.n. = intranasally; MA = mouse-adapted; MoAb = monoclonal antibody; NA = neuraminidase; NP = nucleoprotein

Among three subtypes of viral haemagglutinin (HA) determined in humans, influenza A (H2N2) viruses have caused a major pandemic in 1957 and kept circulating in human population through 1968. After disappearing from humans, H2 influenza viruses have been isolated sporadically from the avian reservoir (Tůmová *et al.*, 1975; Nerome *et al.*, 1984). Moreover, evidence was presented for an increased prevalence of H2 isolates in wild ducks in North America, preceding the appearance of H2N2 viruses in domestic fowl (Webster *et al.*, 1993). Evolutionary relationships among human and avian H2 influenza viruses revealed different lineages (Govorkova *et al.*, 1991; Schäfer *et al.*, 1993), and it was shown that an antigenically conserved counterpart of human Asian pandemic strain of 1957 continued to circulate in the avian population (Schäfer *et al.*, 1993).

An immune response against influenza infection is subtype-specific (Ada and Jones, 1986). This underlines the

importance of serological data showing that anti-H2 antibodies were present in sera of adults in 4.5% while children were found to be without any immune protection against influenza A (H2) subtype (Govorkova et al., 1993). A large susceptible human population and the presence of H2 influenza viruses in avian species that come into direct contact with humans increases the possibility of the appearance of a new pandemic strain of H2 serotype.

In view of emergence of a new pandemic strain of H2 serotype, it is necessary to evaluate and probably to recommend influenza virus strains to be used for development of appropriate influenza vaccines. It is therefore important to determine the level of protection induced by different human and avian influenza A (H2) viruses. In the present study we immunized mice with inactivated influenza A (H2) virus preparations with antigenically different HA and compared their protective efficacy against lethal challenge with MA variants of the same subtype.

Materials and Methods

Viruses. The following influenza A (H2) virus strains were used in these studies: A/Singapore/1/57 (H2N2) (A/Sing/57); A/Leningrad/678/59 (H2N2) (A/Len/59); A/Leningrad/1468/65 (H2N2) (A/Len/65); A/Tokyo/3/67 (H2N2) (A/Tok/67); A/Leningrad/549/80 (H2N2) (A/Len/80); reassortant A/Japan/305/57 x A/Bell/42 (H2N1) (A/Jap x Bell/57); A/duck Germany/1215/73 (H2N3) (A/Germ/73); A/pintail duck/Primorie/695/76 (H2N3) (A/Prim/76); A/duck/Marseille/46/76 (H2N3) (A/Mars/76); A/pintail duck/Alberta/2728/77 (H2N9) (A/Alb/77); A/black duck/New Jersey/1580/78 (H2N3) (A/NJers/78); A/mallard duck/New York/6686/78 (H2N3) (A/NY/78); A/pintail duck/Alberta/211/80 (H2N3) (A/Alb/80); A/duck/Potsdam/80 (H2N3) (A/Pots/80); A/laughing gull/New Jersey/75/85 (H2N9) (A/NJers/85). The viruses were kindly provided by Dr. R.G. Webster, St.Jude Children's Research Hospital, Memphis, TN, and by Dr. S. Yamnikova, the D.I. Ivanovsky Institute of Virology, Moscow, Russia. The viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C for 2 days. The viruses were purified by centrifugation through 25% – 70 % sucrose density gradients (Skehel and Schild, 1971).

MA variants of two pathogenic influenza virus strains – human A/Jap x Bell/57 (H2N1) and avian A/NJers/78 (H2N3) – were prepared by 22 serial lung-to-lung passages. For the 1st passage, an undiluted allantoic fluid was used, while for subsequent passages, 10% lung suspensions prepared from mice 48 hrs post inoculation were used.

Inactivation. Whole influenza A (H2) virus preparations were inactivated by the technique of Budowsky et al. (1993). Briefly, purified viruses (protein concentration of 100 µg/ml) were treated with β-propiolactone (Merck) in 0.015 mol/l phosphate buffer at 20°C for 15 mins. The reaction was stopped by the addition of sodium thiosulphate at final concentration of 0.04 mol/l. The efficacy of virus inactivation was checked by virus titration in embry-

onated chicken eggs. The treatment resulted in the complete loss of infectivity.

Protein assay. The relative HA content was evaluated by determining the ratio of HA units to total viral protein. The protein content was estimated by the method of Bradford (1976).

Haemagglutination-inhibition (HAI) titration was performed with 0.5% chicken erythrocytes and monoclonal antibodies (MoAbs) or mouse sera treated with receptor-destroying enzyme.

MoAbs to the H2 HA of the A/Japan/305/57 (H2N2) strain were obtained from Dr. R.G. Webster. Their HAI titers (HAI reactivities) were expressed as reciprocals of highest positive dilutions of MoAbs (sera).

LD_{50} *titration in mice.* Groups of 10 mice were inoculated intranasally (i.n.) with 10-fold dilutions of virus samples and their survival was monitored for 10 days. Median LD_{50} was calculated by the Kärber method (Kärber, 1931). The $\log_{10} EID_{50}/LD_{50}$ ratio was used as a measure of virulence.

EID_{50} *titration in chicken embryonated eggs* was carried out in a standard way.

Experimental infection. Groups of seronegative F(DWR) mice (6 – 8 g) were immunized intramuscularly (i.m.) and into the base of the tail in the presence of Freund's complete adjuvant (Gibco). Each experimental group consisting of 18-22 animals was immunized with β-propiolactone-inactivated preparations of influenza A (H2) strains (20 µg of viral protein per mice). Three weeks later the animals were bled to obtain postvaccination serum samples and then infected i.n. with 10 LD_{50} of influenza virus variant A/Jap x Bell/57-MA (H2N1) or A/NJers/78-MA (H2N3). This dose caused 100% mortality in mice during the observation period of 15 days. However, in this study, we registered the mortality by the 10th day after the challenge, because this period of observation was sufficient for the determination of different levels of protection provided by inactivated preparations of influenza A virus strains. The protection was expressed as the percent reduction in the mortality rate of the experimental group in relation to the control group.

Results

Infectivity and antigenic properties of MA variants of influenza A (H2) virus strains

Influenza viruses do not cause natural infection in mice, however, these animals are a very common and useful model host for the study of influenza virus pathogenesis and immunity, because bronchopneumonia in the mouse and in the human are pathogenically similar (Sweet and Smith, 1980).

Variants of influenza virus A (H2) subtype virulent for mice were obtained for two strains – human A/Jap x Bell/57 (H2N1) and avian A/NJers/78 (H2N3) (Fig. 1). Low amounts of infectious virus were found in mouse lungs after first 4-5 passages. The capacity of virus to grow in infectious form in lungs appeared to be related to the passage number, and an increase of infectivity was achieved around the 11th – 13th

passage. Additional passages were undertaken to stabilize selected mutants with growth potential in mouse lungs. Both MA variants grew to high infectivity titers in embryonated chicken eggs and mouse lungs. The MA variants exhibited different virulence for mice: A/Jap x Bell/57-MA was more virulent than A/NJers/78-MA ($\log_{10}$(EID_{50}/LD_{50}) were 3.2 and 4.9, respectively) (Table 1).

The MA variants were characterized antigenically by HAI test using a series of HA-specific MoAbs. The HAI reactivity of four MoAbs, representatives of a large series, are shown in Table 1. The reactivity patterns were similar only with MoAb75, while with the three other MoAbs they were different, both for the original influenza A (H2) viruses and their MA variants. So, antigenic variations were observed within both pairs of the tested viruses. A/Jap x Bell/57-MA variant differed from the original strain in HAI reactivity with three MoAbs. HAI titers obtained with MoAb18 and MoAb185 were higher for the original strain than for its MA variant, while the level of MoAb18 reactivity was low for both the original strains and their MA
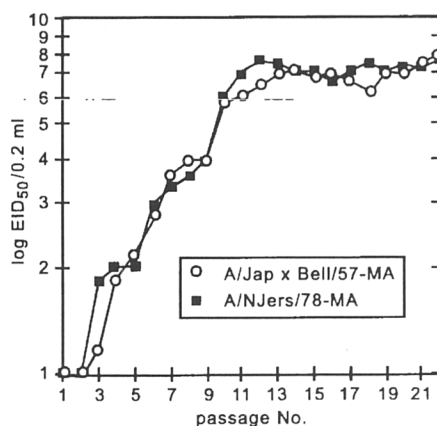

Fig. 1

Changes in the infectivity of influenza A (H2) viruses during their adaptation to mouse lungs

Three days after i.n. infection, the mice were killed and their lungs were titrated for infective virus (EID_{50}).

Table 1. Changes in the infectivity and antigenicity of the original influenza A (H2) viruses and of their MA variants

Viruses		Virus titers			HAI reactivity to A/Japan/305/57[a]			
		-log EID_{50}		-log LD_{50}				
		Chicken embryos	Mouse lungs		MoAb 18	MoAb75	MoAb185	MoAb342
A/Jap x	Original	6.5	ND	ND	400	12800	12800	800
Bell/57	MA	8.3	6.7	5.1	100	12800	400	<100
A/N/Jers/78	Original	6.7	ND	ND	3200	12800	400	12800
	MA	8.0	6.0	3.1	25600	12800	6400	25600

ND = titer not determined (-log LD_{50} below 1.0).
[a]Reciprocals of maximum positive dillutions.

variants. A/Jap x Bell/57-MA variant lost the epitope recognized by MoAb342.

Also the original A/NJers/78 strain and its MA variant were antigenically distinguishable in HAI test with MoAbs: the variant virulent for mice showed higher reactivity with three MoAbs (Table 1).

Thus, serial passages of influenza A (H2) viruses in mouse lungs lead to the acquisition of virulence for mice (i.e. adaptation to a new host) and to changes in the antigenicity of viral HA.

Response to challenge with MA variants following immunization with human influenza A (H2) virus strains

Three weeks after i.m. immunization of F(DWR) mice with inactivated preparations of different human (Table 2) and avian (Table 3) influenza A (H2) viruses, animals were i.n. challenged with virulent MA variants. Immune response was monitored by determination of the level of postvaccination serum HAI antibodies and mortality rates in experimental and control animal groups after challenge. Both human and avian whole virus preparations induced production of serum HAI antibodies in levels of 1:20 – 1:40.

Mice immunized with human A (H2) strains exhibited medium rates of mortality after challenge with A/Jap x Bell/57-MA variant. A similar mortality (33.3% – 38.9%) was found for three strains (A/Sing/57, A/Len/59, A/Len/80). Low mortality was found for the animals immunized with influenza virus homologous to the challenge virus (A/Jap x Bell/57) and with A/Tok/67 strain. Control non-immunized mice suffered a mortality rate of 90.5% after virus challenge.

Virulent A/NJers/78-MA strain was shown to cause different mortality rates after i.n. challenge in mice immunized with human influenza virus strains (Table 2). High rates of mortality (more than 65%) were observed with 3 human viruses (A/Jap x Bell/57, A/Len/65, A/Len/80). Mice vac-

Table 2. Mortality in mice challenged with A/Japan x Bell/57-MA and A/black duck/New Jersey/1580/78-MA variants following vaccination with different inactivated human influenza A (H2N2) viruses

Challenge virus variant	Lethal effect	Virus strains used for vaccination						Control group
		A/Sing/57	A/Jap x Bell/57	A/Len/59	A/Len/65	A/Tok/67	A/Len/80	
A/Jap x Bell/57-MA	Dead/total	7/18	2/18	7/20	9/18	3/24	7/21	19/21
(H2N2)	Mortality (%)	38.9	11.1	35.0	50.0	12.5	33.3	90.5
A/NJers/78-MA	Dead/total	9/22	13/20	4/22	16/20	2/26	14/21	19/20
(N2N3)	Mortality (%)	40.9	65.5	18.2	80.0	7.7	66.7	95.0

Table 3. Mortality in mice challenged with A/Japan x Bell/57-MA and A/black duck/New Jersey/1580/78-MA variants following vaccination with different inactivated avian influenza A (H2) viruses

Challenge virus variant	Lethal effect	Virus strains used for vaccination									Control group
		A/Germ/73	A/Prim/76	A/Mars/76	A/Alb/77	A/NJers/78	A/NY/78	A/Alb/80	A/Pots/80	A/NJers/85	
A/Jap x Bell/57-MA	Dead/total	3/18	1/18	7/21	6/22	2/20	9/18	12/19	10/21	9/20	18/20
(H2N1)	Mortality (%)	16.7	5.6	33.3	27.3	10.0	50.0	63.2	47.6	45.0	90.0
A/NJers/78-MA	Dead/total	1/18	1/21	2/18	3/20	2/21	5/21	10/21	8/18	8/20	19/20
(H2N3)	Mortality (%)	5.6	4.8	11.1	15.0	9.5	23.8	47.6	44.4	40.0	95.0

cinated with influenza A/Sing/57 strain were also rather susceptible to lethal infection with A/NJers/78-MA variant (40.9%).

These studies indicated that mice vaccinated with human influenza A (H2) viruses were satisfactorily protected against challenge with the human A/Jap x Bell/57-MA variant. The survival rate of these mice was in the range of 61.1% – 88.9% and the protection was obtained only against strain A/Len/65 in one half of the animals. A group of mice immunized with the same viral preparations and challenged with avian A/NJers/78-MA variant were protected to a lesser extent. There were three experimental groups of animals vaccinated with A/Jap x Bell/57, A/Len/65 and A/Len/80 strains, respectively, where only 1/3 of mice were protected. For the other three immunized groups (A/Sing/57, A/Len/59, A/Tok/67), the protection was higher and was observed in 59.1% – 92.3% of animals. The differences in cross-protection efficacy suggest that the challenge A/NJers/78-MA variant contains HA antigenically more related to that of the latest human H2N2 viruses, such as

A/Tok/67, than to that of the early human viruses, such as A/Jap/57 and A/Sing/57.

Response to challenge with MA variants following immunization with avian influenza A (H2) virus strains

The pattern of protection of mice vaccinated with avian influenza A (H2) virus strains was distinguishable from that described previously. Challenge with A/Jap x Bell/57-MA variant caused different mortality rates in mice immunized with avian influenza H2 viruses (Table 3). More than 45% of animals died in the experimental groups immunized with several strains isolated in 1978 – 1985. With one of these viruses (A/Alb/80) the highest mortality rate (63.2%) was observed for all 15 strains tested. The strains A/Prim/76 and A/NJers/78 showed the best protection against lethal challenge with A/Jap x Bell/57-MA variant, namely 5.6% and 10.0% mortality, respectively. The other three avian strains tested could be regarded as producing an intermediate level of protection.

Mice immunized with avian strains seemed to be more protected against challenge with virulent A/NJers/78-MA variant. Only three groups of mice tested showed mortality rates of 40.0% – 50% (Table 3). Viruses used for immunization in these three groups were isolated in 1980 – 1985. In the other groups of vaccinated animals mortality rates did not exceed 15%. Like in the cases of A/Jap x Bell/57-MA challenge, the lowest figures were observed for mice vaccinated with A/Germ/73 and A/Prim/76 strains (5.6% and 4.8% mortality, respectively).

These studies indicated that protection efficacy was lower in mice vaccinated with avian influenza A (H2) virus strains isolated in 1978 – 1985 than in mice immunized with viruses isolated earlier. Vaccination of mice with avian influenza A (H2) viruses induced a better protection than that with human viruses both against challenge with A/Jap x Bell/57-MA and A/NJers/78-MA variants.

Discussion

It is widely accepted that new pandemic influenza virus strains emerge by reassortment after double infection of a common host with a prevailing human strain and another influenza A virus strain presumably of animal origin. The influenza A (H2N2) strain, which caused an Asian epidemic in 1957 and circulated in human population in 1957 – 1968, obtained three genes coding HA, NA and PB2 proteins from an avian precursor and other genes from a late human H1N1 strain (Kawaoka et al., 1989). The appearance and prevalence of H2 influenza viruses in domestic and wild birds at modern times (Nerome et al., 1984; Webster et al., 1993) indicate a potential of this subtype to be transmitted to humans. The existence of a large susceptible population (Govorkova et al., 1993) makes the opportunity of virus reintroduction into humans even more real.

The reactogenicity, immunogenicity and protective efficacy of influenza A (H1N1) and (H3N2) virus vaccines has been well characterized (Cate et al., 1982; Belshe et al., 1984; Sears et al., 1988). Influenza A(H2) virus vaccines have almost not been studied at all.

This work was undertaken to study the responses and cross-protection efficacy within H2 subtype of influenza A viruses under lethal infection with two MA variants. Preparation of MA variants of influenza virus strains resulted in changes in the antigenicity of HA, the phenomenon observed also by other authors (Gitelman et al., 1984; Lipatov et al., 1995). These MA variants can serve as a useful tools in the analysis of protective efficacy and in testing different types of vaccines against influenza virus in a mouse model.

The main purpose of this work was to determine what protection could provide immunization of mice with inacti-vated preparations of human or avian influenza A (H2) viruses. It was shown that the inactivated whole influenza A (H2) virus preparations elicited low HAI antibody titers apparently sufficient for protection against lethal challenge with either MA variant. Anti-HA antibodies play an important role in the host recovery and protection against influenza (Schulman, 1975), but the role of other viral proteins, especially NA (Johansson et al., 1993), NP (Yewdell et al., 1985; Stitz et al., 1990) and matrix M2 protein (Treanor et al., 1990) cannot be excluded. The protection induced by inactivated human and avian influenza A (H2) viruses most likely reflect the humoral immune response to HA. The challenge with reassortant A/Jap x Bell/57-MA containing N1 NA excluded the influence of NA antigen on protective efficacy. The other challenge virus, A/NJers/78-MA variant, differed in NA serotype from all human strains containing N2 NA and from two avian strains containing N9 NA. The other avian influenza viruses tested, which contained N3 NA, did not show detectable differences from viruses with other NA subtypes in protection against lethal challenge. Thus, in the present study, the anti-NA immunity didn't play an essential role in protective efficacy.

Under our experimental conditions the role of immunity against NP and M2 proteins was probably very limited. This is supported by the findings that mice vaccinated with human or avian influenza A (H2) viruses were completely susceptible to lethal infection with A/Aichi/2/68 (H3N2) strain (data not shown).

The immunization of mice with inactivated preparations of human influenza A (H2) virus strains provided different levels of protection. There was no correlation between protective efficacy and the year of strain isolation. The highest level of protection against challenge with both MA variants was obtained for influenza A/Tok/67 strain, which probably contains antigenic epitopes related to those of the avian viruses.

Our results suggest that the immunization of mice with avian influenza A (H2) virus strains provides a better protection against lethal infection than that with human strains. The levels of protection in groups vaccinated with avian influenza viruses were higher against challenge with avian than with human MA variant. The level of protection was lower in the groups of animals challenged with strains isolated in 1980 – 1985 (52.4% – 60%). From studies of protective efficacy we could suggest that later avian strains differed from the earlier ones circulating in 1973 – 1975. This supports the findings of phylogenetic analysis, which identified three distinct lineages of H2 HA, one in humans and two in avian species (Schäfer et al., 1993). Attempts were made to correlate antigenic changes in HA found by HAI analysis with polyclonal antisera with protective efficacy against the same influenza strains. The analysis of possible evolution relation-

ships of human and avian influenza A (H2) viruses (Govorkova *et al.*, 1991) revealed that four human viruses (A/Sing/57, A/Len/80, A/Len/59, A/Jap x Bell/57) are antigenically related to three avian viruses (A/Germ/73, A/Prim/76, A/Mars/76). The present results on protective efficacy against human and avian influenza A (H2) viruses did not correlate with the data of the antigenic analysis. However, high mortality rates (more than 40%) were determined for two (A/Len/65, A/NJers/85) out of three HA antigenic variants.

The main conclusion from our studies is that it is more reasonable to use avian than human influenza A (H2) strains for the preparation of influenza vaccines of the H2 serotype. Differences in cross-protection reactivity suggest an utilization of two (or more) avian strains as a possible source of surface antigens for influenza A (H2) virus vaccines.

Newly developed influenza vaccine strains must be properly tested in a model system by one of the most important tests – the determination of the protective efficacy against experimental infection. The approach used in this study could be considered an useful tool for preliminary estimation of protective efficacy of new vaccine candidates.

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