Arch Virol (2000) 145: 1059–1066

Archives of Virology
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Printed in Austria

# Cross-protection and reassortment studies with avian H2 influenza viruses

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Accepted February 7, 2000

**Summary.** In order to assess the degree of immune cross-protection among avian H2 influenza virus strains, mice were immunised with β-propiolactone-inactivated virus preparations and infected intranasally with mouse-adapted variant of A/Black Duck/New Jersey/1580/78 (H2N3) strain. The experiments with 11 avian H2 strains revealed that both Eurasian and American H2 avian influenza viruses exhibit either high or moderate degree of cross-protection. The grouping of the strains in accordance with their cross-protection efficiency does not coincide with H2 phylogenetic branches. Several reassortant clones were obtained with the use of A/Pintail Duck/Primorie/695/76 (H2N3) strain and high-yield X-67 reassortant as parent viruses, among them a high-yield H2N3 reassortant. Taking into account the data on cross-protection among avian H2 strains, the high-yield H2N3 reassortant may be regarded as a prototype strain to be used for the preparation of killed vaccines in the case of a new appearance of avian H2 haemagglutinin in circulation in humans.

## Introduction

Influenza A virus of H2N2 subtype produced a major pandemic in 1957 and circulated in humans until 1968. At present subtypes H1 and H3 circulate in humans, whereas H2, the only other subtype with a definite pandemic record, disappeared from the human population 31 years ago. However, H2 strains continue to circulate in the aquatic birds reservoir [9]. The avian influenza viruses are regarded as a possibile source of HA genes for human pandemic virus variants [11]. Since humans of less than 30 years of age have never encountered the H2 virus, the possibility of a reintroduction of H2 subtype viruses should not be disregarded. As a possible prospective countermeasure, one may consider the development

and stockpiling of anti-H2 inactivated vaccine. This approach, however, would be impractical in the case of a high antigenic diversity within H2 subtype, since one can not predict which antigenic variant of the avian H2 haemagglutinin will be transferred to the human population. The available data [9] suggest that the genetic and antigenic variability of avian H2 strains is moderate. However, to evaluate the chances of successful prophylactic vaccination against a pandemic virus with provisionally produced vaccines, reliable data on immune cross-protection within H2 subtype are required. With the exception of our preliminary results [2], no such data, to our knowledge, have been published. Here, we report the results of cross-protection studies in mice performed with avian H2 strains representing different branches of the phylogenetic tree of H2 haemagglutinin [6, 9]. We also attempted to produce a high-yield reassortant possessing the HA gene of an avian H2 virus, in order to reveal whether such reassortants can produce virus yields similar to those of the high-yield parent virus, so as to be used in the future as one of the prospective anti-H2 vaccine strains.

## Materials and methods

### *Viruses*

Avian H2 influenza viruses used in these studies are listed in Table 1. The viruses were obtained from the Virus Repository of the Department of Virology and Molecular Biology of St. Jude Children's Research Hospital and from the Virus Collection of the D. I. Ivanovsky Institute of Virology. Strains A/Pintail duck/Primorie/695/76 (H2N3) and A/Duck/Marseille/46/76 (H2N3) were formerly designated as A/Pintail/Praimoric/625/76 (H2N2) and A/Mallard/MT/Y61 (H2N2) respectively [9]. The mouse-adapted variant of A/Black Duck/New Jersey/1580/78 (H2N3) strain was prepared by 22 lung-to-lung passages in mice [2]. Strain X-67, a high-yield reassortant between A/Puerto Rico/8/34 (H1N1) and A/USSR/90/77 (H1N1) viruses [4], was obtained from the Virus Collection of D. I. Ivanovsky Institute. The viruses were propagated in 10-day old embryonated chicken eggs. The virus-containing allantoic fluid was stored at 4 °C.

**Table 1.** Avian influenza A viruses used in the cross-protection experiments

| Strain | Subtype | Abbreviation |
|---|---|---|
| A/Black Duck/New Jersey/1580/78 | H2N3 | bdk/NJ/78 |
| A/Pintail duck/Primorie/695/76 | H2N3 | pin/Prim/76 |
| A/Duck/Marseille/46/76 | H2N3 | dk/Mars/76 |
| A/Pintail duck/Alberta/211/80 | H2N3 | pin/Alb/80 |
| A/Laughing gull/New Jersey/75/85 | H2N9 | lgl/NJ/85 |
| A/Mallard duck/New York/6750/78 | H2N2 | ml/NY/78 |
| A/Mallard duck/Potsdam/178-4/83 | H2N2 | ml/Pot/83 |
| A/Herring gull/Delaware/677/88 | H2N8 | hgl/DE/88 |
| A/Gull/Maryland/19/77 | H2N8 | gl/MD/77 |
| A/Ruddy turnstone/Delaware/81/93 | H2N1 | rt/DE/93 |
| A/Chicken/New York/29878/91 | H2N2 | ck/NY/91 |

*Virus concentration and inactivation*

Virus-containing allantoic fluids were layered on top of 3 ml cushion of 20% sucrose and centrifuged in SW27-1 rotor for 90 min at 23000 rpm. The inactivation was performed as described in an earlier publication [1]. Briefly, each sample of purified virus was resuspended in phosphate buffered saline pH 7.4 and treated with 0.015 M β-propiolactone (Sigma Ch. Co., St. Louis, MO, USA) for 15 min at 20 °C. The reaction was stopped by the addition of sodium thiosulphate (final concentration 0.04 M).

*Immunisation and experimental infection of mice*

Unbred albino mice (3 weeks of age) were immunised intramuscularly and into the base of the tail with inactivated whole virus preparations (20 μg total virus protein per mouse). The virus preparations were mixed with an equal volume of complete Freund adjuvant (Calbiochem-Behring). Mock-immunised mice were inoculated with PBS/adjuvant mixture. The mice were infected with challenge virus intranasally 3 weeks after immunisation. The challenge virus dose was 3 to 10 $LD_{50}$ for 6-week-old mice (this is equivalent to 100 $LD_{50}$ for 3 week-old mice). The survivial rate was monitored for 10-day observation period.

*Reassortment procedure and gene identification*

Essentially the technique of Schulman and Palese [10] was used, with modifications described in our earlier publication [5]. Briefly, the avian parnt virus was UV-irradiated so as to lower the infectious titre by 6 $\log_{10}$, mixed with an equivalent amount of live high-yield parent virus and inoculated into the allantoic cavity of embryonated chicken eggs. The eggs were incubated for 14 h at 37 °C, the virus-containing fluid was collected and used for an additional one-cycle egg passage. The yeild was collected, treated with polyclonal antiviral guinea pig serum against the live parent virus (haemagglutination-inhibition (HI) titre 1:10240), and 10- fold dilutions were inoculated into embryonated chicken eggs (15 eggs per dilution). After 48 h of incubation allantoic fluid was collected from the haemagglutination-positive eggs infected with the limiting dilution (separately from each egg) and used for further cloning of the reassortants. The genetic content of the reassortant clones was first characrterised with the use of haemagglutination-inhibition and neuraminidase-inhibition tests and the assessment of the mobility of virus-specific proteins in polyacrylamide gel electrophoresis [5]. In this way the parent origin of HA, NA, NP and NS genes was established. In order to determine the origin of PB1, and PB2, PA and M genes, as well as to confirm the origin of NP and NS genes, the technique of partial sequencing was used. The virus RNA was used as a template for reverse transcription [3] with a 12-base synthetic primer complementary to 3′-end of virus RNA. cDNA was amplified by polymerase chain reaction (PCR), and PCR product was used for sequencing. Sequencing reactions were performed by using dye-terminator cycle-sequening ready-reaction kits (Perkin-Elmer, applied Biosystems, Inc., Foster City, CA). Parts of the genes of both parents and all the reassortants ranging from 300 nucleotides for PA gene to 1000 nucleotides for PB1 were sequenced and compared.

*Assessment of virus protein content*

To ensure the use of identical amounts of virus antigen in the immunisation experiments, as well as to compare the virus yields of the parent viruses and the reassortants, the virus preparation purified by centrifugation through 20% sucrose cushion were dissolved in electrophoresis sample buffer and analysed by SDS-polyacrylamide gel electrophoresis at 15% concentration of acrylamide. As a standard, a range of bovine serum alumin samples (1 to

4 μg) was run in parallel with the experimental samples. The gel slabs were stained with Coumassie R-350 (Pharmacia), scanned with the use of ScanJet 4S (Hewlett Packard) and analysed by the extraction of image intensities using Band Leader software (Version 2.01).

## Results

### *Cross-protection studies*

In the preliminary experiments the ability of the challenge virus stock to kill adult mice was determined. The susceptibility of 6-week-old mice to the mouse-adapted bdk/NJ/78 virus was 1.0 to 1.5 $\log_{10}$ lower than the susceptibility of 3-week old mice used regularly for the determination of $LD_{50}$. Accordingly, the amount equal to 100 $LD_{50}$ for 3-week-old mice was chosen as the challenge dose. In several small-scale immune protection experiments the immunising dose of 20 μg of total virus protein per mice was evaluated to be optimal to compare the extent of protection induced by homologous and heterologous strains.

Two large-scale cross-protection experiments were performed, one with 4 and the other with 6 avian H2 strains heterologous to the challenge virus (Table 2). In each experiment a group of mock-immunised mice and a group of mice immunised with the homologous strain (bdk/NJ/78) were used. Each group of mice immunised with a heterolgous strains contained 18 to 22 animals. The mock-immunised groups of mice and the groups immunised with homologous virus contained 38 to 41 mice. The mortality data in the groups immunised

**Table 2.** Immune protection of mice against intranasal challenge with mouse-adapted variant of influenza A/Black duck/New Jersey/1580/78/ strain

| Exp. | Strain used for immunisation | Number of mice total | Number of mice died | Mortality % | P[a] |
|---|---|---|---|---|---|
| 1 | bdk/NJ/78 | 38 | 1 | 2.6 | – |
| | pin/Prim/76 | 21 | 1 | 4.8 | > 0.5 |
| | dk/Mars/76 | 17 | 1 | 5.9 | > 0.5 |
| | pin/Alb/80 | 21 | 5 | 23.8 | < 0.01 |
| | lgl/NJ/85 | 22 | 5 | 22.7 | < 0.02 |
| | Mock-immunised | 41 | 27 | 65.8 | < 0.01 |
| 2 | bdk/NJ/78 | 39 | 3 | 7.7 | – |
| | ml/NY/78 | 20 | 2 | 10.0 | > 0.7 |
| | ml/Pot/83 | 20 | 3 | 15.0 | > 0.3 |
| | hgl/DE/88 | 20 | 8 | 40.0 | < 0.01 |
| | gl/MD/77 | 18 | 7 | 38.9 | < 0.01 |
| | rt/DE/93 | 19 | 12 | 63.5 | < 0.01 |
| | ck/NY/91 | 18 | 5 | 27.8 | < 0.01 |
| | Mock-immunised | 39 | 34 | 87.2 | < 0.01 |

[a]P for the difference in the mortality of mice immunised with a heterologous strain *vs*. the mortality of mice immunised with homologous virus bdk/NJ/78 as calculated on the basis of $\chi^2$-criterion

**Table 3.** Differences in the amino acid sequences in HA1 subunit distinguishing the low-protecting and high-protecting strains

| Accession number | Viruses | Survival rate (%) | Amino acid position | | | |
|---|---|---|---|---|---|---|
| | | | 141 | 142 | 143 | 144 |
| AF116205 | bdk/NJ/78 | 94.9 | V | S | G | N |
| L11137 | ml/NY/78 | 90.0 | V | S | G | N |
| L11141 | pin/Prim/76 | 95.2 | V | S | G | N |
| L11136 | dk/Mars/76 | 94.1 | V | S | G | N |
| L11139 | ml/Pot/83 | 85.0 | V | S | G | N |
| AF116204 | pin/Alb/80 | 76.2 | V | S | G | S |
| AF116198 | ck/NY/91 | 72.2 | N | L | D | N |
| AF116201 | lgl/NJ/85 | 77.3 | V | Y | G | G |
| L11130 | gl/MD/77 | 61.6 | A | Y | G | G |
| L11132 | hgl/DE/88 | 60.0 | D | Y | G | G |
| AF116208 | rt/DE/93 | 36.5 | A | Y | G | G |

with heterologous strains was compared to the mortality in the mock-immunised mice and to the mortality in mice immunised with homologous virus by means of the computation of $\chi^2$ values for each pair in order to evaluate the probability of random difference between the groups. Since mortality in the mock-immunised mice somewhat differed in two experiments, all the pair-wise comparisons were made within each experiment.

The results demonstrated that all H2 strains tested exhibited a certain degree of protection against the challenge with mouse-adapted bdk/NJ/78 strain. In both experiment the pair-wise comparisons of the mortality in the mock-immunised group against any of the groups of immunised mice revealed that the difference in the mortality between the mock-immunised mice and any group immunised with either homologous or heterologous virus was statistically highly significant. However, the degree of protection was not uniform. Several strains (pin/Prim76, dk/Mars/76,ml/NY/78 and ml/Pot/83) induced the degree of protection similar to the one conferred by the homologous strain: the differences, if any, were too small to be reliably registered as statistically significant variations in mortality. On the other hand, the degree of protection conferred by strains pin/Alb/80, lgl/NJ/85, hgl/DE/88, gl/MD/77, rt/DE/93 and ck/NY/91 was definitely lower than the protection induced by the homologous strain: pair-wise comparisons demostrated highly significant differences in the mortality, with P values from 0.02 to lower than 0.01 (Table 2). An inspection of amino acid sequences [6] suggest that there is a correlation between the degree of cross-protection and the amino acid changes in the region 141–144 of HA1 (Table 3).

### *Production and characterisation of reassortants*

Double infection of chick embryos with UV-irradiated pin/Prim/76 virus and live X-67 virus with subsequent treatment of the yield with immune serum and

**Table 4.** Genetic content of reassortants

| Virus | Genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PB1 | PB2 | PA | HA | NP | NA | M | NS |
| X-67 | U | R | R | U | R | U | R | R |
| Pin/Prim/76 | Pi | Pi | Pi | Pi | Pi | Pi | Pi | Pi |
| PX8 | U | R | R | Pi | R | U | R | R |
| PX9 | U | R | R | Pi | R | Pi | R | R |
| PX14 | U | R | R | Pi | R | U | R | R |

*Pi* Genes of pin/Prim/76 (H2N3); *U* genes of A/USSR/90/77 (H1N1); *R* genes of A/Puerto Rico/8/34 (H1N1)

**Table 5.** Accumulation of the reassortants and parent viruses in embryonated chicken eggs

| Virus | Subtype | Haemagglutination titre | HA protein in allantonic fluid, mg/l |
|---|---|---|---|
| Pin/Prim/76 | H2N3 | 320 | 4.88 |
| PX9 | H2N3 | 1280 | 18.28 |
| PX14-XIII | H2N1 | 640 | 8.98 |
| X-67 | H1N1 | 1280 | 19.28 |

limiting dilution cloning (see Materials and methods) resulted in the selection of several reassortant clones with antigenic formulae H2N1 or H2N3. Two H2N1 reassortants (PX-8 and PX-14) and one H2N3 reassortant (PX-9) were chosen for detailed characterisation. The studies of their genetic content by partial sequencing revealed that 7 genes in both H2N1 reassortants and 6 genes in PX-9 originated from the high yield parent X-67 (Table 4). However, whereas PX-9 produced high yields of virus in the allantoic fluid as revealed by HA titration (similar to the yield produced by X-67), both H2N1 reassortants produced low yields. In our earlier studies we observed that reassortants having NA gene of A/USSR/90/77 virus in combination with H3, H4 or H13 HA gene have a tendency to virion aggregation and produce low yields in chick embryos [7]. Both features, the tendency to aggregation and the low yield, could be abolished by passaging in chick embryos and selection of passage variants [8]. This procedure, when applied to PX-8 and PX-14, resulted in an increase in HA titres, but the yields were still lower than those of X-67 were. The virus protein yield was estimated by partial purification of the viruses with subsequent polyacrylamide gel electrophoresis and scanning. The results (Table 5) revealed that the virus protein yield was similar in X-67 and PX-9, whereas the yields of the passage variants of H2N1 reassortants, although somewhat higher than the yield of pin/Prim/76 parent virus, were lower than the yield of X-67 virus.

## Discussion

Haemagglutinin genes of avian H2 influenza viruses are moderately variable, the maximum difference in the HA amino acid sequences being 15.6% [9]. The HA genes fall into two lineages, Eurasian and American [9]. Several strains isolated in the United States from shorebirds and gulls belong to the Eurasian lineage [6]. The pattern of reactivity of H2 strains with monoclonal antibodies was shown to differ from the phylogenetic grouping: the majority of the strains of both branches, Eurasian and American, reacted with most mAbs in the panel, whereas some strains from both lineages reacted with only two or three mAbs [6, 9].

The results of cross-protection experiments described in the present communication demonstrate that all strains tested exerted a certain degree of protection in mice challenged with mouse-adapted variant of the avian influenza strain A/Black duck/New Jersey/1580/78 (H2N3). The protection was obviously induced by the haemagglutinin: the immunisation with A/Aichi/2/68 (H3N2) virus provided no protection at all against the challenge virus (not shown), and the differences in the degree of protection did not depend on the subtype of virus neuraminidase (Table 2). However, the degree of protection was not uniform. The viruses could be divided into at least two groups: strongly protecting (pin/Prim/76, dk/Mars/76, ml/NY/78/, ml/Pot/83) and moderately protecting (pin/Alb/80, lgl/NJ/85, hgl/DE/88, gl/MD/77, rt/DE/93, ck/NY/91). The degree of protection, when provided by the immunising effect of HA, should reflect the immunological relatedness between the haemagglutinins of the immunising virus and the challenge virus. The grouping on the basis of the degree of protection coincides neither with the grouping on the basis of the general pattern of reactivity with mAbs, nor with the phylogenetic branches [6, 9]. However, it is possible to trace a correlation of the protection data with specific features of the antigenic reactivity and the primary structure. The weakly protecting strains do not react with mAbs 121/7 and 67/7 against the haemagglutinin of influenza A/Japan/305/57 [6, 9]. A comparison of the primary structures of the HA1 [6] reveals that all gull and shorebird H2 strains, when compared with the challenge virus (and with the majority of avian H2 viruses), have amino acid substitutions S142G and N144G in HA1 subunit (Table 3). Strain pin/Alb/80 has N144S substitution, and ck/NY/91 has substitutions S142L and G143D. Thus, all the weakly protecting strains have amino acid substitutions in the region 142–144 of HA1 as compared to the challenge virus. It seems that variations in this region may be important for the immunologic differences revealed by the cross-protection test.

The avian viruses circulating in the waterfowl are generally considered to be the most likely source of the HA genes introduced into the human population [11]. Since the weakly protecting strains had been isolated mostly from shorebirds and gulls, and only occasional H2 strains isolated from the waterfowl demostrated a decreased protection ability (Table 2), it seemed worthwhile to produce a series of high-yield reassortants possessing HA gene originating from a waterfowl H2 strain. The reassortant possessing HA and NA genes of A/Pintail duck/Primorie/695/76 (H2N3) virus produced high yields in the embryonated

chicken eggs, similar to the yields produced by the high-yield parent (Table 5). This reassortant may be regarded as a prototype for the high-yield strains to be used in an emergency program of vaccination of medical personnel in the case of the appearance of an avian H2 haemagglutinin in human circulation.

## Acknowledgements

The research described in this publication was made possible in part by Award RN1-412 of the U.S. Civilian Research & Development Foundation for the Independent States of the Former Soviet Union (CRDF).

## References

1. Budowsky EI, Smirnov Y, Shenderovich SF (1993) Principles of selective inactivation of viral genome. VIII. The influence of beta-propiolactone on immunogenic and protective activities of influenza virus. Vaccine 11: 343– 348
2. Govorkova EA, Smirnov I (1997) Cross-protection of mice immunized with different influenza A (H2) strains and challenged with viruses of the same HA subtype. Acta Virol 41: 251–257
3. Huddleston JA, Brownlee GG (1982) The sequence of the nucleoprotein gene of human influenza A virus, strain A/NT/60/68. Nucleic Acids Res 10: 1 029–1 038
4. Johansson BE, Bucher DJ, Pokorny BA, Mikhail A, Kilbourne ED (1989) Identification of PR8 M1 protein in influenza virus high-yield reassortants by M1-specific monoclonal antibodies. Virology 171: 634–636
5. Kaverin NV, Rudneva IA, Smirnov YA, Finskaya NN (1988) Human-avian influenza virus reassortants: effect of reassortment pattern on multi-cyle reproduction in MDCK cells. Arch Virol 103: 117–126
6. Makarova NV, Kaverin NV, Krauss S, Senne D, Webster RG (1999) Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. J Gen Virol 80: 1 367–1 371
7. Rudneva IA, Kovaleva VP, Varich NL, Farashyan VR, Gubareva LV, Yamnikova SS, Popova IA, Presnova VP, Kaverin NV (1993) Influenza A virus reassortants with surface glycoprotein genes of the avian parent viruses: effects of HA and NA gene combinations on virus aggregation. Arch Virol 133: 437–450
8. Rudneva IA, Sklyanskaya EI, Barulina OS, Yamnikova SS, Kovaleva VP, Tsvetkova IV, Kaverin NV (1996) Phenotypic expression of HA-NA combinations in human-avian influenza A virus reassortants. Arch Virol 141: 1 091–1 099
9. Schafer JR, Kawaoka Y, Bean WJ, Suss J, Senne D, Webster RG (1993) Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194: 781–788
10. Schulman JL, Palese P (1976) Selection and identification of influenza virus recombinants of defined genetic composition. J Virol 20: 248–254
11. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152–179

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Received December 13, 1999