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Phenotypic expression of HA-NA combinations in human-avian influenza A virus reassortants

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Summary. Human-avian and human-mammalian influenza A virus reassortant clones with the neuraminidase (NA) gene of the A/USSR/90/77 (H1N1) strain and hemagglutinin (HA) genes of H3, H4 and H13 subtypes had been shown in an earlier publication to produce low HA yields in the embryonated chicken eggs. The low HA titers had been shown to be due, at least in part, to the formation of virion clusters at 4 °C; the clustering was removed by the treatment with bacterial neuraminidase [Rudneva et al., Arch. Virol (1993) 133: 437–450]. By serial passages of the reassortants in chick embryos non-aggregating variants were selected: the variants produced HA titers of the same order as A/USSR/90/77 parent virus. The assessment of the virus yields by the analysis of the partially purified virus preparations from fixed volumes of the allantoic fluid revealed that actual virion yields of the initial reassortants were lower than the yields of their passaged variants or of the parent viruses. The passaged variant of a reassortant possessing the HA gene of A/Duck/Ukraine/1/63 (H3N2) virus differed from the original (non-passaged) reassortant and from the parent A/Duck/Ukraine/1/63 virus in the reaction with a panel of monoclonal antibodies against H3 hemagglutinin. The data suggest that some HA-NA combinations may lead to an incomplete functional match between HA and NA and to the formation of low-yield reassortants, thus representing a possible limiting factor in the emergence of new HA-NA combinations in natural conditions.

Introduction

Influenza A viruses are abundant in nature and affect a number of mammalian and avian species. The virus glycoproteins, hemagglutinin (HA) and neuraminidase (NA) exist as several antigenic subtypes, 14 of HA and 9 of NA. Many HA-NA gene combinations occur in natural virus strains. New HA-NA combinations may be easily obtained by gene reassortment in laboratory

conditions. However, some combinations of antigenic subtypes of HA and NA were never reported in natural virus strains, and others occur rarely [5]. Besides, some HA-NA combinations seem to be specific for certain hosts, such as H1N1, H2N2 and H3N2 for humans. Although H1N1 and H3N2 subtypes have been co-circulating in the human population for a long time, the appearance of reassortant combinations, such as H1N2 [3] is rare, and such reassortants are not retained in the circulation. The basis for the limitations in the occurrence of HA-NA combinations in natural influenza virus isolates is not understood.

In our previous communication [6] we reported that influenza A virus reassortants possessing the NA gene of the A/USSR/90/77 (H1N1) strain and HA genes originating from avian or mammalian strains belonging to H3, H4, H10 or H13 subtypes produce low HA yields in embryonated chicken eggs. The low yields as measured by HA titrations were shown to be due, at least in part, to the aggregation of virions during the chilling of eggs at 4 °C. The aggregation, as our data suggested, resulted from an incomplete removal of sialic acid residues from the virion surface. However, since the virion aggregates dissociated at 37 °C, the aggregation seemed to present no disadvantage for the growth and dissemination of the reassortant virus in the living organism and could not be regarded as an obstacle to the survival of such reassortants. In order to gain some further insight into this problem we attempted to find out whether the actual virion yield is low in the H3N1 and H13N1 reassortants, whether high-yield mutants can be selected in the course of the passaging of the reassortants, and whether there is a co-variation between the "aggregate-forming" and "low-yield" phenotypes.

Materials and methods

Viruses

Influenza viruses from the D. I. Ivanovsky Institute Virus Collection A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/56 (H4N6), A/Pilot Whale/Maine/328/84 (H13N9) and A/USSR/90/77 (H1N1) as well as the reassortant clones R2, RSB1 and RWB1 were the same as used in the previous communication [6]. The reassortants R2, RSB1 and RWB1 contain HA genes of A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/56 (H4N6) and A/Pilot Whale/Maine/328/84 (H13N9) respectively, all the other genes originating from A/USSR/90/77 [6]. The viruses were passaged in 10-day old embryonated chicken eggs. The eggs were incubated at 37 °C for 48 hours, chilled at 4 °C overnight (if not otherwise stated), the allantoic fluid was collected and stored at 4 °C.

Cells

Madin-Darby Canine Kidney (MDCK) cells were grown in Eagle's MEM supplemented with 10% fetal calf serum. Eagle's MEM with 0.2% bovine plasma albumin and 0.001 mg/ml TPCK-trypsin (Worthington) was used as maintenance medium.

Assessment of virus yields

In order to obtain an estimate of virus yield not biased by virion aggregation we used polyacrylamide gel electrophoresis (PAGE) of partially purified virus. The purification

procedure was modified so as to minimize loss of virus. Virus-containing allantoic or cultural fluid was clarified by centrifugation at 3000 rpm for 5 min, an 8 ml volume of the fluid was layered on top of 6 ml of 20% sucrose in 0.1 M NaCl, 0.01 M Tris-HCl pH 7.4 buffer and centrifuged in an SW-27.1 rotor for 90 min at 21 000 rpm at 4 °C. The pellet was dissolved in dissociation buffer and analyzed in PAGE at 15% acrylamide concentration with diallyltartardiamide linker [8]. The gels were stained with Coomassie Blue and scanned in an ERI-65 densitometer.

Velocity sedimentation analysis

Virus preparations were layered on top of a 15–30% sucrose gradient with a 2 ml 60% sucrose cushion at the bottom of the tube and centrifuged at 24 000 rpm in SW-27.1 rotor at 4 °C for 1 h. The gradient fractions were incubated at 37 °C for 30 min and HA-titrated.

Neuraminidase activity measurement

Fetuin (Serva) was used as a substrate (stock solution 100 mg/ml). The reaction mixture contained 50 µl of purified virus suspension and 50 µl of substrate solution. The amount of free neuraminic acid was determined by the Warren method [9]. The enzyme activity was expressed as micrograms of neuraminic acid cleaved in 1 h at 37 °C per 1 mg of purified virus protein.

Hemagglutinin titrations and serologic reactions

HA titrations and hemagglutination-inhibition reactions were performed in microtiter 96-well plates with 0.5% suspension of chicken red blood cells. Reactivity of NA with monoclonal antibodies was determined in neuraminidase-inhibition (NI) tests and in ELISA [4]. Monoclonal antibodies (mAb) against NA of A/USSR/90/77 virus were kindly provided by Dr. R. G. Webster, mAb against HA of A/Aichi/2/68 (H3N2) were a gift of Dr. J. J. Skehel.

Results

Effect of serial passages in chick embryos on the yields of "single-gene" reassortants

In our previous paper [6] we observed that the reassortants containing HA genes of H3, H4, H10 and H13 subtypes and the NA gene of a A/USSR/90/77 (H1N1) virus produced low HA titers in chick embryos. Three reassortants, R2, RSB1 and RWB1, possessing HA genes of A/Duck/Ukraine/1/63 (H3N8), A/Duck/Czechoslovakia/56 and A/Pilot Whale/Maine/328/84 respectively, and all the other genes of A/USSR/90/77 ("single gene" reassortants) were used for further studies. The reassortants typically produced in chick embryos HA titers ranging from 16 to 64 HAU. All three reassortants were subjected to 21 serial passages in embryonated chicken eggs with the input dose of 1000 EID_{50} per egg. In each case there occurred a shift in HA titer in the course of the passages. With RSB1 the shift occurred at the 7th passage: the yield as measured by HA titer increased from 64 HAU to 512–1024 HAU and remained at this level in the following passages. With RWB1 the increase in titers, also about 8-fold, occurred as late as at the 19th passage. With R2 the increase occurred early: in repeated

series of passages the shift from 32–64 HAU titers to 256–512 titers occurred at the 3rd or 4th passage.

The increased HA titers of the passage variants result both from the loss of the aggregation and from the increase of virion yields

The "single gene" reassortants R2, RSB1 and RWB1 were shown in our previous studies to have a strong tendency to virion aggregation at low temperature. The aggregates dissociated at 37 °C and re-aggregated at 4 °C; the treatment with bacterial neuraminidase resulted in an irreversible desaggregation [6]. In order to find out whether the high-titer passage variants retained the tendency to aggregate, the viruses were subjected to velocity sedimentation in 15–30% sucrose gradients with 60% sucrose cushion, and HA titers were measured in the gradient fractions. The results indicated that whereas the initial reassortants were largely represented by virion aggregates, the high-passage variants sedimented as single virions. The comparison of R2 reassortant with its high-passage variant R2-XXI is presented in Fig. 1. Similar results were obtained with RSB1 and RWB1 reassortants and their high-passage variants RSB1-XXI and RWB1-XXI (not shown).

The dissociation of virion clusters after an incubation of the "single-gene" reassortants" at 37 °C usually leads to a small but definite increase in HA titer [6]. The passage variants, as might be expected on the basis of the sedimentation data, displayed no such increase (not shown). However, it seemed not really obvious whether the difference in HA titers between the passage variants and the

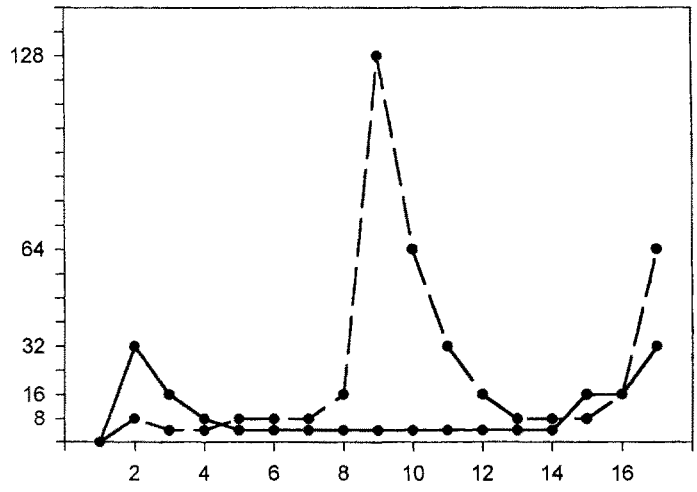

Fig. 1. Velocity sedimentation analysis of the initial R2 reassortant and its passaged variant. Virus-containing allantoic fluid was clarified by low-speed centrifugation, layered on top of a 15–30% sucrose gradient with a 60% sucrose cushion at the bottom of the tube and centrifuged for 1 h at 24 000 rpm at 4 °C in an SW.27.1 rotor. Abscissa: fraction number (from bottom to top), ordinate: HA titer in HAU. ——— R2, — — — R2-XXI

Table 1. Comparison of HA titers of R2 and its passaged variant R2-XXI in individual eggs and the effect of chilling

Virus	Conditions of virus collection	
	chilled eggs	eggs not chilled
R2	32[a]; 64; 64;	128; 256; 256;
R2-XXI	512; 512; 512;	512; 512; 1024;

[a] HAU in individual eggs

initial reassortant clones depended solely on the aggregation, or there was a difference in the actual virion yields as well. When the viruses were collected from the eggs without chilling (so as to avoid the aggregation), the difference in the HA titers between the passage variants and the initial clones, although less expressed than in the preparations collected by standard procedure, still was quite evident (Table 1). In order to evaluate the virion yield the viruses were purified from a standard volume of the allantoic fluid and analysed by PAGE with Coomassie Blue staining and densitometer scanning. The allantoic fluid was collected separately from each egg, and the preparations from several eggs were analysed for each variant. The data indicate (Fig. 2) that the yields of the initial reassortants are lower than those of the passaged variants. The latter are of the same order as the yields of the A/USSR/90/77 parent virus. The quantitative estimates on the basis of the area of the peaks corresponding to M1 protein in the densitometer tracings (Fig. 2C) indicate that the yields of the initial reassortants are 3 to 4 times lower than those of the passage variants or those of A/USSR/90/77 parent virus, which agrees with the data of HA titrations of the viruses collected without chilling (Table 1).

In the next series of experiments the propagation of R2 reassortant and its passage variant in MDCK cells was compared. The maximal level of accumulation of R2 reassortant as measured by HA titration was 8 to 16 times lower than the levels of accumulation of its passaged variant or A/USSR/90/77 parent virus (Fig. 3). The PAGE analysis of virus yields purified from fixed volumes of culture fluid also revealed a clear-cut difference: the virus proteins of the passaged variant R2-XXI were abundant, whereas the amount of the proteins of the initial R2 was negligible, as could be expected with so low HA titers (Fig. 4).

Anti-HA monoclonal antibodies reveal an antigenic change in HA of the passage variant of R2 whereas no changes in the antigenic specificity and enzymatic activity of NA is revealed

The results of the velocity sedimentation analysis and the estimation of virus yields suggested that serial passages of "single gene" reassortants in embryonated chicken eggs resulted in a selection of variants lacking both the "aggregation"

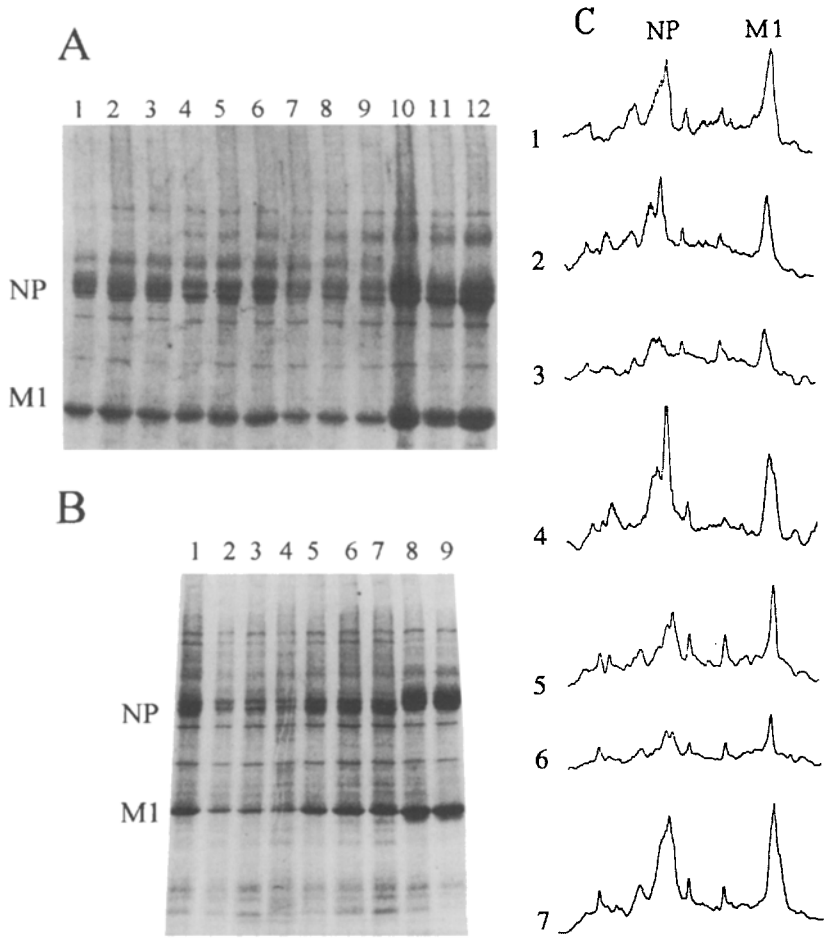

Fig. 2. Assessment of virus yields by PAGE analysis of virus proteins. Allantoic fluid collected from separate eggs was clarified by low-speed centrifugation, the virus was purified from a fixed volume of the fluid as described in Materials and methods, and analysed in PAGE. The gel slabs were stained with Coomassie Blue and scanned with a densitometer. *NP, M1* Positions of virus nucleoprotein and matrix protein, respectively. **A** Comparison of the initial R2 with its passaged variant. *1–3* A/USSR/90/77; *4–6* R2-XXI; *7–9* R2; *10–12* A/Duck/Ukraine/1/63. **B** Comparison of the initial RWB1 with its passaged variant. *1*, *5–7* RWB1-XXI; *2–4* RWB1; *8*, *9* A/USSR/90/77. **C** Densitometer tracings of **A** and **B**. *1* **A**, 1; *2* **A**, lane 4; *3* **A**, lane 7; *4* **A**, lane 10; *5* **B**, lane 1; *6* **B**, lane 2; *7* **B**, lane 8

and the "low yield" characteristics of the initial reassortants. In an attempt to correlate these changes with the characteristics of HA and NA we performed serologic reactions with monoclonal antibodies against HA and NA. The tests with anti-NA mAb were performed both as NI and ELISA with R2, RWB1, R2-XXI, RWB1-XXI and the parent A/USSR/90/77 viruses. In neither case any difference among the viruses could be revealed with a panel of 5 anti-N1 mAb (not shown). In a panel of 9 mAb against HA of A/Aichi/2/68 (H3N2), 6 mAb against site B [2] reacted with HA of A/Duck/Ukraine/1/63. Their titers against

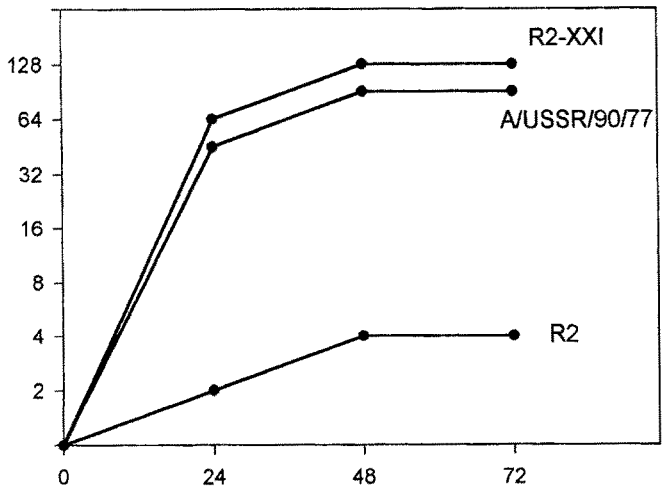

Fig. 3. Accumulation of R2 reassortant and its passaged variant in MDCK cell culture as measured by HA titration. The cells were infected at m.o.i. 0.1 $TCID_{50}$/cell. Abscissa: hours post infection, ordinate: HAU

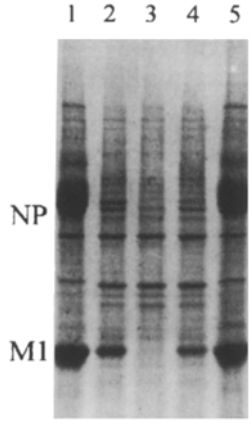

Fig. 4. Virus yields produced by R2 and its passaged variant in MDCK cells. The cells were infected at m.o.i. 0.1 $TCID_{50}$/cell, incubated for 48 h at 37 °C, the virus-containing culture fluids collected and analysed in PAGE as described for the allantoic fluids in the legend to Fig. 2. *1, 5* Allantoic virus A/USSR/90/77 (marker); *2–4* MDCK-propagated A/USSR/90/77, R2 and R2-XXI respectively

Table 2. Reactions of R2 reassortant, its passaged variant and the parent A/Duck/Ukraine/1/63 virus with anti-H3 monoclonal antibodies

Viruses	Monoclonal antibodies					
	31	63	146	152	221	228
R2	<100[a]	800	<100	800	800	800
R2-XXI	12800	12800	6400	3200	12800	6400
A/Duck/Ukraine/1/63	100	1600	<100	800	800	800

[a] Reciprocals of mAb titers

R2-XXI were 4 to 128-fold higher than against the initial R2 or the parent A/Duck/Ukraine/1/63 (Table 2).

The loss of the aggregation in the passaged variants was not correlated with an increase in NA enzymatic activity. The enzymatic activity measured with fetuin as substrate was not increased in the high-passage variants R2-XXI and RSB1-XXI as compared with the original reassortants, R2 and RSB1 respectively (not shown). In the kinetics of the elution from chicken red blood cells no differences in the slopes of the elution curves could be revealed between any one of the three reassortants and its high-passage variant (not shown).

Discussion

Various effects of HA-NA gene combinations on the phenotypic characteristics of influenza A virus reassortants were described, ranging from an impairment of hemagglutinin cleavage [7] to a lack of NA incorporation into virions at an elevated temperature [1]. However, all the reported phenomena were observed with selected pairs of parent viruses, and their significance with respect to the limitations in the formation of HA-NA combinations in natural conditions remained uncertain. In our previous communication [6] we reported that reassortants containing the NA gene of A/USSR/90/77 (H1N1) virus and an HA gene originating from any of the avian or mammalian viruses tested (H3, H4, H10 and H13 subtypes) produced much lower HA titers in chick embryos than any of the parent viruses. The low HA titers of the "single-gene" reassortants (that is, those containing H3, H4, H10 or H13 hemagglutinin genes and all the other genes originating from A/USSR/90/77) were due, at least in part, to the clustering of the virions. Several lines of evidence suggested that the aggregation was a result of an incomplete removal of sialic acid residues by N1 neuraminidase from an "alien" HA (that is, HA derived from the other parent virus). The data suggested a kind of functional mismatch between N1 neuraminidase and the hemagglutinins of the avian and mammalian viruses. However, since the aggregation was not observed at 37 °C, it was unclear in what way it may represent a disadvantage to the virus reproduction and dissemination.

The data reported in the present communication demonstrate that virion aggregation is not the sole cause of the low HA titers of the 'single-gene' reassortants. Their actual virion yields are also lower than those of the parent viruses. One might suppose that the low yields are due to the formation of cell-associated aggregates; however, since the virion aggregates are only formed at 4 °C, and the low yields are observed in the allantoic fluid collected without chilling (Table 1), this explanation seems unlikely. Successive passages of the reassortants in chick embryos have resulted in a selection of high-yield variants. The variants have no tendency to virion clustering; it seems that there is a kind of co-variation between the clustering and the low virion yield. Possibly both features are different phenotypic expressions of the same genotype. The low virion yields of the reassortants, unlike the clustering at low temperatures, may be regarded as an unfavorable feature and a limiting factor for the emergence of

such reassortants under natural conditions. It is noteworthy in this respect that in MDCK cells the effect of the H3N1 HA-NA combination on the virus yield was more expressed than in chick embryos (Fig. 2; Fig. 4).

The results of the reactions with monoclonal antibodies (Table 2) as well as functional tests with neuraminidase seem to indicate that a change in HA rather than in NA results in the loss of the "low-yield" and "aggregation" features in the passaged variants. Sequencing of the HA and NA genes of the reassortants and their passaged variants might provide more insight into the mechanisms of the observed effects. Such studies are now in progress.

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