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COMPARATIVE STUDIES ON BIOLOGICAL PROPERTIES AND GENOME COMPOSITION OF INFLUENZA VIRUS RECOMBINANTS

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Summary. — Some biological properties and the genome composition of antigenic recombinants obtained by crossing of human and animal influenza viruses were studied. Analysis of the recombinants has shown that upon heating of virions in vitro thermostability of the haemagglutinin (HA) does not necessarily correlate with the properties of parent HA; apparently it depended not only on the properties of the HA itself, but also on the peculiarities of other virion proteins. All recombinants obtained by crossing of pathogenic and apathogenic for mice parents either had a reduced pathogenicity for mice or were apathogenic. In some instances, reduction or loss of pathogenicity was observed in recombinants which inherited only one gene from the apathogenic parent; however, the data obtained suggest that pathogenicity involves functions of a number of genes. Human and animal influenza virus strains under study proved to be capable of replication in human embryo tracheal and kidney organ cultures. The degree of reproduction of the recombinants was either lower or higher as compared to the parent strains.

Key words: *influenza virus*; *recombinants*; *genetic markers*; *pathogenicity*

Introduction

Recently some unusual data have been obtained in the studies of influenza virus. (1) The epidemic of 1977 was caused by reappearance of the H1N1 virus. (2) From man epidemic recombinant strains were isolated, the virions of which contained surface antigens of the H1N1 parent and internal proteins of the H3N2 parent (Bean *et al.*, 1980). (3) From whales influenza virus strains were isolated the HA of which was antigenically related to that of strains isolated from man (H0), while its neuraminidase (NA) was similar to that of strains isolated from birds (Nav2) (Lvov *et al.* 1978). (4) From seals an influenza virus was recovered sharing a number of genes with the fowl plague

virus (Webster *et al.*, 1981). In connection with these data, studies of influenza virus recombinants roused general interest.

Development of new techniques for analysis of viral RNAs and proteins made it possible to carry out a more accurate analysis of the genome composition of influenza viruses and to reveal correlation between certain genes and biological properties of the virus.

In the present work we aimed at studying some biological properties and the genome composition of a number of human and avian influenza virus recombinants characterized by various combinations of surface proteins.

Materials and Methods

Viruses. Following influenza viruses were used — human influenza virus reference strains: A/PR/8/34 (H1N1), A/FM/1/47 (H1N1). A/USSR/90/77 (H1N1), A/Singapore/1/57 (H2N2), A/Texas/1/77 (H3N2); avian influenza reference strains: A/turkey/Massachussets/65 (H6N2), A/tern/Turkmeniya/18/73 (H3N2), A/turkey/Wisconsin/66 (H9N2); influenza viruses isolated from various species of animals on the territory of U.S.S.R.: A/pintail/Primorye/695/76 (H2N3), A/redshank/Alma-Ata/760/79 (H10N5), A/tern(Frunze/334/76 (H1N6), A/slender-billed gull/ /Astrakhan/31/76 (H5N3), A/deer/Chukotka/76 (H6N2), A/chick/Tadjikistan/54/77 (H3N2); recombinants obtained earlier by crossing human H1N1 and H2N2 influenza viruses with various animal influenza viruses (Lvov *et al.*, 1979, Podchernyaeva *et al.*, 1980).

The recombinants were obtained in chick embryos (CE) infected with 2 intact influenza viruses (10^7 EID_{50}/0.2 ml of each virus per 1 chick embryo).

Genetic markers (rct 28°, 36°, 40 °C, T_{56}, Pm) were studied as described earlier (Podchernyaeva *et al.*, 1972). A neuraminidase-inhibition (NI) test was performed according to the method recommended by WHO (Aymard-Henry *et al.*, 1973). A haemagglutination-inhibition (HI) test was performed using RDE-treated sera.

Cultivation of influenza viruses in human embryo tracheal and kidney organ cultures was carried out as described earlier (Irzhanov and Khesin, 1972).

RNA-RNA hybridization and analysis of the genome of recombinants were done according to the method of Hay *et al.* (1977). RNA duplexes were analysed by electrophoresis in a 7% polyacrylamide gel containing 5.4 mol/l urea according to a modification of Farashyan *et al.* (1980).

Results

Antigenic characteristics and some biological properties of recombinants

The experiments on recombination between human and animal influenza viruses yielded recombinants containing HA of one parent and NA of another. Antigenic characteristics and results of studies on genetic markers of the parent viruses as well as of the recombinants are summarized in Tables 1 and 2. It can be seen from the Tables that in two experiments on recombination between A/PR/8/34 × A/tern/Frunze/334/78 and A/PR/8/34 × A/deer/Chukotka/76 both direct (H4N1-R17) and reciprocal (H1N6-R10, H1N1-R16) recombinants were selected, which reproduced well in CE at an enhanced (40 °C) temperature of incubation. A correlation between the T_{56}-marker and antigenic type of HA was observed in these recombinants. At the same time no correlation was revealed in a number of other recombinants (R_4, R_6, R_8). In addition, two recombinants were detected (R_{31}, R_{32}) the HA of which proved to be more stable as compared to both parent strains.

Table 1. Antigenic characteristics and biological properties of parent strains and influenza virus recombinants (part I)

Parent strains, recombinants (R) and their antigenic characteristics	Biological properties							
	HA	T_{56}	P_m	rct			HOC	
				28°	36°	40°	Trachea	kidneys
A/PR/8/34 (H1N1)	640	30 min	4.0	5.0	8.0	5.0	3.0	3.5
A/tern/Frunze/334/78 (H4N6)	160	6 hr	< 1.0	3.0	8.2	8.2	5.0	5.8
R_{20} (H1N6)	640	30 min	2.0	3.5	8.2	8.5	4.0	4.0
R_{21} (H1N6)	640	30 min	< 1.0	3.1	8.7	8.5	2.0	2.0
R_{22} (H1N6)	640	30 min	< 1.0	3.0	8.7	8.2	2.2	3.0
R_{17} (H4N1)	160	6 hr	< 1.0	5.1	7.5	7.0	3.0	4.0
A/FM 1047 (H1N1)	320	2 hr	< 1.0	6.0	7.0	6.5	2.0	4.0
A/chick/Tadjikistan/76 (H3N1)	320	10 min	< 1.0	6.0	8.0	7.0	4.0	5.0
R_6 (H1N1)	320	30 min	< 1.0	6.0	7.0	6.8	3.0	3.0
R_8 (H1N1)	320	60 min	< 1.0	6.0	7.2	7.0	n.t.	n.t.
A/pintail/Primorye/695/76 (H2N3)	320	6 hr	< 1.0	7.0	8.5	8.0	5.0	5.0
A/turkey/Massachussets/75 (H6N2)	40	1 hr	< 1.0	6.0	6.8	8.0	4.0	4.0
R_4 (H2N2)	160	3 hr	< 1.0	6.0	8.0	8.0	3.0	3.0
A/pintail/Primorye/695/76 (H2N3)	320	6 hr	< 1.0	7.0	8.5	8.0	5.0	5.0
A/turkey/Wisconsin/66 (H6N2)	160	20 min	< 1.0	7.0	6.0	4.0	4.0	4.0
R_{30} (H2N2)	80	5 hr	< 1.0	7.0	8.0	6.0	3.0	4.0

HA — haemagglutinin titre with chicken erythrocytes; T_{56} — thermostability of HA on heating of virions in vitro at 56 °C (the time of complete reduction of HA titres); P_m — pathogenicity on intranasal inoculation of white mice (log LD_{50}); rct 28, 36, 40° — reproduction in chicken erythrocytes at various incubation temperatures (log EID_{50}); HOC — reproduction in human embryo tracheal and kidney organ cultures (log EID_{50}); n.t. — not tested.

All the recombinants obtained by crossing of pathogenic for mice A/PR/8/34 strain with the avian influenza viruses apathogenic for these animals had a reduced, if any pathogenicity.

Replication in chick embryos and organ cultures

Studies on the reproduction of parent influenza viruses and recombinants were carried out in CE and various incubation temperatures (28, 36, 40 °C) and in human embryo tracheal and kidney organ cultures at 36 °C. The recombinants grew well in CE at optimal temperature (36 °C). Almost all of them inherited the ability to reproduce at an enhanced temperature (40 °C); a lesser portion (except R_{30}) of the recombinants (R_2, R_5, R_{17}, R_{32} and R_{35}) inherited the ability to reproduce efficiently at 28 °C from one of the parents.

Table 2. Antigenic characteristics and biological properties of parent strains and influenza virus recombinants (part II)

Parent strains, recombinants and their antigenic characteristics	Biological properties							
	HA	T_{56}	P_m	rct***			HOC***	
				28°	36°	40°	Trachea	Kidneys
A/Singapore/1/57 (H2N2)	80	6 hr	< 1.0	4.2	6.0	5.0	5.0	3.0
A/tern/Turkmeniya/18/73 (H3N2)	640	6 hr	< 1.0	6.3	8.3	7.0	5.0	5.0
R_5	320	6 hr	< 1.0	6.5	8.0	8.0	4.0	4.0
A/redshank/Alma-Ata/79 (H10N5)	160	1 hr	< 1.0	1.0	7.25	8.75	2.0	1.0
A/PR/8/34 (H1N1)	640	30 min	4.0	5.0	8.0	5.0	3.0	3.5
R_{31} (H10N1)	80	2 hr	2.0	4.25	8.5	8.75	n.t.	n.t.
R_{32} (H10N1)	160	2 hr	1.5	4.25	8.75	7.75	4.0	5.5
R_{33} (H10N1)	80	1 hr	< 1.0	4.5	8.5	8.75	n.t.	n.t.
R_{34} (H10N1)	160	1 hr	< 1.0	4.25	7.75	8.75	n.t.	n.t.
A/duck/Memphis/75 (H11N3)	640	10 min	< 1.0	3.5	8.0	7.0	4.0	5.0
A/PR/8/34 P_m* (H1N1)	640	30 min	< 1.0	5.0	8.0	5.0	3.0	3.5
R_2 (H11N1)	320	10 min	< 1.0	5.2	8.2	7.0	5.0	4.0
R_{10} (H11N1)	320	10 min	< 1.0	n.t	n.t	n.t	5.0	4.0
A/slender billed gull/ Astrakhan/31/76 (H5N3)	640	10 min	< 1.0	1.5	6.0	2.0	3.0	3.5
A/PR/8/34/P_m* (H1N1)	640	30 min	< 1.0	5.0	8.0	5.0	3.0	3.5
R_{35} (H5N1)	1280	10 min	< 1.0	5.25	7.25	7.2	3.0	3.0
A/PR/8/34 P_m* (H1N1)	640	30 min	< 1.0	5.0	8.0	5.0	3.0	3.5
A/deer/Chukotka/76 (H6N2)	320	6 hr	< 1.0	5.2	8.0	7.0	4.5	4.0
R_{16} (H1N2)	640	30 min	< 1.0	5.0	8.0	7.0	3.0	4.0

* A variant of A/PR/8/34 strain apathogenic for mice (Ivanova *et al.*, 1981).
** log LD_{50} *** log EID_{50}
For further explanations see legend to Table 1.

The studies on the reproduction in organ cultures have shown that animal influenza viruses with various types of HA were reproducing in human embryo organ cultures like human influenza viruses. Among human influenza viruses the most efficient reproduction was observed by the epidemic A/USSR/90/77 (H1N1) strain. Among animal influenza viruses, high reproduction was found by A/pintail/Primorye/695/76 (H3N2), A/tern/Frunze/334/76 (H4N6), A/tern/Turkmeniya/18/73 (H3N2) and A/turkey/Wisconsin/66 (H6N2) strains. Some strains and recombinants under study reproduced more efficiently (by 1—2 log) in organ cultures of human embryo kidneys as compared to the reproduction in embryo tracheal cultures. The degree of reproduction in organ cultures did not correlate with the efficiency of reproduction in CE. Reproduction of the recombinants in organ cultures varied greatly: a recombinant was revealed (R_{32}) which reproduced better than both parents, and a reduced reproduction was observed with several recombinants (R_{20}, R_4,

Table 3. Analysis of the genome composition of parent strains and influenza virus recombinants

Parent strains and recombinants	Origin of RNA segments							
	1(P_3)	2(P_1)	(3P_1)	4(HA)	5(NP)	6(NA)	7(M)	8(NS)
A/PR/8/34 (H1N1)	P	P	P	P	P	P	P	P
A/redshank/Alma–Ata/78 (H10N5)	R	R	R	R	R	R	R	R
R_{31} (H10N1)	P	P	P	R	P	P	R	P
R_{32} (H10N1)	P	P	P	R	P	P	P	P
R_{33} (H10N1)	R	R	R	R	P	P	P	P
R_{34} (H10N1)	R	R	R	R	P	P	R	P
A/PR/8/34 (H1N1)	P	P	P	P	P	P	P	P
A/deer/Chukotka/76 (H6N2)	De	De	De	De	De	De	De	De
R_{16} (H1N2)	De	De	De	P	De	De	P	De
A/PR/8/34 (H1N1)	P	P	P	P	P	P	P	P
A/tern/Frunze/78 (H4N6)	T	T	T	T	T	T	T	T
R_{17} (H4N1)	P	P	P	T	P	P	P	P
R_{20} (H1N6)	P	P	P	P	P	T	P	P
R_{21} (H1N6)	P	P	P	P	P	T	T	T
R22 (H1N6)	P	T	P	P	T	T	P	T
A/PR/8/34 (H1N1)	P	P	P	P	P	P	P	P
A/duck/Memphis/75 (H11N3)	D	D	D	D	D	D	D	D
R (H11N1)	P	D	D	D	D	P	D	D
A/FM/1/47 (H1N1)	F	F	F	F	F	F	F	F
A/chick/Tadjikistan/76 (H3N1)	C	C	C	C	C	C	C	C
R_6 (H1N1)	C	C	C	F	C	C	C	C
R_8 (H1N1)	C	C	C	F	C	C	C	C

Letters indicate the strain form which the indicated RNA segment was derived: P — A/PR/8/34, R — A/redshank/Alma–Ata/78, De — A/deer/Chukotka/76, T — A/tern/Frunze/78, D — — A/duck/ Memphis/75, F — A/FM/1/47, C — A/chick/Tadjikistan/76.

R_{21}). Of interest are the recombinants R_{20} and R_{21}: they were obtained in the same recombination experiment, were antigenically similar and their ability to grow in CE correlated; but they differed significantly in the degree of reproduction in organ cultures.

Analysis of genome composition

Analysis of the genome composition of the recombinants and parent influenza virus strains is shown in Table 3. The recombinant R_{16} derived only gene 4 coding for HA from the parent A/PR/8/34 strain, and seven other genes from the second parent — A/deer/Chukotka/76.

Similarity was observed in the genome composition of the recombinants R_6 and R_8 which resulted from crossing of A/FM/1/47 and A/duck/Tadjikistan/76 viruses and inherited gene 4 from A/FM/1/47 strain and all other genes from the second parent. The recombinant R_{10} derived two genes (1 and 6) from one parent (A/PR/8/34) and six other genes from the another (A/duck/Memphis/ 75). Analysis of 4 antigenically closely related recombinants (R_{31}, R_{32}, R_{33}

and R_{34}) obtained by crossing of the A/PR/8/34 strain with another animal influenza virus strain (A/redshank/Alma—Ata/78) showed heterogeneity of the genome composition in spite of similarity of the biological properties. Thus, the recombinant R_{32} inherited gene 4 only from the A/redshank/ /Alma—Ata/78 strain, the recombinant R_{31} derived two genes (4 and 7) from this parent, and the recombinants R_{33} and R_{34} — four (1, 2, 3, 4) or five (1, 2, 3, 4, 7) genes, respectively. The recombinant R_{17} which was obtained by a cross between A/PR/8/34 and A/tern/Frunze/78 strains inherited all genes, except the ones coding for HA, from A/PR/8/34. Heterogeneity of genome compositions was observed in reciprocal recombinants obtained in the same experiment: the recombinant R_{20} derived one gene (6) from A/tern/Frunze/78 strain, the recombinant R_{21} — three genes (6, 7, 8) and the recombinant R_{22} derived four genes (2, 5, 6 and 8) from this parent. It should be noted that the data on genes coding for surface proteins of the recombinants (HA and NA) were always in agreement with the results of protein identification in serological tests.

Discussion

Studies on some biological properties of the antigenic recombinants between human and animal influenza viruses have shown that among the recombinants obtained there were some sharing the biological properties of one parent, as well as someones differing in properties coming from both parent strains. Studies on pathogenicity for mice revealed recombinants which either had a reduced pathogenicity in comparison with parent A/PR/8/34 strain (R_{20}, R_{31}, R_{32}), or proved to be apathogenic like the parent avian influenza virus strains. Of interest was the fact that substitution of a single gene coding for the HA in influenza A/PR/8/34 strain by the corresponding gene of apathogenic avian influenza virus resulted in a recombinant which was completely apathogenic for mice (R_{17}). At the same time, substitution of a single gene of A/PR/8/34 virus coding for the NA (R_{20}) resulted in reduction of pathogenicity of the recombinant. One can assume that the role of the HA in pathogenicity of A/PR/8/34 virus for mice is more important than that of the NA. However, studies of the genome composition of other recombinants suggest that pathogenicity of A/PR/8/34 virus for mice involves functions of other genes as well. Thus, the recombinants R_{21} and R_{22} which, in addition to the gene coding for NA, inherited either the genes 7 and 8 or the genes 2, 5 and 8 from an apathogenic parent, turned out to be completely apathogenic for mice despite the fact that the gene 4 coding for the HA was derived from the pathogenic A/PR/8/34 virus.

Alterations in the pathogenicity may not only depend on substituted genes, but also on their peculiarities in an apathogenic parent, which were inherited by the recombinant. The data show that if the recombinant R_{17} which had derived the gene 4 from the apathogenic A/tern/Frunze/34/78 strain turned out completely apathogenic for mice, then the pathogenicity of the recombinant R_{32} which had the same genome composition except of gene 4 inherited from the apathogenic A/redshank/Alma-Ata/78 strain was decreased rather than completely lost. It should be noted that other

recombinants between A/PR/8/34 and A/redshank/Alma-Ata/78 viruses which derived two (R_{31}), four (R_{33}) and even five (R_{34}) genes from the apathogenic strain, have not fully lost their pathogenicity as it was only to a certain extent reduced.

Studies on the genome composition of influenza virus recombinants revealing a varying degree of pathogenicity for mice confirmed the possible involvement of several genes in manifestation of this property (Scholtissek *el al.*, 1979); they showed that pathogenicity of the recombinants might depend not only on the genome composition, but also on the particular apathogenic strain which the genes had been derived from.

Rather interesting data were obtained in studies of thermostability of HA from recombinants and parent influenza virus strains in a process of heating of virions at 56 °C in vitro. It was shown that this property did not necessarily correlate with the thermostability of the parent HA. Thus, the recombinants R_6 and R_6 inherited the HA of the thermostable parent, but had a significantly lower thermostability as compared to the parent strain; in contrast, several other recombinants (R_{31}, R_{32}) turned out to be thermostable. The analysis of the genome composition showed that among the later there were such recombinants which had inherited from the thermostable parent only the gene coding for HA (R_6, R_8, R_{32}) and nevertheless, the thermostability of HA has changed. These data suggest that thermostability of influenza virus HA in a virion depends not only on the properties of HA itself, but also on these of other viral proteins which are able to influence its stability at higher temperature. On the other hand, such an influence of other proteins on HA thermostability may depend on the peculiarities of these proteins, as it was the case with pathogenicity manifestation. In the recombinant R_{16} which inherited gene 4 from A/PR/8/34 strain and all other genes from A/deer/Chukotka/76 virus, thermostability of HA was exactly the same as that of A/PR/8/34 strain. These data should be taken into consideration while obtaining recombinants possessing a thermostable HA, intended for practical use.

Our data have shown that some of the influenza virus strains isolated from man and animals proved capable of reproducing in human embryo tracheal and kidney organ cultures. Reproduction of various strains in these systems was different and did not correlate with the degree of reproduction in CE. The recombinants obtained reproduced in organ cultures to a various degree — both to lower titres when compared to the parent strains (R_{30}, R_4, R_{21}), as well as to higher ones (R_{32}). The genome analysis of the recombinants studied failed to reveal any dependence between the degree of virus reproduction in human embryo organ cultures and a function of a certain gene. Apparently, the ability of influenza viruses to multiply in this sytem depends on a function of a number of genes, constellation of genes probably being of particular importance. This assumption is confirmed by existence of the recombinant R_{32} which inherited only the gene coding for the HA from one parent and all other ones from the second parent, but multiplied more efficiently than both did.

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