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LETTER TO THE EDITOR

Yields of virus reassortants containing the HA gene of pandemic influenza 2009 virus

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The recent pandemic, due to the swine-origin 2009 H1N1 influenza virus (*11*), raises a concern about the future appearance of drift variants and the new outbreaks caused by the H1N1 subtype. There is a need to develop high-yield strains for the production of inactivated, split, or subunit vaccines. The strains for the production of inactivated or subunit vaccines produced in embryonated chicken eggs usually contain hemagglutinin (HA) and neuraminidase (NA) of an epidemic isolate, and the other genes derived from the high-yield A/Puerto Rico/8/34 (H1N1) strain. The vaccine strains against the 2009 H1N1 virus have been developed on the basis of several 2009 isolates (*10*). Some reassortants contained not only the HA and NA genes of the pandemic virus, but other genes as well. The vaccine strains were produced by both reverse genetics technique and by conventional virus crossing.

We produced a series of reassortants of different genetic content by crossing of A/Moscow/IIV01/2009 (H1N1) pandemic strain with a high-yield H3N2 strain containing the genes of internal and non-structural proteins of the PR8 virus. The H1N1 and H1N2 reassortants were characterized with respect to the virus yield in the embryonated chicken eggs. The initial goal was to produce a candidate vaccine strain against the 2009 pandemic influenza virus (*2*). However, the characterization of the H1N1 and H1N2 reassortants of different genetic content with respect to the virus yield in the embryonated chicken eggs produced some data on the effect of gene constellation on the high-yield properties of influenza vaccine strains.

The strain A/Moscow/IIV01/2009 (H1N1) was isolated during the 2009 pandemic in parallel in MDCK cell culture and in embryonated chicken eggs (*9*). The variant isolated in eggs was used in our studies for the crossing with X-31 (H3N2) reassortant. The X-31 strain contains the HA and NA genes of A/Aichi/2/68 (H3N2) virus and 6 genes of internal and non-structural viral proteins derived from PR8 virus (*1*). The viruses were propagated in 10-day-old embryonated chicken eggs, and the virus-containing allantoic fluid was aliquoted and stored at -80°C. The infectivity titration was determined by limiting dilution method in embryonated chicken eggs. The assessment of the hemagglutination titer was performed using conventional technique. For virus concentration and partial purification the virus-containing allantoic fluid was clarified by low-speed centrifugation and layered on top of 4 ml of 20% sucrose. The virus was pelleted by the centrifugation at 22,000 rpm for 90 min in SW27.1 rotor.

Polyclonal antisera were obtained by immunization of guinea pigs (Laboratory Animal Breeding Institution of the Russian Academy of Medical Sciences, Andreevka, Moscow Region, Russia) weighing 250 g. The purified virus to be used for immunization of animals was treated with 0.1% glutaraldehyde for 7 days at 4°C. The immunization of guinea pigs

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Abbreviations: HA = hemagglutinin; MAb(s) = monoclonal antibody(ies); NA = neuraminidase; PR8 = A/Puerto Rico/8/34 influenza virus

Table. Virus yield of the parent viruses and reassortants as measured by total virus protein and HA content in allantoic fluid

Viruses		HA subtype/virus	NA subtype/virus	PB1 gene	The rest of genes	HA titer (HAU/ml)	Total virus protein (μg/ml)[a]	HA protein (μg/ml)[a]
Parental	Moscow	H1	N1			64-128	4.67 ± 0.45	0.83 ± 0.06
	X-31	H3/Aichi	N2/Aichi	PR8	PR8	1024-2048	44.83 ± 2.96	12.81 ± 0.79
Reassortant	ReM1	H1/Moscow	N2/Aichi	PR8	PR8	256-512	11.14 ± 0.50	2.57 ± 0.13
	ReM8	H1/Moscow	N1/Moscow	PR8	PR8	265-512	15.10 ± 0.60	3.50 ± 0.19
	ReM14	H1/Moscow	N1/Moscow	Moscow	PR8	128-256	6.22 ± 0.35	1.84 ± 0.17

[a]Mean ± SE. Moscow = A/Moscow/IIV01/2009 (H1N1); Aichi = A/Aichi/2/68 (H3N2); PR8 = A/Puerto Rico/8/34 (H1N1). X-31 = X-31 (H3N2) reassortant.

was performed as described in our earlier publication (*14*). The sera were heated for 30 min at 56°C.

MAbs 1E7, 3D9, 5F7, and 6A3 against the HA of influenza A/Moscow/IIV01/2009 (H1N1) virus (*7*) were kindly supplied by Dr. A. Kushch, Laboratory of Cell Engineering, D. I. Ivanovsky Institute of Virology.

The protocol used for reassortment included the mixed infection of embryonated chicken eggs with UV-irradiated and non-irradiated parent viruses (*15*) with modifications described in our earlier publications (*3, 5*).

The assessment of virus protein yields was performed by scanning protein bands after PAGE of virus proteins (*13*), and by the titration of virus antigen in ELISA (*12*).

The choice of the X-31 (H3N2) as a parent virus enabled us to use a polyclonal serum for the selection of reassortants, which would be difficult if the crossings were performed between two H1N1 viruses. The Moscow virus was UV-irradiated to lower the infectious titer by 5 log units, mixed with an equal amount of non-irradiated X-31 virus and used for a one-cycle infection of the embryonated chicken eggs at a high multiplicity of infection. The allantoic fluid was collected, treated with hyperimmune polyclonal guinea pig serum against X-31 virus and used for the cloning of reassortants by limiting dilutions technique in chick embryos. Four reassortant clones containing the HA of H1 subtype were cloned and genotyped by partial sequencing. The genetic content of the reassortants is presented in the table. The reassortants were characterized with respect to their virus yield in embryonated chicken eggs.

Preliminary data obtained by hemagglutination titration suggested that the reassortants ReM8, ReM1, and ReM14 had higher virus yields in allantoic fluid than the parent Moscow 2009 virus. However, the yields of the reassortants were lower than the yield of X-31 virus. The range of the yields produced by the reassortants was confirmed and specified by the comparison of virus protein content in the allantoic fluid as revealed both by the scanning of virus protein bands in stained polyacrylamide gel slabs and the titration of the HA antigen in ELISA (Table). ReM8 having HA and NA genes of the Moscow virus and 6 genes of PR8 produced the highest yield among the reassortants. The yield of the H1N2 reassortant ReM1 was slightly lower than the yield of ReM8. The difference between the yields of ReM8 and ReM1 was small, but reproducible and statistically significant at the probability level $P \leq 0.05$. The reassortant ReM14 having PB1, HA and NA genes of the Moscow virus produced low yields, just slightly higher than the yields of the parent 2009 strain.

The conventional way to produce high-yield reassortants of influenza A virus to be used as vaccine strains is the crossing of epidemic isolates with PR8 virus, or an equivalent plasmid transfection procedure. Thus, the reassortants, which contain the HA and NA genes of a human epidemic virus and the other genes of PR8 virus were shown to be as high-yield as the parent PR8 strain (*6*). Conversely, the reassortants having HA and NA of avian influenza viruses often produce moderate yields in the embryonated chicken eggs, intermediate between the yield of the avian parent virus and the A/Puerto Rico/8/34 (H1N1) strain (*8, 14*). In the present studies we detected a similar situation with respect to the pandemic "swine-like" virus of 2009.

The presence of HA and NA genes having originated from different parents may lead to low yields and a tendency to virion aggregation in avian-human reassortants (*3, 4*). In the present study we have not registered this kind of functional HA-NA mismatch in the reassortant ReM1, having HA and NA originating from different parents. The yield of the H1N1 reassortant was slightly higher than the yield of the H1N2 reassortant, yet the difference, though reproducible, was small. Most likely, the functions of the pandemic 2009 H1N1 virus HA and the human virus N2 NA are better balanced than the functions HA and NA in the avian-human influenza virus reassortants.

In our previous studies with avian-human reassortants we observed an expressed increase of virus yield produced by the introduction of the avian virus PB1 gene in the genetic content of the reassortant (*14*). An enhancement of virus yield by the introduction of the PB1 gene of the 2009 pandemic virus in the genetic content of a reverse genetics-derived reassortant was also reported (*16*). In the present studies the introduction of the PB1 gene of the 2009 pandemic virus decreased the yield. It seems likely that there is no general rule with respect to the effect of the PB1 gene on the yield

of a reassortant, and this effect may vary in different pairs of the parent viruses. The optimal gene constellation for a high-yield production of a reassortant influenza vaccine strain has to be identified for each pair of parent viruses.

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