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ATTENUATED TS-RECOMBINANTS OF INFLUENZA A/USSR/77 (H1N1) VIRUS OBTAINED BY CROSSING WITH THE COLD-ADAPTED DONOR A/LENINGRAD/134/57 (H2N2) VIRUS

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Summary. — Conditions of obtaining attenuated influenza virus recombinants by crossing of a cold-adapted donor with A (H1N1) influenza virus that reappeared in 1977 were studied. Temperature-sensitive recombinants suitable for intranasal immunization of adults with low titres of anti-haemagglutinin and anti-neuraminidase antibodies, and possessing sufficiently high immunogenicity were obtained by crossing of native parent strains and cross-reactivation techniques. It was confirmed that the cold-adapted A/Leningrad/134/17/57 (H2N2) influenza virus variant is a prospective donor of attenuation for obtaining recombinants — — candidates for live influenza vaccine strains.

Key words: *influenza virus*; *recombination*; *live vaccine*

Introduction

Obtaining of attenuated strains for a live influenza vaccine by recombination of current epidemic viruses with a well-known donor of attenuation offers a number of advantages over the method of serial passaging in chick embryos. The recombination technique ensures rapid formation of clones which inherit from the donor of attenuation such useful properties as temperature-sensitivity and safety, and retain the immunogenic potency of epidemic strains. In previous works we showed the possibility of regular obtaining of attenuated recombinants from different H3N2 virus variants that have been circulating in the U.S.S.R. since 1973 (Polezhaev *et al.*, 1974, 1978; Aleksandrova *et al.*, 1979). In these investigations we stressed the particular importance of the use of the cold-adapted A/Leningrad/134/17/57 (H2N2) influenza virus strain, safe for both adults and children on intranasal administration, as a donor of attenuation.

In the present investigations we attempted to obtain attenuated recombinants from the epidemic influenza A virus (H1N1) that reappeared in the human population in 1977.

Materials and Methods

Viruses. The virulent A/Khabarovsk/1/77 (H1N1) strain, related to the A/USSR/1/77 (H1N1) virus, had undergone 2 passages in chick embryos at 32° C. The cold-adapted tempereture-sensitive A/Leningrad/134/17/57 (H2N2) virus was used as a donor of attenuation; its characteristics were described by Aleksandrova *et al.* (1977).

The recombination technique was described by Polezhaev *et al.* (1978). Crossing of parent native viruses or cross-reactivation techniques were used; the latter involved pre-inactivation of the virulent virus by heating at 40 °C for 24 hr. Recombination was carried out by infection of chick embryos with parent viruses in a dose of 7.5 log EID_{50}/0.2 ml. The infected embryos were incubated at 32 °C for 18 hr. The pooled harvests were passed twice and cloned in chick embryos by the limiting dilutions technique at 25 or 32 °C in the presence of serum against the cold-adapted A/Leningrad/134/17/57 (H2N2) strain. The infected chick embryos were incubated for 72 and 48 hr, respectively.

Temperature-sensitivity of the viruses was evaluated by the difference in infectious titres in chick embryos determined after 48 hr of incubation at 32 and 40 °C.

Vaccination technique. Monovaccines prepared from the recombinants with a titre of 7.5 log EID_{50}/0.2 ml and tested for bacterial sterility and absence of extraneous viruses were used. The preparation was given to healthy adults twice, with an interval of 14 days; 0.25 ml volumes of a 1 : 2 dilution were administered intranasally, into each nostril using a sprayer.

Evaluation of reactogenicity. The volunteers were clinically examined daily for 5 days after vaccination, including temperature measurements. In calculations of the reactogenicity index, temperature reactions of 37.6 °C and higher were taken into account. Assessment of vaccine potency was carried out in April and October, 1978 as well as in April and September, 1979.

Virus immunogenicity for humans. Paired sera were taken from the vaccinees before immunization and 21 days after the second vaccination. Anti-haemagglutinin antibody was assayed by standard haemagglutination inhibition (HI) tests, anti-neuraminidase antibody by the inhibition-elution (NIE) test described by Appleyard and Oram (1977) and Topuriya *et al.* (1979). The R-7 A/Eq1/Prague/1/56 — A/Ufa/771/77 (Heq1N1) recombinant, obtained and kindly provided by N. E. Gorev was used as antigen in the NIE test. Briefly, the technique was as follows: twofold dilutions of sera heated at 56 °C for 1 hr were mixed with an equal volume of virus (8 HA units). After 1 hr of contact, a suspension of human erythrocytes was introduced into the mixtures. The plates were incubated in a humid chamber at 36° C for 16 hr. Erythrocytes were then added to the supernatant. The reciprocal of the serum dilution in which no haemagglutination was observed was taken for the antibody titre.

Results

Characterization of the initial viruses used for recombination

The initial virulent A/Khabarovsk/1/77 (H1N1) virus was highly temperature-sensitive, i. e. uncapable of reproducing at 40 °C. The difference in titres at 32 and 40 °C was 5.0 log EID_{50}/0.2 ml; it could not be differentiated from the cold-adapted donor of attenuation by this marker (Table 1). The initial A/Khabarovsk/1/77 virus was cloned at 40 °C; as a result the A/Khabarovsk/1/77 K virus was obtained. It possessed a high capcity to reproduce at 40 °C, the difference in titres at 32 and 40 °C decreased to 0.

Conditions of obtaining attenuated H1N1 recombinants

To obtain H1N1 recombinants, crossing of native parent strains or cross-reactivation techniques were used; the latter involved reactivation of a heat-inactivated wild strain with a native attenuated virus.

Recombination of the native parent viruses resulted in clones H/25/5 and H/25/17, while clone H/32/5 was obtained by cross-reactivation. All

Table 1. Temperature-sensitivity of parent and recombinant influenza A virus strains

Virus	log EID_{50}/0.2 ml at 32 °C	log EID_{50}/0.2 ml at 40 °C	Difference in titres at 32 and 40 °C (RTC_{40} marker)
A/Khabarovsk/1/77 (H1N1)	6.5	1.5	5.0
A/Khabarovsk/1/77 K	6.25	6.25	0
A/Leningrad/134/17/57 (H2N2)	8.5	1.5	7.0
H/25/5 (H1N1)	7.75	2.5	5.25
H/25/17 (H1N1)	8.25	3.0	5.25
H/23/5 (H1N1)	7.5	1.5	6.0

these viruses possessed the H1N1 surface antigens, like the virulent A/Khabarovsk/1/77 strain.

The selected recombinants possessed a high temperature-sensitivity, i. e. their capacity to reproduce at 40 °C was lowered (Table 1). The difference in titres at 32 and 40 °C was 5.25-6 log EID_{50}/0.2 ml, approaching that of the donor of attenuation, the A/Leningrad/134/17/57 (H2N2) virus.

Immunizing properties of the recombinants

Reactogenicity of the recombinants H/25/5, H/25/17 and H/32/5, obtained by crossing of the A/Khabarovsk/1/77 (H1N1) and A/Leningrad/134/17/57 (H2N2) viruses, was studied in groups of persons aged 18-20 years who had different levels of anti-haemagglutinin and anti-neuraminidase antibodies. All the recombinants tested were characterized by low reactogenicity (based on trials in large groups of volunteers irrespective of the initial level of humoral immunity) (Table 2). Intranasal administration of monovaccines from the H/25/5, H/32/5 and H/25/17 recombinants caused only occasional transient febrile reactions with temperatures up to 37.6 °C and higher.

Table 2. Reactogenicity of influenza virus recombinants A/Khabarovsk/1/77 (H1N1) and A/Leningrad/134/17/57 (H2N2) for persons with different levels of anti-haemagglutinin and anti-neuraminidase immunity

Recombinant	Anti-haemagglutinin antibody titre ≦ 16			Anti-neuraminidase antibody titre ≦ 16			Irrespective of initial antibody level		
	I	II	III	I	II	III	I	II	III
H/25/5	25	0	0	25	0	0	41	0	0
H/25/17	47	0	0	47	1	2.1	76	1	1.3
H/32/5	54	1	1.8	25	0	0	69	1	1.4

I — No. of vaccinees with the indicated antibody titre.
II and III — No. (II) and % (III) of vaccinees with a temperature rise (37.6 °C and higher).

Table 3. Immunogenicity of the A/Khabarovsk/1/77 (H1N1) and A/Leningrad/134/17/57 (H2N2) influenza virus recombinants depending on anti-haemagglutinin and anti-neuraminidase antibody titres

Recombinant	Initial antibody titre	Changes in anti-haemagglutinin antibody titres			Changes in anti-neuraminidase antibody titres		
		I	II	III	I	II	III
H/25/5	≤ 8	37	28	75.7	26	4	15.4
	≥ 256	89	52	58.4	31	5	16.1
H/25/17	≤ 8	39	21	53.8	33	8	24.2
	≥ 256	76	39	51.3	36	10	27.8
H/32/5	≤ 8	141	98	69.5	121	45	37.2
	≥ 256	275	121	44.0	214	63	29.0

I — No. of vaccinees.
II and III (No. II) and % (III) of vaccinees showing 4-fold or higher seroconversion.

Immunogenicity of the recombinants was determined after a two-dose immunization of the volunteers based on increases of anti-haemagglutinin and anti-neuraminidase antibody in paired blood sera. The HI test revealed the highest seroconversion rates (69.5 and 75.7% for persons with antibody titres from 0 to 8) with the H/32/5 and H/25/5 recombinants (Table 3). When determined for the whole group of the vaccinees irrespective of the initial antibody titres, these rates were 44.0 and 58.4%, respectively. The H/25/17 recombinant proved to be the least immunogenic, causing a 4-fold increase of anti-haemagglutinin antibody in 53.8% of seronegative volunteers.

A rise in anti-neuraminidase antibody was frequently observed in volunteers vaccinated with the H/32/5 recombinant (37.2%). According to the NIE test, seroconversion among volunteers vaccinated with the H/25/5 and H/25/17 recombinants occurred only in 15.4 and 24.2% of persons, respectively, this percentage having been significantly lower than that obtained in assays of anti-haemagglutinin antibody.

The highest immunogenicity was thus shown by the recombinant H/32/5 that actively induced antibody to both surface antigens of influenza virus, haemagglutinin and neuraminidase.

Discussion

Previously (Polezhaev *et al.*, 1973, 1978; Aleksandrova *et al.*, 1979) we reported data about the conditions of obtaining attenuated influenza A and B virus recombinants safe for intranasal immunization of adults. This was achieved by crossing different wild influenza A (H3N2) virus strains (A/Victoria/35/72, A/Port Chlamers/1/73, A/Victoria/3/75) and the B/Hong Kong/5/72 strain with attenuated cold-adapted strains A/Leningrad/134/17/

/57 (H2N2), A/Leningrad/9/37/46 (H0N1) and B/Leningrad/14/17/55. We showed that attenuated recombinants can be regularly obtained by crossing native parent strains and by the cross-reactivation technique, when a virulent parent had been inactivated by heating.

In the present work we studied the conditions of obtaining attenuated recombinants from influenza A (H1N1) virus that reappeared in the human population in 1977. The epidemic A/Khabarovsk/1/77 (H1N1) strain related to the A/USSR/1/77 variant used in our experiments was characterized by a temperature-sensitivity unusual in virulent strains, i. e. it was a natural ts mutant; it did not differ in its ts-phenotype from the donor of attenuation, the A/Leningrad/134/17/57 (H2N2) virus. This circumstance could have hampered the selection of attenuated recombinants based on the ts marker. Therefore, before carrying out the main experiment on obtaining temperature-sensitive A (H1N1) recombinants, we attempted to obtain a temperature-resistant clone of the epidemic A/Khabarovsk/1/77 (H1N1) virus. For this purpose the initial virus was passed once in chick embryos at 40 °C, following which we could rapidly decrease the temperature-senstitivity of the initial virus and obtain the ts- variant A/Khabarovsk/1/77 (H1N1) K that reproduced well both at high (40 °C) and optimal (32 °C) temperatures. This variant of the epidemic A/Khabarovsk/1/77 (H1N1) virus was used for obtaining H1N1 recombinants by crossing with the donor of attenuation, the A/Leningrad/134/17/57 (H2N2) virus. The three temperature-sensitive clones obtained proved to be safe on intranasal administration to susceptible persons.

The present data confirmed once again that the cold-adapted A/Leningrad/ /134/17/57 virus is a prospective donor of attenuation for rapid obtaining of areactogenic recombinants. The advantage of this virus over the similar cold-adapted A/Ann Arbor/6/60 strain obtained in the U.S.A. by Dr. Maassab (Maassab *et al.*, 1977; Spring *et al.*, 1977; Cox *et al.*, 1981) is the presence of multiple (at least three) ts-mutations in the genome, and well-documented safety even for children aged 1-3 years (Ghendon *et al.*, 1981). Crossing of epidemic viruses with the indicated donor ensures immediate elimination of their pathogenicity and does not require additional passages which are necessary when the A/PR8/34 virus is used (Rubin *et al.*, 1976).

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