Acta virol. 24: 273—278, 1980

PECULIARITIES OF OBTAINING ATTENUATED THERMOSENSITIVE RECOMBINANTS OF INFLUENZA A VIRUS AT THE END OF THE H3N2 EPIDEMIC CYCLE

F. I. POLEZHAEV, L. M. GARMASHOVA, V. I. RUMOVSKY, G. I. ALEKSANDROVA, A. A. SMORODINTSEV

Dept. of Virology, Research Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences, 197022 Leningrad, U.S.S.R.

Received January 16, 1980

Summary. — The conditions of obtaining thermosensitive recombinants of the virulent A/Leningrad/82/76 (H3N2) virus with two donors of attenuation, A/Leningrad/134/17/57 (H2N2) and A/Leningrad/9/37/46 (H0N1), were evaluated. The recombinants were obtained by various methodical approaches (hybridization of native viruses, cross-reaction and selection of recombinants at temperatures of 25, 32 and 40 °C) to study their effects on the degree of attenuation and the regularity of transmission of the ts marker. The recombinants examined varied in thermosensitivity which depended on the conditions of recombination of parent viruses. All recombinants studied, irrespective of their RCT_{40} marker, were innocuous for men.

Key words: influenza A virus; recombination; attenuation; ts marker

Introduction

Our method of genetic recombination, based on crossing of virulent viruses with cold-adapted strains of influenza virus innocuous for children and possessing a ts marker, makes it possible to obtain regularly attenuated recombinants innocuous for adults and possessing high immunogenicity (Polezhaev *et al.*, 1974, 1978; Aleksandrova, 1977; Polezhaev and Aleksandrova, 1978; Aleksandrova *et al.*, 1979*a*, *b*). The prospects of using the donors mentioned for rapid attenuation of virulent viruses was first demonstrated on their crossing with epidemic strains of A (H3N2) influenza virus circulating from 1972 to 1975, as well as with influenza virus B/Hong Kong/3/72.

In the present work we evaluated the conditions of obtaining and the results of a broader study of 9 thermosensitive recombinants of the virulent A/Leningrad/82/76 (H3N2) virus with two donors of attenuation, A/Leningrad/134/17/57 (H2N2) and A/Leningrad /9/37/46 (H0N1).

The A/Leningrad/82/76 (H3N2) virus corresponded by its antigenic properties to the A/Victoria/3/75 (H3N2) strain and was one of the last variants that completed the epidemic cycle of H3N2 influenza by the end

of 1976 and beginning of 1977, i. e. 1 year before the reappearance of the A(H1N1) (A/USSR/77) subtype (Ivanova *et al.*, 1977).

The recombinants of A/Leningrad/82/76 virus were prepared by various methods of crossing with thermosensitive donors of attenuation to study the effects of the methods on the degree of attenuation and on the regularity of transmission of the ts marker.

Materials and Methods

Viruses. The virulent A/Leningrad/82/76 (H3N2) virus was isolated in our Department after two chick embryo passages at 32 °C. Two thermosensitive viruses, A/Leningrad/134/17/57 (H2N2) and A/Leningrad/9/37/46 (H0N1), were used as donors of attenuation. The characteristics of the strains were reported by Aleksandrova (1977).

Methods of recombination. The recombinants were obtained by crossing native parent viruses (recombination) or by reactivation of heat-inactivated virulent virus by native attenuated virus (cross-reactivation). Groups of 5 developing chick embryos each were inoculated with a mixture of virulent and thermosensitive virus with titres of 7.5 log EID_{50}/0.2 ml. In recombination, equal volumes of the two viruses were mixed. In cross-reactivation experiments, the virulent virus had been previously heated at 40 °C for 18 hr until complete loss of infectivity. The inoculated embryos were incubated for 18 hr at 32 °C. The allantoic fluids were then harvested and pooled. Cloning was done by the limiting dilution method in the presence of antiserum to the thermosensitive virus and at three temperatures of incubation (25, 32 and 40 °C). The inoculated embryos were incubated for 72, 48 and 24 hr. Threreafter clones with heamagglutinin and neuraminidase corresponding to the virulent strain (H3N2) were selected. The method of determination of surface antigen specificity was described (Polezhaev *et al.*, 1973).

Thermosensitivity of the virus strains and recombinants (RCT_{40} marker) was determined based on the difference in infectivity titres at 32 and 40 °C.

Vaccination of humans. Monovaccines from the recombinants obtained with a titre of 7.5 log EID_{50}/0.2 ml, checked for bacterial sterility and absence of contaminating viruses were used. They were diluted 1 : 2 and administered to healthy volunteers intranasally twice at a 14-week interval in 0.25 ml volumes into each nostril using a sprayer.

Evaluation of reactogenicity. The vaccinees were subjected to medical examination and their temperature was measured daily for five days. The virus was considered reactogenic if 2 or more per cent of the vaccinees showed a temperature reaction (over 37.5 °C). The tests were carried out in November — December, 1976, and February — March, 1977.

Immunogenicity of the viruses for humans. Paired sera were obtained from the vaccinees before immunization and 21 days after the second vaccination. Titres of specific antibody were determined by routine haemagglutination inhibition (HI) tests. We calculated the frequency of 4-fold or higher increases in antibody titres in the blood, geometric mean titre values and the value of the antibody increase after the second vaccination.

Results

Effects of hybridization methods on thermosensitivity of recombinants

The virulent parent strain A/Leningrad/82/76 (H3N2) used for recombination replicated actively at the nonpermissive temperature of 40 °C, i. e. it was thermoresistant as distinct from the donors of attenuation — the thermosensitive cold-adapted viruses A/Leningrad/134/17/57 (H2N2) and A/Leningrad/9/37/46 (H0N1). RCT_{40} values for the virulent virus and the donors of attenuation were 1.0, 6.5 — 7.0 and 6.0 — 6.5 log EID_{50}/0.2 ml, respectively.

Table 1. Effects of the conditions of crossing on the thermosensitivity of recombinants of virulent influenza virus A/Leningrad/82/76 (H3N2) with cold-adapted strains

Donor of attenuation	Clones selected at °C	Recombinants obtained by cross-reactivation		Recombinants obtained by crossing of native viruses	
		Clone No.	RCT_{40}*	Clone No.	RCT_{40}*
A/Leningrad/9/37/46	25	13	6.0	13	4.75
(H0N1)		14	5.0	14	5.0
(RCT_{40} 6.0—6.5)		15	6.25	15	5.25
	32	22	6.5		
		23	5.5	21	1.75
		24	4.75		
	40	4	3.25	1	2.25
		5	3.5	3	0
A/Leningrad/134/17/57	25	2	6.75	16	6.0
(H2N2)		5	6.75	17	6.0
(RCT_{40} 6.5—7.0)	32	17	6.0	13	3.75
		18	4.25	14	3.25
				15	3.0
	40	18	5.75	1	1.0
				3	1.5

* RCT_{40} marker expressed as difference in log EID_{50}/0.2 ml values at 32 and 40 °C.

The conditions of recombination and the temperature at which subsequent selection of recombinant clones was carried out had a significant effect on the RCT_{40} values of the viruses obtained. As shown in Table 1, the highest RCT_{40} values were found in recombinants obtained by cross-reactivation (heat-inactivated virulent virus reactivated with native cold-adapted strain). In this case thermosensitivity was the highest in clones selected at 25 °C (RCT_{40} 6.25—6.75 log EID_{50}) as compared with recombinants selected at optimal or (especially) nonpermissive temperature.

The effect of selection temperature on the RCT_{40} values of the recombinantss was the most marked when the latter were obtained by hybridization of native viruses. Thermosensitivity of the clones thus obtained varied from very high RCT_{40} values (6.0—4.75 log EID_{50}) corresponding to the ts marker of the attenuated donors, to very low values (1.0—2.25 log) approaching that of the virulent strain A/Leningrad/82/76. Like in cross-reactivation experiments, a high thermosensitivity was found in recombinants obtained following selection at 25 °C. No thermosensitive variants were obtained by cloning at optimal or nonpermissive temperature (maximal RTC_{40} value of 2.25 log EID_{50}).

Reactogenicity and immunogenicity of recombinants obtained under different conditions

Since hybridization conditions markedly affected the ts phenotype of the recombinants obtained, it could be assumed that viruses differing in thermosensitivity would also differ in the degree of their reactogenicity in humans. But examination of recombinants possessing different RCT_{40} markers in

Table 2. Vaccinal properties of recombinants obtained under different conditions

Donor of attenuation, recombination method[1]) and temperature of selection of the clone		Clone No.	Reactogenicity for persons with original blood antihaemagglutinin titres 0—8	Reactogenicity for persons with original blood antihaemagglutinin titres total	Immunogenicity for persons with original blood antihaemagglutinin titres 0—8 I[2])	II[3])	III[4])
A/Leningrad/9/37/46 (H0N1)	N, 25 °C	14	56/0/0[5])	115/0/0[5])	24/21/87.5	8.0	39.0
	N, 40 °C	1	53/1/1.8	87/1/1.1	50/43/86.0	7.5	32.0
	CR, 25 °C	14	34/0/0	61/0/0	29/25/86.2	8.0	39.0
	CR, 32 °C	22	52/0/0	106/0/0	40/32/80.0	8.0	42.0
A/Leningrad/134/17/57 (H2N2)	N, 25 °C	17	43/0/0	85/0/0	30/24/80.0	11.3	69.9
	N, 32 °C	15	12/0/0	16/0/0	12/8/66.6	7.5	34.0
	N, 40 °C	1	17/0/0	22/0/0	11/9/81.8	6.1	39.0
	CR, 25 °C	2	33/0/0	53/0/0	21/19/90.4	7.5	34.0
	CR, 32 °C	17	60/0/0	111/0/0	39/18/46.15	8.0	19.7

[1]) N = recombinants obtained by crossing of native viruses; CR = cross-reactivation of heat-inactivated parent virus.
[2]) Total No. of persons examined/No. of persons with a 4-fold or higher increase in blood antibody titres/% of the latter persons.
[3]) Geometric mean increase in antibody titre (-fold).
[4]) Geometric mean antibody titre in 2nd serum samples.
[5]) Total No. of vaccinees/No. of vaccinees with a temperature reaction of 37.6 °C and higher/% of vaccinees with a temperature reaction of 37.6 °C and higher.

rather large groups of adults, including persons without antihaemagglutinins in their blood before vaccination, showed that all the recombinants had lost virulence for humans. Eight of the nine recombinant clones studied caused no febrile reaction of medium or high intensity even in highly susceptible persons with antibody titres from 0 to 8 (Table 2). Only on vaccination of 53 volunteers with recombinant No. 1, obtained by crossing of native viruses A/Leningrad/82/76 and A/Leningrad/9/37/46 and selection at 40 °C, one person had elevated temperature of 37.6 °C for a short time. But the general index of reactogenicity of this recombinant was rather low (1.8%).

Most of the recombinants studied were highly immunogenic, causing seroconversion in more than 80% of vaccinees with initial antibody titres of 8 or lower. The mean increase in antibody titres after two immunizations was from 7.5- to 8-fold and the geometric mean antibody titre in the second serum samples was at least 32.0. An exception was recombinant No. 17, obtained by cross-reactivation of the epidemic virus A/Leningrad/82/76 with the cold-adapted strain A/Leningrad/134/17/57 and selected at 32 °C. This recombinant characterized by a rather low immunogenicity caused seroconversion in only 46.15% of vaccinees.

Discussion

Cold-adapted vaccine strains of influenza virus A/Leningrad/134/17/57 (H2N2) and B/Leningrad/14/55, innocuous for children, obtained by long-term passaging at optimal temperature and subsequently adapted to lowered

temperature (25 °C) (Aleksandrova, 1977), proved to be prospective donors in obtaining recombinants innocuous for adults (Aleksandrova *et al.*, 1979*a*, *b*). But in view of the low numbers of persons examined these preliminary experiments could not fully characterize the vaccinal properties of the recombinants.

The present investigations carried out on rather large groups of susceptible adult persons concerned 9 recombinants of the virulent A/Leningrad/82/76 (H3N2) virus, a variant of A/Victoria/3/75, with two cold-adapted donors of attenuation, namely A/Leningrad/134/17/57 (H2N2) and A/Leningrad/9/ /37/46 (H0N1), innocuous for children and possessing a ts marker.

The recombinants examined differed from each other with respect to the conditions under which they were obtained in two ways: method of crossing and temperature of cloning. Some recombinants were obtained by crossing of two native parent viruses, while others were obtained by cross-reactivation (inactivated virulent parent virus was reactivated with native cold-adapted strain). Selection of the recombinants was carried out at 25, 32 and 40 °C.

The conditions under which the recombinants were obtained exerted a significant influence on their thermosensitivity. The latter was the highest in clones obtained by cross-reactivation. On crossing native viruses, similar RCT_{40} values were only obtained in recombinants isolated at 25 °C.

It could be assumed that recombinants differing in the degree of thermosensitivity would also differ in the degree of attenuation and immunogenicity would also differ in the degree of attenuation and immunogenicity for humans. But all the clones proved to be areactogenic on intranasal administration to adult vaccinees and were characterized by high immunogenicity. No relationship could be established between thermosensitivity and avirulence of the viruses because complete attenuation was found both in recombinants with a ts marker and in those with a moderate thermosensitivity, as well as in those with a ts^+ phenotype.

One of the possible causes of the full areactogenicity of all recombinants studied without any relationship to the degree of thermosensitivity could have been the epidemiological situation under which the investigations were performed. The parent virulent virus A/Leningrad/82/76 corresponded to the A/Victoria/3/75 (H3N2) variant and was isolated by the end of 1975 in the course of the last epidemic of influenza A (H3N2). The high level of collective immunity to these viruses had been formed after repeated epidemics caused by related H3N2 variants sharing a common neuraminidase component and differing from each other basically by the antigenic properties of their haemagglutinin (A/Victoria/35/72, A/Port Chalmers/1/74 and A/Victoria/3/75).

The seronegative adults, selected based on the absence of antihaemagglutinins, in whom the recombinants of A/Leningrad/82/76 were tested, could have shown different levels of antibody against neuraminidase (Murphy *et al.*, 1979). The degree of attenuation of influenza virus strains under consideration as candidate vaccine strains for live influenza vaccine at the end of an epidemic cycle could thus be lower than in a period of appearance of a quite new influenza virus subtype when the population is devoid of antibodies to both surface antigens of influenza virus.

References

Aleksandrova, G. I. (1977): The use of the method of genetic recombination for obtaining vaccine strains of influenza virus (in Russian). *Vop. Virus.* **22**, 387–395.

Aleksandrova, G. I., Garmashova, L. M., Golubev, D. B., Kolyak, L. I., Medvedeva, T. E., Polezhaev, F. I., Rumovsky, V. I., and Smorodintsev, A. A. (1979*a*): Attempt at selection of innocuous thermosensitive recombinants of influenza virus A (in Russian). *Vop. Virus.* **24**, 342–345.

Aleksandrova, G. I., Medvedeva, T. E., Polezhaev, F. I., Garmashova, L. M., Grinus, A. M., Rumovsky, V. I., Kolyak, L. I., Blinova, T. E., Polyakov, Yu. M., and Golubev, D. B. (1979*b*): Live influenza vaccine from thermosensitive recombinants (in Russian), pp. 120–123. In A. A. Smorodintsev (Ed.): *Aktualnye Problemy Immunologii, Virusologii, Khimioterapii i Epidemiologii infektsionnykh Zabolevanii*, Meditsina, Leningrad.

Ivanova, N. A., Luzyanina, T. Ya., Taros, L. Yu., Grinbaum, E. B., Rumel, N. B., and Smorodintsev, A. A. (1977): Antigenic drift of contemporaneous influenza A viruses (in Russian). *Vop. Virus.* **22**, 168–172.

Murphy B. R., Chanock, R. M., Levine, M. M., Van Blerk, G. A., Beiquist, E. J., Douglas, R. G., Betts, R. F., Couch, R. B., and Cate, Th. R. (1979): Temperature-sensitive mutants of influenza A virus: evaluation of the A/Victoria/75-ts/A2 temperature-sensitive recombinants virus in seronegative adult volonteers. *Infect. Immun.* **23**, 249–252.

Polezhaev, F. I., and Aleksandrova, G. I. (1978): Temperature-sensitive recombinants of *Influenzavirus* B. *Acta virol.* **22**, 253.

Polezhaev, F. I., Garmashova, L. M., and Aptekareva, M. N. (1973): Attempt at obtaining psychrophilic recombinants of influenza virus A/England/42/72 (in Russian). *Problemy Grippa i virushnykh ORZ (Leningrad)* **9**, 117–121.

Polezhaev, F. I., Garmashova, L. M., Aleksandrova, G. I., Voitsekhovsky, B. L., and Romanov, Yu. A. (1974): Obtaining of "cold" recombinants of influenza virus, possessing properties of attenuated strains. *Acta virol.* **18**, 364.

Polezhaev, F. I., Garmashova, L. M., Polyakov, Yu. M., Golubev, D. B., and Aleksandrova, G. I. (1978): Conditions for production of thermosensitive attenuated influenza virus recombinants. *Acta virol.* **22**, 263–269.