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ATTENUATED INFLUENZA B VIRUS RECOMBINANTS OBTAINED BY CROSSING OF B/ENGLAND/2608/76 VIRUS WITH A COLD-ADAPTED B/LENINGRAD/14/17/55 STRAIN

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Summary. — Crossing of an attenuated influenza B virus strain (B/Leningrad/14/17/55) passaged at a low temperature with a virulent influenza B virus strain (B/England/2608/76) yielded recombinants similar in the antigenic specificity of their haemagglutinin (HA) and neuraminidase (NA) to B/England/2608/76 strain, but possessing an $RCT_{37.5}$ marker alike to the attenuated donor. Analysis of the genome composition of 2 recombinants has shown that they inherited genes coding for P (1, 2, 3) and M (7) proteins from the attenuated parent, but genes coding for HA (4), NA (6), NP (5) and NS (8) proteins from the virulent parent. All recombinants proved to be areactogenic for adult volunteers with no pre-existing antibody to the corresponding HA (≤ 8); however, they had a reduced immunogenicity as compared to parent viruses.

Key words: *influenza B virus*; *recombinants*; *attenuation*; *RCT marker*; *reactogenicity*; *immunogenicity*

Introduction

Considerable progress has been recently made in developing recombinant influenza A virus vaccine strains for live influenza vaccines. Cold-adapted A/Leningrad/134/17/57(H2N2), A/Leningrad/9/37/46(H1N1) and A/Ann Arbor/6/60 strains proved to be the most perspective donors of attenuation (Maassab *et al.*, 1969; Murphy *et al.*, 1980; Polezhaev *et al.*, 1978, 1980; Ghendon *et al.*, 1981). Studies on developing attenuated recombinants of influenza B virus were also based on the use of either highly-passaged B/Lee/40 and B/Setagaya/3/56 strains with no known laboratory passage history, or of cold-adapted B/Leningrad/14/17/55 and B/Ann Arbor/1/60 strains, as attenuated donors strains (Beare *et al.*, 1977; Miller *et al.*, 1977; Polezhaev and Aleksandrova, 1978).

Evaluation of reactogenicity and immunogenicity of influenza B virus recombinants, which was carried out so far only in limited trials, revealed variability of reactogenicity indices of the recombinants obtained from

high-yielding strains, and a reduction in immunogenic potency of the recombinants obtained on the basis of cold-adapted strains as compared to an epidemic virus used in recombination experiments.

The present paper describes the genome composition, reactogenicity and immunogenicity of influenza B virus recombinants obtained by crossing of the virulent B/England/2608/76 influenza virus strain (a variant of B/Hong Kong/5/72) with the cold-adapted strain B/Leningrad/14/17/55.

Materials and Methods

Viruses. The virulent B/England/2608/76 strain was supplied by Dr. Pereira, England, after 2 passages in embryonated eggs. This virus corresponded to the reference B/Hong Kong/5/72 strain in the antigenic specificity of its HA and NA. The B/Leningrad/14/17/55 strain was isolated from an ill child in 1955; it had undergone 20 passages in embryonated eggs at a temperature of 32 °C followed by 17 passages at 25 °—26 °C (Alexandrova and Smorodintsev, 1965).

Temperature-sensitivity ($RCT_{37.5}$ marker) of the parent strains and recombinants was determined by comparison of infectious titres in chick embryos (egg infection doses = EID) at 32 °C (Medvedeva *et al.*, 1980).

Recombination technique. Two approaches to recombination of viruses were used: in the first instance the virulent B/England/2608/76 virus (at a dose of 6.0 log EID_{50}/0.2 ml) was partially inactivated by heating (at 36 °C for 72 hr) reducing its infectivity by 2.0 log, and supplemented with an equal amount (6.0 log) of the native B/Leningrad/14/17/55 virus; the second approach involved crossing of native virulent and vaccine strains at a dose of 6.0 log EID_{50}/0.2 ml. Chick embryos infected with a mixture of the viruses were incubated at 32 °C for 48 hr. The recombinants were selected after two passages in the presence of 16—32 neutralizing units of antiserum to the B/Leningrad/14/17/55 virus followed by cloning in embryonated eggs at 32 °C for 72 hr using limiting dilutions technique. Clones were then selected with antigenic characteristics of the B/England/2608/76 virus.

Analysis of the genome composition of the recombinants was carried out as described elsewhere (Ghendon *et al.*, 1979, 1981) by analysis of influenza virus double-stranded RNAs (Hay *et al.*, 1977*a*, *b*).

Vaccination of the volunteers. Monovaccine tested for sterility, absence of PPLO and extraneous viruses and containing not less than 6.5 log EID_{50}/0.2 ml was used at a dilution of 1 : 2. Volunteers aged 20—25 revealing anti-HA titres to influenza virus of $\leq$8 were immunized with 0.25 ml of the preparation into each nostril by means of a sprayer. The vaccinees were observed for 5 days following vaccination for febrile reactions (temperature $\geq$37.5 °C).

Evaluation of immunogenic potency of viruses. Sera of the vaccinees collected prior to vaccination and 2—3 weeks following revaccination, were inactivated by heating at 58 °C for 30 min and tested for antibody to HA by haemagglutination-inhibition (HI) test using chicken erythrocytes which were added after 1 hr of contact of serum dilutions with 4 haemagglutination units (HU) of the virus.

Results

Reproduction of parent viruses in chick embryos at various temperatures

The virulent B/England/2608/76 strain grew in chick embryos at 37.5 °C nearly as efficiently as it did at 32 °—36 °C (Table 1). A difference in infectious titres at 37.5 °C did not exceed 0.5 log as compared to the optimal temperature (32 °C). However, reproduction of this strain at 25 °C was reduced drastically. The B/Leningrad/14/17/55 strain which had been passaged at a low temperature, reproduced in chick embryos at optimal temperature (32°—36 °C) to titres of 7.25 log EID_{50}/0.2 ml; at elevated temperature (up to 37.5 °C) its reproduction was reduced to 1.25 log, suggesting it was tem-

Table 1. Reproduction of virulent and cold-adapted influenza B virus strains at various temperatures

Strain	Virus titres* at given temperature					Difference in titre**	$RCT_{37.5}$
	25 °C	32 °C	34 °C	36 °C	37.5 °C		
B/England/2608/76	1.5	6.75	6.75	6.75	6.25	0.5	+
B/Leningrad/14/17/55	5.0	7.25	7.25	7.25	1.25	6.0	−

* log EID_{50}/0.2 ml; ** log EID_{50} at 32 °C − log EID_{50} at 37.5 °C

perature-sensitive. This strain grew rather efficiently in chick embryos at 25 °C and was thus cold-adapted.

Temperature-sensitivity and antigenic specificity of the recombinant influenza B virus strains

Table 2 shows that ts recombinants, i.e. clones with a reduced ability to grow at 37.5 °C as compared to 32 °C resulted both from crossing of native parent viruses (2 from 7), and from cross-reactivation (5 from 7), when the virulent B/England/2608/76 strain was partially inactivated by heating at 36 °C (see Materials and Methods).

On crossing of the native viruses the degree of reproduction at the elevated temperature was reduced in 2 clones (8H and 9H) only by 1.5—2.0 log, in

Table 2. Indices of reproduction of the recombinants in chick embryos at 32 °C and 37.5 °C

Parent virus	Recombinant	Virus titre*** at temperature of		Difference in titres***	$RCT_{37.5}$ marker
		32 °C	37.5 °C		
B/England/2608/76		7.26	6.5	0.75	+
B/Leningrad/14/17/55		8.25	1.5	6.75	−
	12R*	7.5	1.5	6.0	−
	13R	7.75	1.25	6.5	−
	15R	7.0	2.25	4.75	−
	17R	7.75	1.5	6.25	−
	24R	7.5	4.5	3.0	±
	28R	6.0	4.5	1.5	±
	25R	6.5	0.5	6.0	−
	2H**	7.25	4.25	3.0	±
	3H	7.5	1.75	5.75	−
	4H	7.25	4.5	2.75	±
	5H	7.5	4.25	3.25	±
	7H	7.75	1.5	6.25	−
	8H	7.5	5.5	2.0	+
	9H	7.75	6.25	1.5	+

* R — The virulent virus is partially inactivated;
** H — crossing of the native viruses.
*** For explanation see legend to Table 1.

Table 3. The genome composition of recombinants 12R and 13R

Recombinant	Genes							
	1	2	3	4(HA)	5(NP)	6(NA)	7(M)	8(NS)
12R	C	C	C	V	V	V	C	V
13R	C	C	C	V	V	V	C	V

C — inherited from the cold-adapted parent;
V — inherited from the virulent parent.

other clones (2H, 4H, 5H) it was reduced at 37.5 °C by approximately 3 log ($RCT_{37.5\pm}$), and 2 another clones (3H and 7H) reproduced at 37.5 °C very poorly (32°/37.5 difference in titres being 5.75—6.25 log) and were characterized by a distinct $RCT_{37.5-}$ marker.

A greater portion of the recombinants which resulted from crossing of a partially inactivated virulent strain and native cold-adapted strain, was characterized by ts marker. Only 2 clones (24R and 28R) failed to inherit the ts marker from the B/Leningrad/14/17/55 donor strain.

These data suggest that frequency of the appearance of ts recombinants may be dependent on the recombination technique. The use of partially inactivated virulent parent strain for recombination increased the frequency of formation of ts clones.

Studies on the antigenic specificity of HA and NA have shown that antisera to the B/Hong Kong/5/72 strain reacted to similar titres in HI and NA inhibition tests with the HA and NA of the B/England/2608/76 and B/Hong Kong/5/72 strains, respectively, which confirms the similarity of antigenic specificity of outer membrane proteins of these strains. At the same time the B/Leningrad/14/17/55 strain, which was similar in the antigenic specificity

Table 4. Reactogenicity and immunogenicity of the parent strains and recombinants 12R, 13R and 7H in persons with pre-existing anti-HA antibodies ≤ 8

Parent virus	Recombinant	Febrile reactions*	Reisolation of virus	Immunogenicity		
				No. of paired sera	No. of vaccinees with a 4-fold and higher antibody rise	
					abs	%
B/England/2608/76		2/20**	19/20[a]	19	14	73.6
B/Leningrad/14/17/55		0/16	n. t.[b]	16	10	62.5[c]
	12R	0/10	3/10	7	2	28.6
	13R	0/156	23/30	84	47	55.9
	7H	0/18	0/3	3	1	33.3

* $\geq$37.5 °C; [a] denominator = number of persons tested; numerator = number of persons from which the virus was recovered; [b] not tested; [c] according to the data of Rumovsky *et al.* (1971).
** Numerator = number of persons showing temperature $\geq$37.5 °C; denominator: number of vaccinees.

of its HA and NA to an influenza B strain which had been circulating in 1952—1955, failed to react with antiserum to the B/Hong Kong/5/72 strain in HI and NA inhibition tests. Studies of the antigenic specificity of HA and NA of the recombinants have shown that sera of mice immunized with each of the 14 recombinants obtained reacted in HI and NI tests only with the B/England/2608/76 strain, but not with B/Leningrad/14/17/55. Therefore, all the recombinants obtained were similar in the antigenic specificity of their HA and NA to the virulent B/England/2608/76 strain.

Analysis of the genome composition of recombinants 12R and 13R

Genome composition of the recombinants 12R and 13R was studied by means of the analysis of influenza virus double-stranded RNAs (Hay *et al.*, 1977; Ghendon *et al.*, 1979, 1981). It has shown that both recombinants inherited 3 genes coding for proteins of the polymerase complex (P1, P2 and from the cold-adapted strain P3), the gene coding for the M protein from the same strain, while other 4 genes coding for the envelope proteins, namely HA, NA (4 and 6), NP (5) and for NS proteins (8) came from the virulent parent (Table 3).

Reactogenicity and immunogenicity of influenza B virus recombinants

The ts recombinants 12R and 13R obtained by cross-reactivation and the clone 7H — a product of crossing between native parent viruses — were studied for their reactogenic properties and immunogenic potency in a limited number of volunteers aged 20—25 with pre-existing anti-HA antibody titres (≤ 8). Results of vaccination trials are summarized in Table 4.

According to regulations currently valid in the U.S.S.R., we had no possibility to test the virulent B/England/2608/76 strain after 2 passages in chick embryos which was used for producing recombinants. The B/England/2608/76 strain was tested after 6 passages in chick embryos at 32 °C. After administration of this virus to 20 volunteers with pre-existing anti-HA antibody titres of ≤ 8, two showed moderate vaccination reactions with a fever 37.5 °C or higher, headache, catarrhal symptoms in the upper respiratory tract. Reisolation of the virus was observed in 19 from 20 vaccinees (95%). A 4-fold and higher antibody rise was found in 14 from 19 vaccinees, i.e. in 73.6% instances. The cold-adapted B/Leningrad/14/17/55 strain caused febrile reactions in none of 16 seronegative individuals and induced antibody formation in 62.5% of the vaccinees, i.e. was nearly as efficient as the B/England/2608/76 virus with respect to this index.

The ts recombinants 12R, 13R and 7H proved to be areactogenic for adults and did not cause any clinical vaccination reactions with a fever of more than 37.5 °C. These viruses differed in their ability to grow in organism of the vaccinees and in the indices of immunogenic potency. Minimum indices of antibody rises were observed with the recombinants 12R and 7H (seroconversion in 2 from 7 vaccinees and in 1 from 3 vaccinees, respectively). We failed to reisolate the recombinant 7H, and the recombinant 12R was reisolated from 3 out of 10 vaccinees. The recombinant 13R which, unlike

the above recombinants, reproduced in the mucous membrane of the upper respiratory tract of 23 from 30 volunteers (76.6%) and induced a 4-fold and higher antibody rise in 55.9% of the vaccinees, possessed a higher antigenic potency. However, immunogenicity levels of this recombinant were somewhat lower than those of the parent B/England/2608/76 virus.

Discussion

The present paper shows a possibility of obtaining areactogenic and sufficiently immunogenic recombinant influenza B virus strains by recombination between the epidemic B/England/2608/76 strain and the vaccine B/Leningrad/14/17/55 strain revealing obsolete antigenic specificity of HA. The above viruses differed in their ability to reproduce in chick embryos at 37.5 °C. The data obtained that this technique of recombination between a virulent temperature-resistant virus and a ts vaccine strain allows to select areactogenic clones by a laboratory test indicating attenuation (RCT marker). Immunogenicity levels of $RCT_{37.5}$-recombinants for man was normally lower than that of parent strains. Even the most potent recombinant 13R turned out to be somewhat less immunogenic as compared to the virulent parent B/England/2608/76 virus and to the donor of attenuation — B/Leningrad/14/17/55 virus. It should be noted that neither the sufficiently immunogenic recombinant 13R, nor the poorly immunogenic one — 12R — differed in their genome compositions: both inherited genes 4, 5, 6 and 8 from the virulent parent, and genes 1, 2, 3, 7 from the attenuated one. The reason for a reduction in immunogenicity of recombinants obtained by crossing of influenza B virus strains, is not yet clear.

References

Alexandrova, G. I., and Smorodintsev, A. A. (1965): Obtaining of an additionally attenuated vaccinating cryophil influenza strain. *Rev. Roum. D'inframicrobiol.* **2**, 179—186.

Beare, A. S., Sherwood, J. E., Callow, K. A., and Craig, J. W. (1977): Selection of influenza B virus recombinants and their testing in humans for attenuation and immunogenicity. *Infect. Immunity* **15**, 347—353.

Ghendon, Y. Z., Klimov, A. I., Blagoveshenskaya, O. V., and Genkina, D. B. (1979): Investigation of recombinants of human influenza and fowl plague viruses. *J. gen. Virol.* **43**, 183—191.

Ghendon, Y. Z., Klimov, A. I., Alexandrova, G. I., and Polezhaev, F. I. (1981): Analysis of genome composition and reactogenicity of recombinants of cold-adapted and virulent virus strains. *J. gen. Virol.* **53**, 215—224.

Hay, A. J., Lomniczi, B., Bellamy, A. R., and Skehel, J. J. (1977*a*): Transcription of influenza virus genome. *Virology* **83**, 337—355.

Hay, A. J., Bellamy, A. R., Abraham, G., Skehel, J. J., Brand, C. M., Webster, R. G. (1977*b*): Procedures for characterization of the genetic material of candidate vaccine strains. *Develop. Biol. Stand.* **29**, 15—24.

Maassab, H. F., Francis, T., Davenport, F. M., Hennessy, V., Minuse, E., and Anderson, G. (1969): Laboratory and clinical characteristics of attenuated strains of influenza virus. *Bull. Wrld. Hlth. Org.* **41**, 589—594.

Medvedeva, T. E., Gordon, M. A., Alexandrova, G. I., and Smorodintsev, A. A. (1980): Experimental basis of enhancing the immunogenicity of influenza B virus by genetic recombination. *Acta virol.* **24**, 385—390.

Miller, L. W., Togo, G., and Hornick, R. B. (1977): Clinical and serological effect of live attenuated serum inhibitorresistant influenza B vaccine in seronegative adults. *J. med. Virol.* **1**, 193—199.,

Murphy, B. R., Rennels, M. B., Douglas, R. G., Betts, F., Couch, R. B., Cate, T. R., Chanock R. M., Kendal, A. P., Maassab, H. F., Suwangool, S., Sotman, S. B., Cisneros, L. A., Anthony, W. C., Nalin, D. R., and Levine, M. M. (1980): Evaluation of influenza A/Hong Kong/123/77 (H1N1) ts-1A2 and cold-adapted recombinant viruses in seronegative adult volunteers. *Infect. Immunity* **29**, 348—355.

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

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

Polezhaev, F. I., Garmashova, L. M., Rumovsky, V. I., Alexandrova, G. I., and Smorodintsev, A. A. (1980): Peculiarities of obtaining attenuated thermosensitive recombinants of influenza A viruses at the end to the H3N2 epidemic cycle. *Acta virol.* **24**, 273—278.

Rumovsky, V. I., Panteleev, V. G., Vasiljev, B. Ya., Evdokimov, N. M., Romanov, Yu. A., Zlydkinov, D. M., and Aleksandrova, G. I. (1971): Characteristics of reactogenic and immunogenic properties of live standard influenza vaccine on aerosol application, (in Russian) pp. 117—128. In *Immunology and specific influenza prophylaxis in children*, Leningrad.