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Selection of Influenza B Virus Recombinants and Their Testing in Humans for Attenuation and Immunogenicity

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The selection of influenza B virus recombinants from plaques in bovine kidney cell monolayers is described. Two sets of recombinants were each derived from parents of high and low virulence for humans, respectively. Recombination frequency was apparently high, and reassortment of genes made it possible to obtain attenuated recombinants containing the surface antigens of the virulent parents. Attenuation and immunogenicity were demonstrated in a series of volunteer trials. However, the technique proved less statisfactory than for influenza A viruses, which periodically undergo antigenic shift and for which there is a wide choice of parent viruses with distinctive surface antigens. In our two influenza B recombinant series there was appreciable antigenic overlap in the neuraminidases of the parents, even though in both cases these were chronologically widely separated. Another marker used was comparative titer at 35 and 38°C. In practice, technical problems might sometimes make it difficult to ensure rapid production of live influenza B vaccines by recombination.

The high recombination frequency of influenza A viruses is now widely applied to the making of live vaccines from fully characterized parents (4, 19, 20, 26), but so far no one system has been able to meet all specifications for the mass vaccination of humans (2). We have therefore continued to experiment with different parent strains and, when rapid antigenic change in epidemic influenza A viruses has made human trials difficult to carry out, have turned our attention to the chemically and biologically similar type B viruses. Perry and Burnet (22) showed in mouse experiments that influenza B viruses readily recombined with each other. However, they and Gotlieb and Hirst (13) saw no evidence of recombination between influenza A and influenza B viruses. Interest in genetic recombination of influenza B viruses has recently been revived by a report from Tobita and Kilbourne (27) that they were able to produce recombinants in tissue culture after they had found that a high plaquing efficiency could be achieved by adding trypsin to the agar overlay. This presumably facilitated the cleavage of precursor hemagglutinin into HA1 and HA2 (1, 15, 17). We have not ourselves adopted this system, since we already had a technique with a high efficiency of plating for influenza B (but not influenza A) viruses in primary bovine kidney cell monolayers (5, 6), and this was presumably an example of variation of hemagglutinin cleavage of individual influenza viruses under different cultural conditions (15). In this paper we shall describe the double infection of bovine kidney monolayers with selected parent influenza B viruses and the characterization of the progeny as recombinants from an examination of their laboratory properties and of the responses they elicited from volunteers.

MATERIALS AND METHODS

Parent viruses. B/Lee/40, originally received from the World Influenza Centre, Mill Hill, London, had had numerous unrecorded passages in ferrets, mice, and eggs. Although attenuated, it was still infective for humans (2).

B/Hannover/1/70, provided by Hildegard Willers, Medizinaluntersuchungsamt, Hannover, had had two passages in monkey kidney tissue culture and two in embryonated eggs. It was highly virulent for humans.

B/Setagaya/3/56, an attenuated live vaccine virus (21), was obtained from Y. Okuno, Osaka, after it had had 120 egg passages.

B/Hong Kong/8/73 (antigenically equivalent to the prototype B/Hong Kong/5/72) was received from the World Influenza Centre after it had had a few egg passages only. It was of moderate human virulence.

In Salisbury, viruses were initially reisolated in specific pathogen-free (SPF) eggs from volunteers' nasal washings and were then triple cloned by plaque selection in calf kidney cell monolayers (5). Only specific pathogen-free eggs were used thereafter to propagate viruses for human trials.

All influenza B viruses formed plaques with high efficiency in calf kidney cells as previously described (6).

Recombinant viruses. Recombination was performed in bovine kidney cells in 50-mm plastic petri dishes. Monolayers were inoculated with 5.0 ml of Eagle minimal essential medium (MEM) containing 10 plaque-forming units (PFU) each of attenuated and virulent viruses per cell. The first recombinant series was B/Lee/40-Hannover/1/70, and the second was B/Setagaya/3/56-Hong Kong/8/73 (Table 1). Mixed-virus inocula were adsorbed to cells for 3 h with occasional shaking. Fluid was then withdrawn, and cells were washed three times with MEM containing antibiotics. Hyperimmune rabbit sera prepared against both parent viruses (0.5 ml of each) were added to the cells, and the cells were allowed to stand at room temperature for 1 h. The sera were then removed, the cells were again washed, and 5.0 ml of MEM was added to each plate. The plates were incubated at 33°C for 21 h. At the end of the incubation period, the supernatant fluid was harvested and titrated in 1-log dilution steps in bovine kidney cell monolayers, four to five plates per dilution, using L15 medium (18) in a final agarose concentration of 0.75% (6). Incubation was performed at 33°C for 5 days, after which 3.0 ml of a second overlay, similar to the first but also containing neutral red to a final concentration of 0.004%, was superimposed on the first overlay. The following day, well-segregated plaques were cut out of the agarose, designated as individual potential recombinants, and dropped into 1.0 ml of MEM with 0.2% bovine plasma albumin, which was allowed to stand overnight at 4°C. Harvested plaques were stored in glass ampoules, sealed with a flame, and kept at −70°C until required.

Each recombinant was then cloned by plaque selection as shown in Table 1. Clones of B/Setagaya/3/56-Hong Kong/8/73 containing the hemagglutinin of the wild parent, were designated 101 to 105, and those of B/Lee/40-Hannover/1/70 were designated 232 to 235. Numerous other clones were stored but were not examined further because of a lack of volunteers for a study of their human virulence.

Characterization of parent viruses and recombinants. Three properties were used to characterize parental viruses and recombinants, antigenic characters of hemagglutinins and neuraminidases, comparative virus titers in embryonated eggs at 35 and 38°C, and virulence for humans. Details of methods are given below.

(i) Study of hemagglutinins and neuraminidases. Hemagglutinins were identified in a conventional hemagglutination-inhibition (HI) test with rabbit hyperimmune sera treated with cholera filtrate. Neuraminidase-inhibition (NI) tests were performed as described elsewhere (10) by calculating percentage NI on a standard substrate with a single serum dilution. A modification was the substitution of 0.1 M imidazole buffer, pH 6.9, containing Ca^{2+} (10^{-3} M) for phosphate buffer, pH 5.9 (24). Antiserum for B/Lee/40-Hannover/1/70 recombinants was used at 1:30, and that for B/Setagaya/3/56-Hong Kong/8/73 recombinants was used at 1:100. Normal rabbit serum at the same dilution was used as a control. Reciprocal titers were calculated from the equation $[V_0/V_1) \times T] - T$, where V_0 is the extinction with normal rabbit serum, V_1 is the extinction with antiserum, and T is the dilution factor, according to the method of Siekmann and colleagues (24).

(ii) Comparative virus titers at different temperatures. Serial 10-fold dilutions of virus were each inoculated in duplicate into the allantoic cavities of four to five embryonated eggs that were incubated at 35 and 38°C. Harvested fluid was tested for hemagglutinin with 0.5% fowl erythrocytes, and infectivity titers were recorded for each virus.

(iii) Volunteer trials. Only volunteers with initial low reciprocal HI titers (24 or less) to the hemagglutinins of the inoculated viruses were used in the trials. Methods of isolating the volunteers, of grading and scoring the clinical reactions, and of measuring the serological responses have been described elsewhere (3, 4). In brief, clinical reactions were

TABLE 1. *Passage histories of parent viruses and recombinant clones used in experiments and in volunteer trials*

Virus	Laboratory passages[a]
B/Setagaya/3/56	E 122 (including 2 egg passages in this laboratory)
B/Hong Kong/8/73	A few egg passes on receipt; 2 egg passages in this laboratory.
B/Setagaya/3/56-Hong King/8/73	
Clone 101	X E1 BK1 E1 BK1 E2
Clone 102	X E1 BK1 E1 BK1 E2
Clone 103	X E1 BK1 E1 BK1 E2
Clone 104	X E1 BK1 E2 BK1 E3
Clone 105	X E1 BK1 E1 BK1 E2
B/Lee/40	Unknown; 2 egg passages in this laboratory + cloning
B/Hannover/1/70	MK E4 HU1 E3
B/Lee/40-Hannover/1/70	
Clone 232	X E1 BK1 E1 BK1 E2 BK1 E1
Clone 233	X E1 BK1 E1 BK1 E2 BK1 E1
Clone 234	X E1 BK1 E1 BK1 E2 BK1 E4
Clone 235	X E1 BK1 E1 BK1 E2 BK1 E2

[a] Abbreviations: X, Mixed infection of bovine kidney monolayers; E, egg passages; BK1, passages in bovine kidney monolayers; MK, passages in monkey kidney tissue culture; HU, passage in humans.

graded severe (influenza-like), moderate (local symptoms and constitutional upset), mild (local symptoms only), very mild (trivial symptoms), and nil. In addition, an arbitrary scoring method was used based on pyrexia, handkerchief count, coryza, and subjective symptoms. For volunteer inoculation, viruses were titrated in embryonated whole eggs and diluted in Hanks' balanced salt solution containing 0.2% bovine plasma albumin. Each volunteer received 1.0 ml of fluid (0.5 ml in each nostril), with the head fully extended and hanging vertically downwards over the end of a couch. This position was maintained for 1 min, and the volunteer was asked not to blow his nose for 1 h afterwards. Nasal washings for virus isolation were collected 3 and 4 days after virus administration and were each inoculated into four 11-day embryonated eggs for virus recovery. HI tests were performed simultaneously on sera collected before the trials and 2 to 3 weeks afterwards by standard methods.

The maximum possible dose of virus was given before a virus was designated "attenuated," i.e., producing no more than "mild" reactions (2).

(iv) Absorption of hyperimmune sera for identification of neuraminidases. The absorption of antibodies from sera (see Table 5) was performed with concentrated suspensions of virus by a method described earlier (10).

RESULTS

Passage histories of parent viruses and of virus clones selected from calf kidney monolayers that had undergone mixed infections are shown in Table 1. Cloning by direct plaque-to-plaque passage was sometimes impracticable because single plaques contained relatively little infectious virus, and intervening egg passages were therefore frequently made between the passages in bovine kidney cells. All the viruses used produced indistinguishable circular plaques of about 5-mm diameter (5, 6). Potential genetic markers for parent viruses and recombinants were sought in comparative virus titers at 35 and 38°C, in the antigenic characters of hemagglutinins and neuraminidases, and in relative virulence for volunteers with reciprocal antihemagglutinin antibody titers of 24 or less. In our experience, the laboratory passages themselves were most unlikely to have exercised any appreciable attenuating effect on the viruses (3), and reductions in virulence observed in the progeny of mixed infections as compared with those of wild parents almost certainly reflected the attenuating effect of the recombination process itself.

The B/Lee/40-B/Hannover/1/70 system is illustrated in Tables 2 through 6. The parent viruses were chosen for two reasons: first, because the older virus was known to be highly attenuated for humans and the newer virus highly virulent (Table 6); and second, because it was thought that the 30-year interval between their respective isolations would ensure an absence of antigenic crossing. In their relative infectivities at 35 and 38°C, B/Lee/40 and B/Hannover/1/70 seemed to be easily distinguishable (Table 2), and the former quite clearly had a higher titer at both temperatures. However, only one of the mixed-infection clones, 235, closely resembled either parent by this criterion, resembling B/Hannover/1/70. Since 235 also resembled this virus antigenically (Tables 3 and 5) and in its virulence for humans (Table 6), it probably was not a recombinant at all.

Attempts to compare the antigenic characters of hemagglutinins and neuraminidases in parent viruses and in mixed-infection clones encountered difficulties. Chakraverty (11) found that some degree of crossing persisted in influenza B virus hemagglutinins even when the strains were chronologically widely separated. Furthermore, degrees of relationship did not necessarily depend upon the time intervals between isolation. She found even greater difficulty in grouping the neuraminidases according to the dates on which the viruses had appeared (12). There appeared to be a minor de-

TABLE 2. *Infectivity in eggs of B/Lee/40, B/Hannover/1/70, and their recombinants at 35 and 38°C*

Virus	Titer (log_{10} EID_{50}/ml)[a] at:	
	35°C	38°C
B/Lee/40	8.7	7.0
B/Hannover/1/70	7.8	2.0
Recombinant clones		
232	6.5	4.0
233	5.8	4.0
234	6.3	3.0
235	7.4	3.0

[a] EID_{50}, 50% egg-infectious dose.

TABLE 3. *Antigenic characterization of hemagglutinins and neuraminidases of B/Lee/40, B/Hannover/1/70, and their recombinants*

Virus	Antiserum titer			
	B/Lee/40		B/Hannover/1/70	
	HI	NI	HI	NI
B/Lee/40	1,151	464	36	17
B/Hannover/1/70	40	32	576	66
Recombinant clones				
232	36	41	3,072	93
233	48	37	2,304	108
234	36	28	2,304	82
235	24	49	1,344	117

TABLE 4. *Cross-HI tests between B/Lee/40, B/Hannover/1/70, clone 12, and clone 28*

Virus	Antiserum titer			
	B/Lee/40	B/Hannover/1/70	Clone 12 [B/Mass/71 (H)-Lee/40 (N)]	Clone 28 [B/Lee/40 (H)-Mass/71 (N)]
B/Lee/40	2,304	192	84	3,072
B/Hannover/1/70	12	3,072	768	144
Clone 12 [B/Mass/71 (H)-Lee/40 (N)]	84	3,072	3,072	384
Clone 28 [B/Lee/40 (H)-Mass/71]	2,304	288	192	12,288

TABLE 5. *Attempt to render rabbit antisera specific for neuraminidases by absorbing them with antigenic hybrid viruses*[a]

Virus	Antiserum titer	
	Anti-neuraminidase B/Lee/40[b]	Anti-neuraminidase B/Hannover/1/70[c]
B/Lee/40	132	7
B/Hannover/1/70	8	35
Clone 232	11	63
Clone 233	9	38
Clone 234	9	37
Clone 235	6	40

[a] The hemagglutinins of these hybrids were similar to those of the viruses used for preparing the sera, but the neuraminidases were different.

[b] Anti-B/Lee/40 serum absorbed with clone 28, B/Lee/40 (H)-Massachusetts/71 (N).

[c] Anti-B/Hannover/1/70 serum absorbed with clone 12, B/Massachusetts/71 (H)-Lee/40 (N).

gree of antigenic crossing between the hemagglutinins of B/Lee/40 and B/Hannover/1/70, and clones 232, 233, 234, and 235 quite clearly contained the hemagglutinin of B/Hannover/1/70 (Table 3). Using a rabbit antiserum prepared against whole virus and a percentage NI test with a single serum dilution converted into titers (10, 24), the neuraminidases also were probably those of B/Hannover/70. However, it seemed possible that steric hindrance might have resulted from antibodies acting on the hemagglutinin (23), and an attempt was therefore made to absorb these out with antigenic hybrid strains provided by E. D. Kilbourne, New York (27). These hybrids were B/Massachusetts/1/71 (H)-Lee/40 (N), known as clone 12, and its reciprocal B/Lee/40 (H)-Massachusetts/1/71 (N), known as clone 28. It seemed likely that the hemagglutinins of the New York parent strains would prove similar to those of our own, and that it might therefore be possible to absorb out cross-reacting HI antibodies, leaving NI antibodies intact. This proved to be the case, and the effects of steric hindrance seemed to have been eliminated. The use of specific anti-neuraminidase antisera produced in this way indicated that all the clones contained the B/Hannover/1/70 neuraminidase (Table 5). Relationships between the hemagglutinins of B/Lee/40, B/Hannover/1/70, clone 12, and clone 28 are shown in Table 4.

The results of volunteer trials with the clones of the B/Lee/40-Hannover/1/70 series are summarized in Table 6. Susceptibility was judged by initial reciprocal HI antibody titers of 24 or less, and the largest possible dose was given before a virus was diagnosed as attenuated. The results suggested that clone 233 was fully attenuated, that clone 232 was rather less so, and that clone 235 was virulent. Clone 234 did not grow sufficiently well to enable a high dose of virus to be given and was therefore not properly characterized.

Experiments with a second recombination series are shown in Tables 7 through 9. The Japanese live vaccine strain B/Setagaya/3/56 (21) was chosen as the attenuated parent, and the influenza B serotype of current epidemiological importance, B/Hong Kong/8/73 (equivalent to B/Hong Kong/5/72), was the wild strain. Although the time interval between the isolation of these two viruses was shorter than that between B/Lee and B/Hannover, there was evidently less cross between the hemagglutinins. HI and NI titers obtained with the five recombinant clones, 101 through 105, using antisera prepared against whole virus, were similar to those obtained with the wild parent (Table 8). Although the hemagglutinins were undoubtedly those of the wild virus, the probable increase in NI titers because of steric hindrance made it difficult to identify the neuraminidases with equal certainty. Analysis of the infectivity titers at two different temperatures (Table 7) showed apparent clear-cut inheritance of parental characteristics in only one prospective recombinant, 105, which resembled the wild parent, whereas four other strains, 101, 102, 103, and 104, yielded figures intermediate between those of the two parents. In the trial results, 101 and 105 were appreciably less virulent than the wild parent, whereas 102, 103, and 104 were not (Table 9). Taking growth

TABLE 6. *Inoculation of volunteers with the parent viruses, B/Lee/40 and B/Hannover/1/70, and their recombinants*

Virus	Dose (log_{10} EID_{50})[a]	Virus excretions[b]	Grading of clinical reactions					Avg clinical score in people with laboratory evidence of infection[c]	HI antibody rises[b]
			Severe	Moderate	Mild	Very mild	Nil		
B/Lee/40	6.0	3/5	0	0	0	2	3	2.0	2/2
B/Hannover/1/71	6.5	3/3	3	0	0	0	0	42.0	3/3
B/Lee/40-Hannover/1/70									
Clone 232	6.3	3/6	0	1	1	3	1	17.1	5/6
Clone 233	6.3	2/6	0	0	3	1	2	6.7	5/5
Clone 234	3.9	3/9	0	0	4	4	1	15.3	4/9
Clone 235									
1st trial	6.5	2/2	0	0	2	0	0	27.0	2/2
2nd trial	7.2	6/7	2	1	1	1	2	24.5	5/7

[a] EID_{50}, 50% egg-infectious dose.
[b] Number positive/number tested.
[c] Virus excretion, HI antibody rise, or both.

TABLE 7. *Infectivity titrations of B/Setagaya/3/56, B/Hong Kong/8/73, and their recombinants at 35 and 38°C*

Virus	Titer (log_{10} EID_{50}/ml)[a] at:	
	35°C	38°C
B/Setagaya/3/56	8.6	6.7
B/Hong Kong/8/73	9.2	2.5
Recombinant clones		
101	8.7	2.0
102	9.0	2.0
103	7.4	2.0
104	8.5	4.8
105	9.0	2.4

[a] EID_{50}, 50% egg-infectious dose.

TABLE 8. *Antigenic characterization of hemagglutinins and neuraminidases of B/Setagaya/3/56, B/Hong Kong/8/73, and their recombinants*

Virus	Antiserum titer			
	B/Setagaya/3/56		B/Hong Kong/8/73	
	HI	NI	HI	NI
B/Setagaya/3/56	1,152	805	6	251
B/Hong Kong/8/73	6	155	288	445
Recombinant clones				
101	12	260	1,152	812
102	12	274	768	821
103	12	159	672	397
104	12	166	576	406
105	12	202	600	796

characteristics and human virulence in combination, it seemed that clones 101 and 105 inherited characteristics from the two parents and should therefore be considered recombinants, but clones 102, 103, and 104 did not differ significantly from B/Hong Kong/8/73.

DISCUSSION

To prove that recombination has taken place between viruses, it is necessary to show that at least some of the cloned progeny of mixed infections have inherited properties from each of the parents. The suitability of parent strains is therefore very largely dependent upon their possession of distinctive properties that can serve as genetic markers. Such markers have in the past included virulence for mice (7–9), plaquing characteristics under defined conditions (25), nature of envelope antigens (16), hemagglutination titer and growth capacity (14), human virulence (4), and genetic defects that have render the viruses temperature sensitive (20). McCahon and Schild (19), using four markers (nature of hemagglutinin and neuraminidase antigens, production of hemagglutinin over a range of temperatures, and mouse virulence), found that these markers segregated independently in recombinants obtained from two sharply contrasting influenza A viruses. Hence they concluded that each character reflected a different primary gene function, although it was possible that hemagglutinin production depended on more than one primary gene function, since values intermediate between those of the parents were common in recombinants.

Our own special interest in recombination has centered on its potential uses for producing live influence vaccine viruses. Parent viruses are selected for their ability to donate to recombinant progeny the following properties: high

TABLE 9. *Inoculation of volunteers with the parent viruses, B/Setagaya/3/56 and B/Hong Kong/8/73, and their recombinants*

Virus	Dose (log_{10} EID_{50})[a]	Virus excretions[b]	Grading of clinical reactions					Avg clinical score in people with laboratory evidence of infection[c]	HI antibody rises[b]
			Severe	Moderate	Mild	Very mild	Nil		
B/Setagaya/3/56	6.5	0/4	0	0	0	0	4	NA[d]	0/4
B/Hong Kong/8/73	6.3	5/7	0	2	2	1	2	15.8	6/7
B/Setagaya/3/56-Hong Kong/8/73									
Clone 101									
1st trial	6.0	1/6	0	0	0	0	6	0	3/6
2nd trial	7.0	1/5	0	0	0	3	2	4.6	4/5
Clone 102									
1st trial	6.0	2/5	0	1	2	1	1	12.6	5/5
2nd trial	7.0	3/4	2	1	1	0	0	37.9	4/4
Clone 103									
1st trial	5.8	3/4	0	1	1	1	1	14.3	1/4
2nd trial	6.8	5/5	1	1	2	0	1	16.5	5/5
Clone 104									
1st trial	6.2	4/4	0	1	2	1	0	16.0	0/4
2nd trial	7.2	4/6	0	1	3	2	0	21.5	5/6
Clone 105									
1st trial	6.0	5/6	0	0	3	1	2	5.9	3/4

[a-c] See Table 6.
[d] NA, Not applicable.

infectivity for humans, attenuation, correct surface antigens, good growth, and reasonable genetic stability. However, some of these characters cannot as yet be identified in the laboratory, and final selection of viruses is subject to the results of volunteer experiments.

Little previous work has been done on the recombining of influenza B viruses, and our approach was therefore made ab initio. Influenza B viruses of low virulence for humans were crossed with those that produced human disease. In this connection it should be noted that the native virulence of wild influenza B viruses for humans is much more variable than that of corresponding influenza A viruses (2). If recombination took place with the frequency described by Perry and Burnet (22) and by Tobita and Kilbourne (27), then the ready reassortment of genes would easily modify a polygenic property like virulence. This did in fact happen, but completing the experiment by identifying laboratory markers in the parent viruses and in the clones selected after these had been incubated together proved more difficult and less conclusive than in the case of the influenza A viruses. Tobita and Kilbourne selected as parents two influenza B viruses that were antigenically distinguishable and, in a subsequent characterization of 31 clones obtained from mixed infection without selection pressures, found that no fewer than 20 were antigenic hybrids and had derived their hemagglutinins and neuraminidases from different parents. The recombination rate must therefore have been abnormally high. The limitation in our own case that one virus of each series had to be virulent for humans and the other attenuated left little room for considering other distinctive properties or even desirable vaccine properties such as good growth. Plaque morphology provided no help since, although our plaquing system was highly efficient, all influenza B viruses produced a similar type of plaque. We therefore did what we could with surface antigens and comparative titers at two different temperatures. Surface antigens were not fully demarcated, and this would seem to be a feature of the influenza B group generally (11, 12). Hence, although our experiments support the view that recombination between influenza B viruses readily takes place, it is doubtful whether live vaccines can be reliably produced in the future by the recombination of attenuated laboratory strains with new virulent antigenic variants. The fact that influenza B viruses do not undergo antigenic shift and that antigenic drift is not necessarily progressive suggests that it may not always be possible to have parent viruses that are antigenically sufficiently distinctive to meet technical requirements.

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