Laboratory properties of cold-adapted influenza B live vaccine strains developed in the US and USSR, and their B/Ann Arbor/1/86 cold-adapted reassortant vaccine candidates

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The adaptation of two influenza B strains (B/Leningrad/14/55 and B/Ann Arbor/1/66) to replication at 25°C is described. Comparison of the two viruses indicates that both also exhibit temperature sensitive phenotypes, although that of the virus B/Leningrad/14/55 is less pronounced. When inoculated into ferrets both viruses replicate well in the trachea, but only the B/Leningrad/14/55 cold-adapted virus replicates in the lungs. This virus exhibited a moderate level of attenuation in the animals, in contrast to the B/Ann Arbor/1/66 cold-adapted virus, which was fully attenuated. Reassortant viruses deriving the surface antigens of the contemporary wild type virus B/Ann Arbor/1/86 and most or all of their other genes, from one or other cold-adapted parent, were virtually indistinguishable from their respective cold-adapted parents. The B/Leningrad/14/55 reassortant was slightly more attenuated than its cold-adapted parent in ferrets. These studies extend knowledge of the properties of viruses used to prepare experimental live influenza B human vaccines.

Keywords: Influenza; attenuated vaccine; virus

Introduction

A recent method for preparation of attenuated vaccine strains of influenza virus is based on the recombination of contemporary wild-type (wt) viruses with a cold-adapted (ca) donor strain. Reassortant viruses which inherit the genes coding for haemagglutinin and neuraminidase from the epidemic virus and most of the other genes from the ca donor strain are then selected by antigenic and molecular analysis[1–3].

Most studies to date with reassortant ca influenza viruses have involved type A strains. The progress with the techniques used has prompted us to extend these procedures to type B influenza vaccine. Presently, the following ca type B influenza strains exist: B/Leningrad/14/55, B/USSR/69/60, B/Dushanbe/62 and B/USSR/67, developed in the USSR (Ref. 4 and unpublished results), and B/Massachussetts/66, B/Ann Arbor/1/66 and B/Tecumseh/1/69 developed in the US[5]. Although these viruses have been available for several years, only recently have reassortants begun to be prepared from them for laboratory and clinical analysis.

In 1985/86 major epidemics of influenza B occurred in some countries, including Eastern Europe and the US, in association with high levels of morbidity in children and adults[6,7]. The epidemic was caused by a new antigenic variant, for which the reference strain B/Ann Arbor/1/86 was selected for production of the subsequent inactivated vaccine[8]. This opportunity was taken simultaneously to prepare ca reassortant viruses from candidate ca vaccine strains in the US and USSR, and the same wt reference strain, for comparison of their properties. Such a comparison of laboratory properties is described here, with clinical evaluation of the reassortant derived in the USSR described elsewhere[9].

Materials and methods

Viruses

The wt B/Ann Arbor/1/86 strain was isolated during the 1985/86 epidemic in the US. Virus was isolated in specific pathogen free (SPF) eggs, during a single passage, then plaque-purified twice at a temperature of 37°C in primary chick kidney cells (PCKC) prepared from SPF embryos, and finally passaged twice more in SPF eggs to prepare a seed pool used as the starting material for preparation of candidate vaccines. The B/Leningrad/14/55 strain was isolated from an ill child in 1955; it had undergone 20 passages in embryonated eggs at a temperature of 32°C before being adapted to grow at the

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normally sub-optimal temperature of 25°C by 17 passages in embryonated eggs at 25–26°C[4,10]. Derivation of the B/Ann Arbor/1/66 ca virus was by passage in PCKC at successively lower temperatures. Including initial isolation, the virus had received seven PCK passages before being considered adapted to growth at 25°C[5]. This was plaque-purified by seven sequential passages in PCKC from SPF embryos, before preparing an egg pool for use in the studies described.

Preparation of ca reassortant viruses

For preparation of a reassortant virus with the ca B/Leningrad/14/55 parent, virulent B/Ann Arbor/1/86 virus (at a dose of 6.25 $\log_{10}$ EID_{50}/0.2 ml) was partially inactivated by heating (at 36°C for 48 h), reducing its infectivity by 3.75 logs. This preparation was mixed with an equal volume of untreated ca B/Leningrad/14/55 virus, containing 6.25 logs of infectivity. Chick embryos were infected with a mixture of the viruses and incubated at 34°C for 48 h. The reassortant was selected after two passages in the presence of 16–32 neutralizing units of antiserum to the B/Leningrad/14/55 virus followed by cloning in embryonated eggs at 34°C for 72 h using limited dilution technique. Clones were then selected with antigenic characteristics of the B/Ann Arbor/1/86 virus, and their genome composition screened as described below. A clone designated LEN-B/14/5/1 was used for subsequent studies, after verification it contained only the HA, NA and NS genes from the wild-type parent.

Derivation of the reassortant of wt B/Ann Arbor/1/86 and ca B/Ann Arbor/1/66 was accomplished by mixed infection of PCKC at 25°C, and selection in the presence of immune serum to B/Ann Arbor/1/86, as previously described for influenza A viruses[1]. After two cycles of plaque purification at 25°C, genome composition of reassortant viruses was determined as described below, and the clone designated AA-CRB117 selected for further studies. This reassortant contained only HA and NA of the wt parent virus.

Effect of temperature on replication. Cold-adaptation and temperature sensitivity were determined by either titration in embryonated eggs or PCKC cultures at 25°C, 32°–34°C, and 37°C, 38°C or 39°C, as previously described[11,12]. End points were determined as either median egg infectious doses (EID_{50}) or, in the case of tissue culture, p.f.u. ml^{-1}.

Animal studies. The ability of the parental and reassortant viruses to replicate in the upper and lower respiratory tracts of ferrets was determined as previously described[13].

Genome analysis. Electrophoresis of single-stranded virion RNA was performed for both the LEN-B/14/5/1 and AA-CRB/117 reassortant viruses, and their respective parents, as previously described[14,15]. In addition, the technique of cRNA:vRNA hybridization followed by electrophoresis of nuclease S1-treated homologous and heterologous double stranded RNA's[16,17] was used to confirm results with the reassortant LEN-B-14/5/1.

Table 1 Change of phenotype of B/Leningrad/14/55 after cold-adaptation

No. of egg passages			$\log_{10}$ p.f.u. ml^{-1} in chick kidney cells		
32°C	25°C	Total	25°C	32°C	38°C
20	0	20	3.5	6.4	3.2
20	17	37	5.9	6.8	0

Table 2 Adaptation of influenza B/Ann Arbor/1/66 to growth at 25°C in primary chick kidney cells (PCKC)[a]

No. of passages required for optimal growth				Maximum infectious titre (p.f.u. ml^{-1})
33°C	30°C	27°C	25°C	
2	2	1	1	3×10^7

[a]B/Ann Arbor/1/66 originally isolated in PCKC

Table 3 Comparison of ca influenza B viruses for replication at different temperatures in primary chick kidney cells (PCKC)

	Infectivity titre (p.f.u. ml^{-1}) in PCKC			
	25°C	33°C	37°C	39°C
Parental viruses				
wt B/Ann Arbor/1/86	$<10^3$	7×10^6	5×10^6	$<10^4$
ca B/Leningrad/14/55	1×10^8	2×10^8	8×10^7	$<10^4$
ca B/Ann Arbor/1/66	1×10^8	3×10^8	$<10^3$	N.D.
Reassortant viruses				
LEN-B/14/5/1	9×10^7	2×10^7	4×10^7	$<10^4$
AA-CRB117	2×10^7	5×10^7	$<10^3$	N.D.

Results

Cold-adaptation and ts phenotypes of parental viruses

Virus B/Leningrad/14/55 was sequentially passaged 20 times in embryonated eggs at 32°C and then 17 times at 25°C to yield the modified virus designated B/Leningrad/14/17/55. This virus could be differentiated from its progenitor in having higher levels of replication at 25°C, and lower replication at 38°C in PCKC (*Table 1*). The virus B/Ann Arbor/1/66 was passaged at sequentially lower temperatures in PCKC until the derived ca virus replicated efficiently at 25°C (*Table 2*).

When the ca viruses derived in the US and USSR were compared, they were equally adapted to growth at 25°C (*Table 3*). However, in these studies, which utilized temperatures of 37°C and 39°C to detect temperature sensitivity, only the B/Ann Arbor ca virus experienced a cut-off in replication at a lower temperature than the wt B/Ann Arbor/1/86 included as a control. Thus, it appears probable that the degree of temperature sensitivity of the ca B/Leningrad/14/17/55 virus is somewhat less pronounced than for the ca B/Ann Arbor/1/66 virus.

Cold-adaptation and ts phenotypes of reassortant viruses

Comparison of the replication in PCKC of parental and ca reassortant viruses at different temperatures indicated that the LEN-B/14/5/1 reassortant virus was indistinguishable from its B/Leningrad/14/17/55 ca parent, and the AA-CRB117 virus was indistinguishable from its B/Ann Arbor/1/66 ca parent (*Table 3*). Thus, both ca reassortants replicated much better than their contemporary wt parent B/Ann Arbor/1/86 at 25°C, whereas only the AA-CRB117 virus, but not the LEN-B/14/5/1 virus, was restricted in replication at 37°C.

Table 4 Replication of ca B/Leningrad/14/55 virus and its c.a. reassortant LEN-B/14/5/1 in embryonated eggs at different temperatures

Virus	Titre (log_{10}. EID_{50})			
	25°C	34°C	37°C	38°C
wt B/Ann Arbor/1/86	2.25	7.25	6.25	1.25
ca B/Leningrad/14/17/55	6.0	7.5	7.0	1.0
ca LEN-B/14/5/1	6.5	8.5	7.75	2.5

Titration of the Leningrad viruses in embryonated eggs confirmed the cold-adaptation phenotype of the parental and reassortant ca viruses (*Table 4*). In this host system, as in PCKC, temperature sensitivity was not detected at 37°C for the ca Leningrad parent or reassortant. Because even wt influenza B virus does not replicate reliably in eggs at 38°C no meaningful evidence of ts phenotype could be obtained from the ca B/Leningrad viruses in eggs. Egg infectivity titrations at different temperatures were not carried out for the B/Ann Arbor/1/66 ca virus, or its ca reassortant. Clone LEN-B/14/5/1 was passed in eggs at 34°C five times to determine the possible alteration in ca phenotype. Titration in eggs after the fifth passage showed no changes (not shown).

Replication in ferrets

In two experiments a total of four ferrets each were infected in parallel with wt virus B/Ann Arbor/1/86, ca parental viruses B/Leningrad/14/17/55 and B/Ann Arbor/1/66, and the ca reassortant viruses LEN-B/14/5/1 and AA-CRB117. The wt contemporary virus B/Ann Arbor/1/86 was detected in both trachea and lungs at 3 days postinfection, with lung titres being about 2 logs less than in the trachea (*Table 5*).

Infection was associated with fever lasting 2 days in all animals. The cold-adapted B/Leningrad/14/17/55 virus replicated in lungs to a titre about 2 logs lower than in the trachea. Only two of four ferrets experienced fever, of one day duration in both cases.

Reassortant ca virus LEN-B/14/5/1 differed from both parent viruses in replicating only in trachea, not lungs. Two of four ferrets experienced low level fever of 39.9°C, a borderline increase compared with the normal baseline of 39.7°C for ferrets. Thus the reassortant LEN-B14/5/1 appeared to be closer to having full attenuation than its ca parent, although evidence of marginal pathogenicity was evident in this animal model.

Neither the ca B/Ann Arbor/1/66 parent virus, nor its reassortant AA-CRB117, replicated to detectable levels in ferret lungs, despite quite high levels of replication in trachea. Likewise, neither of these two viruses elicited evidence of febrile reaction, or coryza. Thus, both were highly attenuated.

Discussion

Live influenza B vaccines were used in the USSR for many years, and cold-adapted influenza B vaccine was developed by 1965[10,11]. However, relatively little information exists about such vaccines, and most vaccine development and research has focused on influenza A.

Although one reason for this may be the perception that influenza B poses a lower risk than influenza A, beginning in 1979 influenza B has been responsible for quite major epidemics, involving high risk adults, as well as children. Excess mortality has been consistently evident[18,19]. In addition, the use of inactivated influenza B vaccine may on many occasions provide inadequate protection, particularly in the elderly[20,21]. This may be due to low immunogenicity of influenza B vaccine[21,22], or the failure to include the optimum virus strain in the vaccine on account of virus genetic and antigenic heterogeneity, and the rapid spread of new variants[23].

The availability of live attenuated influenza B vaccine might contribute to improved ability to control this disease, at least in some segments of the population. This would particularly be so, were such a vaccine to offer broader or more long-lasting immunity than inactivated vaccine, as has been hoped for in the case of type A virus.

This report indicates that in theory it is possible to prepare new vaccinal strains containing contemporary surface antigens, by reassortment with ca parental virus. The biological marker of cold-adaptation was reproducibly transferred to reassortants, as was the ts phenotype if the appropriate host system was used for its detection. Ferret virulence was also reproducibly transferred in the case of the B/Ann Arbor/1/66 virus, but for the B/Leningrad/14/55 virus the modest residual virulence in the ca parent was reduced slightly further in its reassortant.

Based on the *in vitro* and animal model data, it might be expected that the B/Ann Arbor/1/66 ca virus would prove more acceptable as a donor strain for making live vaccines, in view of its slightly higher temperature sensitivity, and lower levels of replication in ferret lungs than the B/Leningrad/14/55 virus.

Unfortunately, it is still not possible to extrapolate directly from *in vitro* or animal studies to human clinical experience. For example the reassortant vaccine LEN-B/14/5/1 was in fact highly attenuated[9]. Clinical studies are being undertaken in the US with the B/Ann Arbor reassortant virus.

Considerably more laboratory and clinical experience is needed before it will be known how reproducibly live attenuated influenza vaccines can be prepared, and their general efficacy determined. The only reported field study of efficacy in a natural challenge indicated some beneficial effect from a ca vaccine, but the virus was of a different origin from that studied here[24]. Small scale volunteer studies with a ca reassortant of B/Ann Arbor/1/66 did demonstrate safety and immunogenicity, as well as efficacy against artificial challenge with a wt strain[25,26]. It will also be necessary to evaluate the ability to combine influenza B with one or more type A components without interference occurring, or dilution of the type B virus below its infectious threshold in man. Finally, genetic stability must be established. The availability now of data on the genome sequence of at least the B/Ann Arbor/1/66 vaccine candidate, and preliminary data on gene constellations associated with attenuation, will assist this process[27,28].

In conclusion, the data obtained support from the theoretical concept that live attenuated influenza B vaccines can be readily obtained by reassortment with cold-adapted parents, including the transfer of defined laboratory markers. Such vaccines warrant further evaluation of their practical application in man.

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