

A Comparative Study of Attenuated Influenza Viruses

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Influenza A and influenza B viruses were adapted to growth at 25°C. When given to volunteer subjects, the viruses were attenuated but remained infective and antigenic. The minimum immunizing dose of an egg-adapted virus appeared to be 10^{5.0} EID₅₀. Cloning by plaque selection at 25°C gave seed cultures of relatively low infectivity titres. These titres were increased when necessary by passage at 33°C. No reversion to virulence was observed.

Viruses attenuated in the United Kingdom and the USA were compared in volunteer trials with vaccine strains that had already been used in the USSR for mass immunization. Results were broadly similar. Currently available methods of attenuation and work with temperature-sensitive mutants are reviewed.

Workers in the USSR originally suggested that influenza A viruses resistant to serum inhibitors were attenuated for man and that they could be used for mass vaccination (Solov'ev et al., 1961, 1968; Smorodincev et al., 1965, 1967). This has since been confirmed in the United Kingdom where volunteer trials showed that inhibitor-resistant strains were less virulent than the inhibitor-sensitive stocks from which they were derived and were potentially immunogenic and protective (Beare, 1969; Beare & Bynoe, 1969). The trials in the United Kingdom also involved a preliminary study of virus attenuation by passage at 25°C. The latter method has received greater attention in the USA (Maassab, 1967, 1968, 1969) where it was shown that influenza A and influenza B viruses, initially virulent for ferrets and mice, could be attenuated for these animals. In this article the responses of volunteer subjects to viruses manipulated in different laboratories by a variety of techniques are compared and an attempt is made to assess these viruses as potential human vaccines.

MATERIALS AND METHODS

Viruses

The adaptation of A2/Hong Kong/1/68 to growth at 25°C was carried out at the Common Cold Unit as previously described (Beare & Bynoe, 1969), the virus being passaged 17 times in embryonated eggs at that temperature with 3-day incubation periods. Infectivity titres were generally low and a final passage was therefore made at limit dilution at 33°C. This pool, which had a titre of 10^{8.5} 50% egg-infecting doses (EID₅₀) per ml of allantoic fluid, was used in a volunteer trial.

The A2/Aichi/2/68 strain of the Hong Kong virus was attenuated at the Michigan University School of Public Health. Monolayers of chicken kidney in plastic Petri dishes were infected and overlaid by methods described elsewhere (Maassab, 1968). After incubation at 25°C, a plaque was selected from which the virus was propagated in series in embryonated eggs at the same temperature (Maassab et al., 1969). In the later passages the incubation periods were 5–6 days. The virus grew reasonably well with moderate titres of haemagglutinin and the plaquing system appeared to have hastened the adaptation process. Virus that had received 8 such passages showed a titre of 10^{6.0} EID₅₀ in eggs incubated either at 33°C or at 25°C. This virus was given to volunteer subjects in two trials. The virus was then passaged again at 33°C

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at a dilution of $10^{-4.0}$ and new seed was obtained with an infectivity titre of $10^{7.3}$ EID₅₀ per ml. This seed was used in a third trial.

Virus strain B/AA/1/66 was attenuated at the Michigan University School of Public Health by the technique used for strain A2/Aichi/2/68. A plaque selected at 25°C was passaged 10 times in eggs at this temperature. The pool given to volunteer subjects had a titre of $10^{6.5}$ EID₅₀ per ml.

Three viruses from the USSR were also given to volunteers. They were not attenuated by us and were supplied as vaccine strains by the D. I. Ivanovsky Institute of Virology of the USSR Academy of Medical Sciences. Their names and passage histories are as follows:

(1) A2/Istra/10/69, an inhibitor-resistant Hong Kong strain, received 8 egg passages (3 of them in the presence of horse serum), 4 passages in chick-embryo tissue-culture, and 2 further egg passages. The temperature of incubation was 33°–35°C.

(2) A2/Hong Kong/1/68, an inhibitor-sensitive vaccine strain of the prototype virus, was prepared independently in Moscow. A laboratory virus that had initially received 2 passages in monkey-kidney tissue-cultures and 3 in eggs was passaged a further 21 times in eggs at 35°C.

(3) B/Dushanbe/1/66 received 22 egg passages at 35°C, 2 being made in the presence of horse serum.

Volunteer subjects

Isolation and inoculation of volunteers and methods used in the grading of clinical reactions have already been described (Tyrrell, 1963). Diluted viruses were given in 1.0-ml amounts intranasally by dropper or in 0.5-ml amounts with a coarse hand-sprayer. An arbitrary scoring system, based on the incidence of pyrexia, coryza, and subjective symptoms and on handkerchief counts on successive days following virus inoculation, was also used to compare the severity of reactions induced by the different viruses.

Assessment of the trials

Criteria were generally the incidence and severity of clinical reactions, the presence of virus in nasal washings taken on the 2nd, 3rd, and 4th days after inoculation, and rises in haemagglutination-inhibiting (HI) antibody titres in the blood 2–3

weeks after the trials. Serological and virus isolation techniques have been described previously (Beare et al., 1968). Because of the relative insensitivity of monkey-kidney tissue-culture for the isolation of Hong Kong virus, the allantoic cavity of embryonated eggs was sometimes used instead.

RESULTS

Influenza viruses had previously been attenuated for animals by the methods used for the attenuation of the viruses at the Michigan University School of Public Health (Maassab, 1969), but since these had not been inoculated into man, the Aichi variant was first given in 3 different dilutions. Groups of volunteers received 1.0-ml amounts of virus diluted $10^{-4.0}$, $10^{-3.0}$, and $10^{-2.0}$, and these contained doses of $10^{2.0}$, $10^{3.0}$, and $10^{4.0}$ EID₅₀, respectively. The results are shown in Table 1. Although a few infections occurred and there were some clinical reactions, it was clear that even the highest virus dose had failed to infect most of the susceptible volunteers. This could have been due to over-attenuation or to insufficient dosage. Since the seed had already been diluted $10^{-2.0}$, it was decided to prepare a new pool with a higher infectivity titre. Passage at 25°C might have achieved this, but as it might also have resulted in over-attenuation, the next passage was made in 6 leucosis-free eggs at a dilution of $10^{-4.0}$ at an incubation temperature of 33°C. The new pool had a titre of $10^{7.3}$ EID₅₀ per ml, which when diluted 1:10 and given to 11 more volunteers proved adequately infective and antigenic (Tables 1 and 2). Only 1 susceptible volunteer now failed to become infected. A detailed examination of the responses showed antibody rises comparable with those obtained with viruses attenuated by other means (Beare & Bynoe, 1969) and no apparent increase in virulence. The responses of volunteers to the Salisbury "cold" virus (Table 1) showed that a 1:1000 dilution, which provided an individual dose of $10^{5.5}$ EID₅₀, was also infective and antigenic and, like the Aichi variant, relatively non-reactive. Lastly, in this trial series, the attenuated B/AA/1/66 strain was given to 19 volunteers. Most of the susceptibles were infected by doses ranging from $10^{5.0}$ to $10^{5.5}$ EID₅₀ and clinical symptoms were trivial. Nevertheless, the titre of the seed ($10^{6.5}$ EID₅₀ per ml) was probably low for clinical use and needed to be elevated, if possible, by further passage at 33°C.

Table 1. Responses of volunteer subjects to inoculation with influenza A and influenza B viruses adapted to growth at 24°C at the Common Cold Unit, Salisbury and at the Michigan University School of Public Health, Ann Arbor

Virus strain	Dilution of seed	Virus dose (EID ₅₀ in 1 ml)	Significant clinical reactions ^{a, b}	Serum haemagglutination-inhibiting antibody rises:	
				in those with initial titres ≤24	in all volunteers
A2/AICHI/2/68 (Ann Arbor)	10 ^{-4.0}	10 ^{2.0}	1/7	1/6	1/7
	10 ^{-3.0}	10 ^{3.0}	2/7	2/3	2/7
	10 ^{-2.0}	10 ^{4.0}	3/21	3/12	4/20 ^c
A2/Aichi/2/68, after passage at 33°C	10 ^{-1.0}	10 ^{6.3}	6/11	6/7	8/11
A2/Hong Kong/1/68 (Salisbury)	10 ^{-3.0}	10 ^{5.5}	3/9	4/4	4/9
B/AA/1/66 (Ann Arbor)	10 ^{-1.0}	10 ^{5.0-10^{5.5}}	2/19	10/15	10/19

^a Number of responses/number of specimens tested.

^b All reactions shown were graded "mild".

^c One serum specimen was lost in this series.

Table 2. Responses of volunteer subjects to inoculations with strain A2/Aichi/2/68 virus *

Volunteer	Grading of clinical reactions	Reciprocal haemagglutination-inhibiting antibody titres before and after the trial
1	Mild	<6; 72
2	Mild	<6; 84
3	Nil	<6; 12
4	Mild	48; 192
5	Nil	48; 192
6	Nil	144; 192
7	Nil	6; 48
8	Nil	<6; <6
9	Mild	<6; 24
10	Nil	36; 36
11	Mild	<6; 48

* Virus dose: 10^{6.3}EID₅₀. The virus had had 8 passages at 25°C and 1 at 33°C. Significant antibody rises are shown in bold-face type.

The responses of volunteers to inoculation with the USSR vaccine strains are shown in Table 3. Both the influenza A viruses were given in doses of 10^{3.0} and 10^{5.0} EID₅₀, neither of which was fully

immunogenic. However, since they have been used in large-scale field trials in the USSR, they appear to merit further study. Strain A2/Istra/10/69 (the inhibitor-resistant strain) seemed less antigenic than A2/Hong Kong/1/68 but the difference was not statistically significant; strain B/Dushanbe/1/66 produced antibody rises in 5 out of 6 initially susceptible volunteers. There were no reports of undesirable clinical effects during mass vaccinations in the USSR, but in the closely supervised trials of the Common Cold Unit a number of reactions were recorded, and although most were "mild", those following inoculation with B/Dushanbe/1/66 were graded "moderate".

An attempt to make a closer comparison of the clinical responses induced by the viruses is shown in Table 4. Each of the gradings "mild", "moderate", and "severe" covered a wide range of symptoms, and initial efforts to give numerical scores to individual reactions suggested that values below 20 signified mild (and therefore acceptable) responses. While a high degree of accuracy is not claimed for the system, this additional analysis appears to provide useful information. Strain B/Dushanbe/1/66 was clearly under-attenuated, while strain A2/Aichi/2/68 and the Salisbury and Moscow strains of A2/Hong Kong/1/68 were of roughly similar pathogenicity and were all adequately attenuated. Strains B/AA/1/66 and A2/Istra/10/69 seemed to produce virtually no reactions in the volunteers infected with them.

Table 3. Responses of volunteer subjects to inoculation with live influenza vaccines prepared in the USSR *

Virus strain	Incubation temperature during attenuation (°C)	Sensitivity to horse serum inhibitor	Estimated virus dose (EID ₅₀)	Clinical reactions ^a	Virus isolations ^b	Serum haemagglutination-inhibiting antibody rises:	
						in those with initial titres ≤24 ^b	in all volunteers ^b
A2/Istra/10/69	33-35	Resistant	10 ^{3.0}	0/6	2/6	1/2	2/6
			10 ^{5.0}	3/8	2/8	1/3	2/8
A2/Hong Kong/1/68	35	Sensitive	10 ^{3.0}	0/5	2/5	2/3	2/5
			10 ^{5.0}	6/9	6/9	4/7	5/9
B/Dushanbe/1/66	35		10 ^{5.0}	3/9 ^c	5/9	5/6	6/9

* At the D. I. Ivanovsky Institute of Virology, Moscow.

^a Number of responses/number of specimens tested.

^b These three reactions were graded " moderate ". All others in this table were graded " mild ".

Table 4. Comparison of clinical responses in volunteer subjects infected by the attenuated viruses

Virus strain	Virus (EID ₅₀)	No. of infected subjects ^a	Mean peak handkerchief count	Mean no. of reactions per infection	Mean score of symptoms and signs per infection ^b
A2/Aichi/2/68 (Ann Arbor)	10 ^{2.0} -10 ^{4.0}	7	6.9	0.43	13.3
A2/Aichi/2/68 (Ann Arbor)	10 ^{6.3}	8	11.1	0.6	14.4
B/AA/1/66 (Ann Arbor)	10 ^{5.0} -10 ^{5.5}	11	3.2	0.18	4.8
A2/Hong Kong/1/68 (Salisbury)	10 ^{5.5}	4	21.3	0.75	14.8
A2/Istra/10/69 (Moscow)	10 ^{3.0} -10 ^{5.0}	4	6.3	0.25	6.3
A2/Hong Kong/1/68 (Moscow)	10 ^{3.0} -10 ^{5.0}	8	7.8	0.63	11.8
B/Dushanbe/1/66	10 ^{5.0}	6	21.2	0.6	32.8

^a Volunteers with virus excretion or antibody rise or both.

^b Based on number of days on which there was pyrexia, coryza, increased use of handkerchiefs, and subjective symptoms.

DISCUSSION

The experiments show that influenza viruses passaged repeatedly at 25°C, or propagated from plaques selected at this temperature, are relatively attenuated for man. Although none of the viruses obtained by these means was of a sufficiently high titre to be used for human immunization, titres were subsequently increased by passage at 33°C. There was then no apparent reversion to virulence. However, this latent possibility stresses the desirability, where possible, of careful initial cloning.

During the attenuation of influenza viruses the influenza-like symptoms which they evoke are replaced by those of afebrile coryza. All the vaccine strains that we have so far produced cause symptoms of this kind in at least a proportion of the people who are successfully immunized.

It was interesting to compare the clinical effects of the vaccines from the USSR with those of vaccines from the United Kingdom and the USA. The degree of reaction with most of the influenza A viruses seems to have been about the same, and since strains from the USSR had been used for

large-scale immunization it is probably an acceptable level. Strain B/Dushanbe/1/66 may, however, have been under-attenuated. The attenuation methods employed in the USSR reflect the views held in that country on the attenuating effect on influenza viruses of simple egg-passage at ordinary temperatures of growth (Smorodincev, 1969; Ždanov, 1967). It has, however, not been possible to attenuate viruses by these means at the Common Cold Unit (Beare et al., 1968; Beare, unpublished data), where influenza viruses are held to be relatively stable genetically. The attenuation of strain A2/Istra/10/69 may well be due to its inhibitor-resistant character since such strains are habitually attenuated (Beare & Bynoe, 1969; Slepúškin et al., 1968) and, in our opinion, are a potentially valuable source of live vaccines providing they do not become over-attenuated. For the moment, it is considered that low-temperature adaptation is the most generally useful of the attenuation methods since it can be applied both to influenza A and influenza B serotypes and can, if necessary, be made independent of plating ability.

In conclusion, reference may be made to publications from other laboratories on the possibility of using temperature-sensitive (*ts*) conditional lethal mutants as live influenza vaccine strains (Mackenzie, 1969, 1970; Mills et al., 1969a). These mutants fail

to grow at the upper temperature limits of the parent strains and are produced by the use of chemical mutagens. Mills and his colleagues demonstrated that two such mutants were attenuated for hamsters and attributed this to the existence of a temperature gradient within the respiratory tract, which confined the replication of the viruses to the upper part of the tract. While we accept the evidence that *ts* mutants are attenuated, this explanation seems much over-simplified. Mackenzie's work showed that *ts* defects in WSN virus were associated with diminished virulence for mice and that the mutants were infective and antigenic. He was unable to correlate lack of virulence with physiological changes in the haemagglutinin and neuraminidase and confirmed the opinion of others that virulence in influenza viruses is polygenic. In addition, he thought that there was no direct connexion between the level of "cut-off" temperature and the degree of attenuation but, in this respect, his views differ from those of Mills et al. (1969b), which were also based on Mackenzie's own results. The production of *ts* mutants seems unlikely at present to replace low-temperature adaptation as a practical means of obtaining attenuated viruses for live vaccines. Nevertheless, it provides a system for the genetic analysis of influenza viruses, which must ultimately prove of great importance to the development of live vaccines.

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RÉSUMÉ

ÉTUDE COMPARATIVE DE VIRUS GRIPPAUX ATTÉNUÉS

Des virus grippaux A et B, atténués par différents procédés dans des laboratoires du Royaume-Uni, des États-Unis d'Amérique et d'URSS, ont été expérimentés sur des volontaires à la Common Cold Unit, de Salisbury (Angleterre). On se proposait d'étudier: a) les modalités de l'infection et de l'immunisation éventuelle, d'après

l'excrétion du virus et la production d'anticorps; b) la fréquence et la gravité des réactions cliniques chez les sujets vaccinés; c) l'infectivité des diverses souches virales et la possibilité de les utiliser pour la vaccination de masse.

En Angleterre, par passages répétés sur œuf, à 25°C, suivis d'un dernier passage à 33°C, on a obtenu une

souche atténuée de virus A2/Hong Kong/1/68 titrant $10^{8.5}$ DIE₅₀ (dose infectant 50% des embryons de poulet) par millilitre. Aux Etats-Unis, la souche A2/Aichi/2/68 du virus de Hong Kong et une souche de virus grippal B(B/AA/1/66) ont été atténuées par inoculation au rein de poulet en couche monocellulaire et incubation à 25°C pendant 5-6 jours. Après repiquage sur œuf, on a récolté un virus A titrant $10^{8.0}$ DIE₅₀/ml et un virus B titrant $10^{8.5}$ DIE₅₀/ml. Trois virus ont été atténués en URSS: la souche A2/Istra/10/69 (virus de Hong Kong résistant aux inhibiteurs); une souche A2/Hong Kong/1/68 atténuée par 21 passages sur œuf, à 35°C; une souche B/Dushanbe/1/66 atténuée par 22 passages sur œuf, à 35°C.

Les essais sur volontaires ont montré que les différents virus, à l'exception peut-être du virus B/Dushanbe/1/66, étaient convenablement atténués. La technique de la sélection de plages permet d'accélérer le processus d'atténuation, mais les virus ainsi obtenus ne font preuve que

d'une infectivité relativement faible; cependant, le passage à 33°C accroît les titres sans augmentation concomitante de la virulence.

Si l'on en juge d'après les effets cliniques, l'atténuation des virus obtenus au Royaume-Uni et aux Etats-Unis est du même ordre que celle des virus d'URSS, déjà utilisés pour la vaccination de masse.

Selon les auteurs, l'adaptation des virus à la croissance aux basses températures (25°C) apparaît comme le procédé le plus intéressant parmi les méthodes d'atténuation dont on dispose actuellement. Elle peut en effet être appliquée aux sérotypes A et B et elle ne dépend pas nécessairement des possibilités de sélection de plages. Quant à la production de mutants sensibles à la température, bien qu'offrant de l'intérêt pour l'étude génétique des virus grippaux, elle ne semble pas pouvoir être substituée à l'adaptation aux basses températures, car elle entraîne généralement une diminution nette de la virulence.

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