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Laboratory and Clinical Characteristics of Attenuated Strains of Influenza Virus*

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Prior to 1967, attenuation of influenza virus was achieved by gradually lowering the incubation temperature until optimal growth at 25°C was obtained. The process of attenuation of a Hong Kong strain was modified and considerably shortened. The temperature of incubation was changed abruptly from 35°C to 25°C and a cold variant was selected using the plaque-assay system.

A set of genetic markers was developed for assessing the potential virulence of cold-passaged variants. The cold variant of the Hong Kong strain was temperature-sensitive, acid-labile and produced a small plaque in primary chick kidney cells incubated at 35°C. Additional differentiating biological properties relating to the adaptation of the virus to growth at 25°C and to loss of virulence in a susceptible host are presented.

The cold-adapted variant was found to be relatively avirulent and highly antigenic for mice and ferrets, and virus was recovered from the nasopharynx of infected ferrets during the first 3 days. The virus recovered was still unable to grow well at 41°C (rct/41 −), was sensitive to acid pH and produced small plaques at 35°C and larger ones at 25°C.

After a series of plaque purifications, the cold variant showed further loss of virulence to mice, more vigorous growth at 25°C, complete failure to grow at 41°C and good antigenic potency.

The genetic markers were stable in the plaque-purified cold variant after at least 10 consecutive passages either in tissue culture at 35°C, or in mice.

Cold variants of type B influenza virus have a narrower range of temperature sensitivity compared with type A strains. Reduced plaquing efficiency and reproductive capacity occurred at 35°C (rct/35 −) with the attenuated type B strains instead of at 41°C as with the type A strains.

Clinical trials with the attenuated Hong Kong strain of influenza virus (A2/Aichi/2/68) have demonstrated the acceptability and immunogenicity of the strain in man.

Attenuated strains of the influenza virus have been obtained through adaptation of the virus to growth at 25°C by gradually lowering the incubation temperature (Maassab, 1967). The cold variants of influenza A and B infected susceptible hosts, such as ferrets and mice, without causing illness or pathological pulmonary symptoms and with the induction of high antibody levels. However, this process took 6–8 months to produce a non-reactive but immunogenic strain.

A new procedure based on the plaque technique (Maassab, 1968) shortened this process considerably. The incubation temperature was reduced abruptly from 35°C to 25°C, instead of lowering it gradually, and plaques that appeared in primary chick kidney tissue culture held at 25°C were selected as the source of virus for further passages in eggs or tissue cultures at the suboptimal temperature. The steps followed for cold adaptation of A2/Aichi/2/68 are shown in

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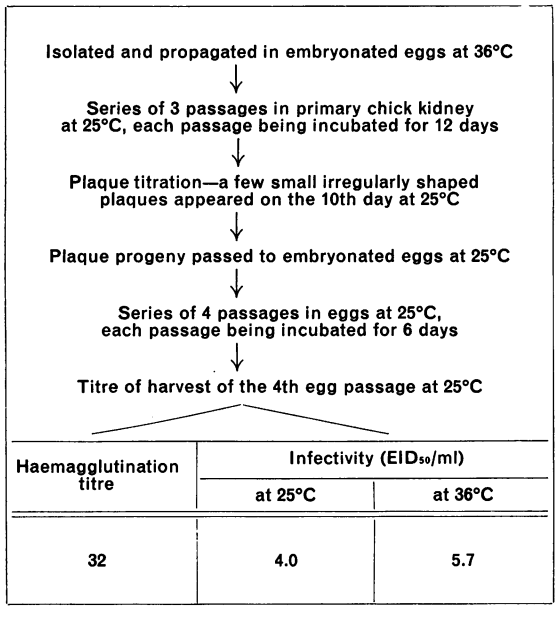
the accompanying flow diagram. Thus it was possible in 7 passages to adapt this Hong Kong strain to growth at 25°C. The time required was 3 months.

FLOW DIAGRAM OF THE ADAPTATION OF A2/Aichi/2/68 TO 25°C



Haemagglutination titre	Infectivity (EID_{50}/ml)	
	at 25°C	at 36°C
32	4.0	5.7

USE OF GENETIC MARKERS TO CHARACTERIZE THE HONG KONG COLD VARIANT

A set of genetic markers of cold variants has been developed in this laboratory for assessing the potential virulence of cold-passaged lines (Maassab, 1969). The appropriate tests are applied to the Hong Kong cold variant and its "wild" counterpart. Table 1 shows the results of a typical set of experiments. The findings suggest that the cold variant A2/Aichi/2/68 has become attenuated since (1) its reproductive capacity is reduced at 41°C, rct/41(−), (Sabin, 1960), (2) it is acid-labile and (3) it has a characteristic size of plaque. Without exception so far, cold variants exhibit these typical features.

The use of other properties of virus such as heat-stability of haemagglutinin, sensitivity to inhibitors, capacity to agglutinate red blood cells derived from different species or differences in enzymatic activities have failed to differentiate consistently between cold variants and their parent lines.

TESTS FOR AVIRULENCE OF COLD-ADAPTED A2/AICHI/2/68

A pool of cold-adapted virus was prepared in 11-day embryonated eggs held at 25°C and titrated for both haemagglutination and infectivity. The values found were 1/64 and 25×10^6 plaque-forming units per ml (PFU/ml) respectively. Aliquots of serial dilutions were given intranasally to ferrets and mice. The results of intranasal inoculation of ferrets are given in Table 2. Nasopharyngeal swabs were taken daily from each of the ferrets for a period of 5 days. Clinical response manifested by fever or coryza was recorded and each ferret was bled before and 2 weeks after infection. In ferrets inoculated with the undiluted preparation, the virus persisted for 4 days in the nasopharynx. There was a slight fever of 1 day's duration and coryza which was apparent on the 2nd day after infection lasted for 48 hours. In the ferrets receiving 10^{-1} dilution, the virus persisted for 3 days, with 1 day of fever and no coryza. Inoculation with 10^{-2} dilution of the virus elicited a good antibody response without clinical symptoms, and the virus could be recovered from the nasopharynx for the first 2 days after infection.

The virus isolated from these ferrets was tested for the set of genetic markers presented in Table 1. It was found that the recovered virus was still temperature-sensitive, acid-labile and produced small plaques in primary chick kidney incubated at a temperature ranging between 35°C and 36°C.

In other experiments, 5 groups of 30 mice each were inoculated with the same dilutions used in ferrets. Every day 2 mice were sacrificed and examined for lung lesions. Lung lesions were not observed during the 3 weeks of observation. Infection of mice elicited a graded antibody response ranging from 1024 after administration of the undiluted preparation to 1/16 after use of the 10^{-4} dilution. The findings in animals confirm predictions made on the basis of the genetic-marker analysis. The cold-adapted line was found to be relatively avirulent and highly antigenic.

THE USE OF PLAQUE PURIFICATION FOR THE MODIFICATION OF A COLD VARIANT

Plaque purification has been found useful in previous studies for verification of the stability of the genetic markers of cold-adapted lines (Maassab, 1968). It was recognized that serial plaque passages at 25°C might alter the behaviour of cold-adapted lines by preferentially selecting clones which grew

TABLE 1

MARKERS OF THE COLD-ADAPTED HONG KONG STRAIN (A2/Aichi/2/68)

Virus strain	Virus titre at [a]					Plaque formation		
	Temperature			pH				
	25°C	35°C	41°C	5.7–6.3	7.0–7.2		At 35°C	At 25°C
Cold variant	22×10^6	28×10^6	1×10^{2} [b]	2×10^{3} [b]	28×10^6	Size [b]	<3 mm	>4 mm
						Shape	Irregular boundary	Irregular boundary
" Wild " type	18×10^2	32×10^6	30×10^6	30×10^6	32×10^6	Size	>4 mm	No plaque
						Shape	Irregular boundary	Irregular boundary

[a] All titrations are expressed as plaque-forming units per ml (PFU/ml).
[b] The cold variant is thus temperature-sensitive (rct/41–), acid-labile and has plaques of a distinctive size.

TABLE 2

RESPONSE OF THE A2/Aichi/2/68 COLD VARIANT IN FERRETS

Infection dilution	Virus isolation (days after infection)					Homologous HI response		Clinical manifestations	
	1	2	3	4	5	Pre-infection	Post-infection	Fever (°F; °C)	Coryza
Undiluted	+	+	+	+	0	<8	2 048	+ (104.3; 40.2)	+
10^{-1}	+	+	+	0	0	<8	1 548	+ (104.0; 40.0)	0
10^{-2}	+	+	0	0	0	<8	512	0 (102.0; 38.9)	0
10^{-3}	+	0	0	0	0	<8	128	0 (102.0; 38.9)	0
10^{-4}	0	0	0	0	0	<8	32	0 (101.8; 38.8)	0

more vigorously at the lower temperature. Studies designed to examine this possibility were carried out with the A2/Ann Arbor/2/65 strain. A sample of A2/Ann Arbor/2/65 yielding 10^5 PFU/ml was plaque purified 6 times and the progeny, obtained from the 1st, 3rd and 6th serial plaque isolates, were passed once in embryonated eggs. Serial dilutions of this passage were then titrated in embryonated eggs at 3 different temperatures of incubation in primary chick kidney cultures at 25°C and were also given to mice. The results from a typical experiment are presented in Table 3. While the cold variant grew to a reasonable titre prior to plaque purification there was a marked increase in infectious yield at 25°C in embryonated eggs and tissue cultures, especially noticeable upon infection with the progeny derived from the 6th plaque isolate. It is also apparent that after plaque purification, there was further loss of virulence for mice with no changes in antibody titre and practically complete failure of growth in eggs at 41°C. An experiment of the same design using the parent strain passaged at 36°C did not show such trends.

The progeny derived from the 6th serial plaque purification at 36°C was still virulent for mice, did not grow at 25°C and grew equally well at 35°C and 41°C. The findings demonstrate that serial plaque purification can improve the properties of a cold-adapted line, emphasizing those that would seem desirable characteristics of vaccine strains.

STABILITY OF COLD VARIANTS IN PLAQUE-PURIFIED LINES

The stability of lines purified by 6 serial plaque purifications was tested after additional passages

TABLE 3

BEHAVIOUR OF THE COLD VARIANT A2/Ann Arbor/2/65[a] in DIFFERENT HOSTS AFTER A SERIES OF PLAQUE PURIFICATIONS AT 25°C

No. of serial plaque purifications	Embryonated eggs (EID_{50}/ml)			Primary chick kidney (PFU/ml at 25°C)	Mice	
	25°C	35°C	41°C		LD_{50}/ml	HI titre
1	$10^{5.3}$	$10^{7.3}$	$10^{2.5}$	2×10^{5}	$10^{1.7}$	1 024
3	$10^{6.0}$	$10^{7.0}$	$10^{1.3}$	4×10^{6}	$10^{0.7}$	512
6	$10^{7.0}$	$10^{7.7}$	$<10^{1.0}$	3×10^{7}	0	1 024

[a] The strain was isolated in primary chick kidney and was adapted to growth at 25°C in tissue culture prior to undergoing plaque-purification.

at 36°C in primary chick kidney cells, or in mice, or after 30 passages in embryonated eggs at 25°C. Results of earlier studies with the A2/Ann Arbor/6/60 strain showed that markers of plaque-purified cold variants were stable after at least 10 consecutive passages either in primary chick kidney tissue cultures incubated at 36°C or in mice infected intranasally. Moreover, there was no reversion of virulence (Maassab, 1969). In further studies with the same virus, 30 additional passages of the plaque-purified line were made in embryonated eggs or tissue culture at 25°C. The yields were tested for the 3 genetic markers characteristic of attenuated strains and were found to be unchanged. As illustrated in Table 4, the response of mice to infection with the material passaged in eggs 30 times at 25°C was excellent in terms of the absence of pathological pulmonary symptoms (not shown), deaths and protection upon challenge with a virulent strain. Clearly 30 passages at 25°C had not effected the stability of this line. In contrast, reversion of the genetic markers and reacquisition of virulence has been observed by serial passage at 35°C of non-serial-plaque-purified lines.

TABLE 4

RESPONSE OF MICE TO INFECTION WITH THE COLD VARIANT OF TYPE A INFLUENZA VIRUS (A2/Ann Arbor/6/60) AFTER 30 PASSAGES AT 25°C

Dilution used to infect mice	Mortality ratio	Homologous HI[a] response after 2 weeks	Mortality ratio after challenge[b]
10^{-1}	0/20	1 024	0/10
10^{-2}	0/20	512	0/10
10^{-3}	0/20	64	2/10
10^{-4}	0/20	8	6/10
Uninfected controls	0/20	8	10/10

[a] HI = Haemagglutination inhibition.

[b] Two weeks after infection 10 mice of each dilution group were bled while the other 10 were used for challenge with a 10^{-2} dilution of mouse-adapted A2/AnnArbor/6/60 (titre $10^{-4.3}$ MLD_{50}/ml).

STUDIES WITH THE COLD VARIANTS OF TYPE B INFLUENZA VIRUS

Development of cold variants of type B influenza virus was accomplished by gradually lowering the incubation temperature. As a general rule, type B influenza virus required fewer passages at the intermediate temperatures of incubation before achieving optimal growth at 25°C.

Typical findings with the B/Ann Arbor/1/66 strain are shown in Table 5. The development of the cold variant in chick cells was followed until complete cellular lysis was evident. Further incubation of cells resulted in decrease in titre due to thermal inactivation of virus. It is evident that the infectious yields were highest at 25°C and 33°C incubation, but that the development at 25°C was slower as evidenced by PFU titre and time of appearance of cytopathology. At 35°C incubation, cellular lysis occurs at the same time as at 33°C, but the infectious yield is 97% lower than that at 33°C and 25°C incubation. Hence, type B cold variant had an impaired reproductive capacity at 35°C (rct/35−). The development of the virus in cells infected with the "wild" type was studied in the same fashion. As expected, the parent line did not grow at 25°C. Note that maximal yields of the "wild" type at 35°C and 33°C are comparable and are not greater than that of the cold variant at 25°C. Thus, the reproductive capacity of the parent line at 35°C was not impaired (rct/35+).

TABLE 5

DEVELOPMENT OF 2 STRAINS OF INFLUENZA VIRUS TYPE B (B/Ann Arbor/1/66) AT DIFFERENT TEMPERATURES [a]

Time after infection (days)	Titre of cold variant [b]			Titre of " wild " type [b]		
	25°C	33°C	35°C	25°C	33°C	35°C
0	12×10^2	15×10^2	12×10^2	13×10^2	14×10^2	13×10^2
2	13×10^3	24×10^4	23×10^4	14×10^2	20×10^5	15×10^5
4	14×10^5	$\underline{\underline{30 \times 10^6}}$	$\underline{\underline{5 \times 10^5}}$	25×10^2	$\underline{\underline{27 \times 10^6}}$	$\underline{\underline{23 \times 10^6}}$
6	$\underline{\underline{28 \times 10^6}}$	—	—	20×10^2	—	—
	(rct/35−)			(rct/35+)		

[a] $\underline{\underline{\quad}}$ Denotes when complete cytopathic change was evident.
[b] Titre is expressed as PFU/ml.

EXPERIENCE WITH ATTENUATED HONG KONG VIRUS AS AN IMMUNIZING AGENT IN MAN

Because of success in rapidly obtaining a cold-adapted line of Hong Kong virus, in promptly characterizing its genetic markers, and the results of tests for virulence and immunogenicity in animals, we explored the feasibility of using this line for immunization of man.

Live attenuated vaccine was prepared by the Michigan State Department of Health Laboratories using leucosis-free and RIF-resistant eggs. The final product was subjected to sterility tests and tests for extraneous viral agents according to the usual requirements for live virus vaccines. Well-educated volunteer adults in the State of Michigan were the subjects employed in the study. A dose of 0.5 ml of vaccine containing $10^{4.5}$ EID_{50} per ml was dropped into each nostril. Blood for serum antibody determination was obtained on the day of inoculation and at 2 and 6 or 10 weeks after inoculation. White blood cell counts were done on day 0 and for a further 4 days. Throat washings for virus isolation attempts were obtained on day 1, day 2, day 3 and day 4. Morning, afternoon and evening temperatures were taken and a symptom chart was kept by each volunteer for the first 4 days. The subjects were seen daily during this period.

Vaccine was given first to 4 individuals convalescent for several months from Hong Kong influenza. Next, 4 individuals who had previously received inactivated Hong Kong vaccine were inoculated. Finally 2 individuals who had a negative history for illness and vaccination were given the attenuated line. The studies were carried out between 14 April 1969 and 23 July 1969 after influenza had left the area.

Virus was not isolated from any of the 4 individuals with a history of prior illness. From 1 of the 4 subjects with a history of vaccination, virus was isolated at 24, 48, and 72 hours after intranasal instillation. Both individuals with a negative history of vaccination or illness shed virus at 48 hours, and one of them was still shedding at 72 hours.

Table 6 shows that 6 of the 10 volunteers, including the 3 who had shed virus, showed a 4-fold or greater increase in serum HI antibody levels. For the 2 subjects with a negative history for vaccination or illness, an increase from 0 to 128 was observed in the 2-week post-inoculation serum specimens. A 4-fold or greater increase was demonstrable only in the 6-week to 10-week sera of 4 other individuals. Since 2 of these persons had a previously documented illness and the other 2 had received inactivated vaccine some months before, it is possible that infection was more limited and in consequence the antibody response was delayed. Whatever the explanation, this finding makes a 6-week bleeding schedule mandatory when attenuated influenza virus vaccines are being studied in man.

None of the 10 volunteers experienced any unusual symptoms after vaccination and all temperatures were below 100° F (37.8°C). There were no marked changes in the white blood cell counts following intranasal installation.

The importance of the problem, the lack of untoward effects and the demonstration of antibody

TABLE 6
HI RESPONSE OF VOLUNTEERS RECEIVING LIVE ATTENUATED A2/Aichi/2/68 VACCINE INTRANASALLY

Volunteer	History	HI titre with A2/Aichi/2/68 Virus		
		Before intranasal vaccination	After vaccination 2 weeks	6–10 weeks
L. A.	Confirmed HK illness	128	256	1 024
J. C.	Confirmed HK illness	64	128	512
K. C. [a]	Confirmed HK illness	4 096	4 096	2 048
F. D. [a]	Confirmed HK illness	1 024	1 024	512
E. E.	HK vaccination	16	16	512
R. E. [b]	HK vaccination	32	32	1 024
H. K.	HK vaccination	32	32	32
H. M.	HK vaccination	256	256	256
K. F. [b]	No HK illness and no HK vaccination	<8	128	128
T. K. [b]	No HK vaccination	<8	128	16

[a] These volunteers had received Hong Kong vaccine 3 and 6 weeks, respectively, before their illness.
[b] These volunteers shed virus for 1–3 days after intranasal vaccination.

increase in a majority of the subjects after administration of the vaccine in this small sample, clearly warrants further studies with this attenuated influenza virus vaccine.

DISCUSSION

The attenuation of influenza virus using cold-adaptation procedures has been shown to offer some promise in the development of live influenza virus vaccine for use in man (Maassab, 1967, 1969; Alexandrova & Smorodincev, 1965; Medvedeva, Alexandrova & Smorodincev, 1968). At present the methods employed are not standardized. Two factors seem to influence the successful adaptation of virus to 25°C. One is the passage level of the strain prior to adaptation and the kind of host in which it was isolated. For instance, a strain with fewer passages at 36°C can be adapted to growth at 25°C with fewer passages at the intermediate temperature than a strain with a high passage level at 36°C. It is also true that strains isolated in primary monkey kidney tissue culture require more passages in embryonated eggs or primary chick kidney tissue culture for successful adaptation to growth at 25°C than strains isolated directly in embryonated eggs or chick kidney cells. Present studies suggest that the development of genetic markers is of major importance, not only to identify the degree of attenuation of cold variants, but to suggest their utility for monitoring the production of live virus vaccines.

The ability to obtain attenuated B strains encourages us to believe that polyvalent live influenza virus vaccines may be feasible (Maassab, 1969).

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