Evaluation of Low Temperature Grown Influenza A2/Hong Kong Virus in Volunteers (35187)

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The methods of immunization against influenza virus developed over the past 37 years, using either inactivated vaccines given parenterally, or live vaccines administered by the respiratory route, have not significantly altered the pattern of epidemic illness. Despite this experience, the development of live, attenuated, respiratory virus vaccines remain of interest because of several potential advantages over inactivated vaccines presently in use in the U.S.A. Live attenuated vaccines could be produced rapidly and in quantities sufficient for adequate mass immunization, and would be relatively easy to administer by nasopharyngeal spray. Most importantly, this approach would have the advantage of effecting direct stimulation of local secretory antibody which is presently thought to play a major role in resistance to viral respiratory disease (1, 2).

Adaptation of viruses to growth at suboptimal temperatures has been used successfully to produce attenuated mutants of poliovirus, Japanese B encephalitis virus and measles virus (3–5), and similar success has been reported with influenza A virus (6). The appearance of a major antigenic variant of influenza A virus in the summer of 1968 (A2/Hong Kong/68) afforded us the opportunity to test the efficacy of cold adaptation as a method for developing a live, attenuated strain of influenza A virus, and to assess both its clinical and immunological effects on volunteers lacking detectable homologous neutralizing antibody to this virus.

Materials and Methods. Virus Suspensions. The virus strain used was obtained from a throat swab specimen collected in Hong Kong on August 8, 1968, isolated in primary human embryonic kidney (HEK) tissue culture, lyophilized, and sent to us through the kindness of Dr. N. Wiebenga. The patient yielding the virus was a 9-year-old girl who, during the epidemic, had symptoms of headache, fever, and bone ache, of 2 days duration. All subsequent tissue culture passages were carried out in primary bovine kidney cells (BK), using Eagles-199 (1:1) medium containing glutamine and chlortetracycline (25 μg/ml). Kidneys used for preparation of tissue cultures were obtained from calves. Except for the two passages preceding preparation of the virus for use in volunteers, penicillin (200 units/ml) and streptomycin (200 μg/ml) were also added to the medium. Four virus suspensions were prepared for evaluation in the laboratory; three of these were prepared in such a manner that they were suitable for administration to volunteers. The history and designations of these materials is shown in Table I.

The low passage challenge pool was prepared after 4 BK passages at 34° and was not cloned, so as to favor retention of natural virulence.

The low temperature-adapted pool was derived from the second BK passage material by abruptly shifting the temperature of incubation from 34 to 25°. Each of the subsequent passages was carried out at 25°. At both the eighth and ninth passages in BK, an attempt was made to obtain a homogeneous virus population through use of the terminal dilution technique. During these passages, 3-fold dilutions were prepared, and each dilution was inoculated into 20 roller tube cultures. The virus subsequently used was chosen from the dilution yielding 1 to 3 hemadsorption positive tubes out of 20 inoculated. The 25° virus (BK-12) evaluated in volunteers was grown in 32-oz prescription bottle cultures and harvested after 7 days of incu-

TABLE I. Passage History of Viruses Employed.

Virus designation	Passage history			Cloned by terminal dilution	Safety tested[a]
	Human embryonic kidney (34°)	Primary bovine kidney	Egg allantois		
Original strain	HEK-1	BK-2 (34°)	—	No	No
Low passage	HEK-1	BK-4 (34°)	—	No	Yes
Low temp. adapted	HEK-1	BK-2 (34°) BK-10 (25°)	—	8th and 9th passages	Yes
Parallel passage	HEK-1	BK-12 (34°)	—	8th and 9th passages	Yes
Laboratory antigen	HEK-1	BK-2 (34°)	ALL-4 (34°)	No	No

[a] Prepared for evaluation in volunteers.

bation. The supernatant fluid and cells were pooled after sonication at 10 kc for 30 sec, and gelatin was added to a final concentration of 0.5%. The mixture was passed through an 800 mμ membrane filter to remove cellular debris, sealed in glass ampoules, and stored at —70°.

The parallel passage suspension was derived in exactly the same manner as the cold-adapted volunteer pool except that it was grown at 34°, and was passaged at 3-day intervals.

The virus suspensions destined for evaluation in volunteers were safety tested by standard techniques to detect microbial contaminants (7). The two suspensions tested in volunteers were found to be free of extraneous infectious agents.

The laboratory antigen pool was likewise derived from the original strain but was carried through four subsequent passages in embryonated eggs. During the second and third passages in eggs this virus was grown in the presence of heated horse serum to obtain a serum insensitive (S—) strain for use in measurement of neutralizing antibodies in serum and nasal secretions (8).

Virus titration. Three of the virus suspensions, including the 2 tested in volunteers, were titered several times in BK and rhesus monkey kidney (RMK) tissue culture. Serial 10-fold dilutions were prepared, and 5 roller tube cultures were inoculated with each dilution. The inoculated cultures were incubated at 34° on a roller drum and were read by the hemadsorption technique at 72 hr using 0.1% guinea pig erythrocytes.

Assay of hemagglutinin and neuraminidase. Hemagglutinin assays were performed using standard microtiter technique (9), 2-fold serial dilutions, and 0.5% chick red blood cells. The titer was considered to be the highest dilution which produced 50% agglutination of chick erythrocytes. Neuraminidase activity was measured by the method of Laver and Kilbourne on a sample of each virus pool (10).

Plaque titration. The ability of virus to produce plaques in BK monolayers at 34, 37, 38, 39, and 40°C was determined. Each strain was serially diluted from 10^{-1} to 10^{-4} and 20 plate cultures/dilution were inoculated with each sample. Adsorption was permitted for 2 hr before the residual inoculum was removed and each plate overlayed with a mixture of equal parts 2% agarose and 2× concentrated L-15 medium containing glutamine and antibiotics. Four plates/strain were incubated at each temperature for 5 days and examined periodically for plaque formation. Plaques were easily visualized without staining.

Infection of hamsters. Because their internal body temperature approximates that of man, 7-week-old Syrian hamsters were chosen as the animal model for determining the pattern of viral growth in the respiratory tract following intranasal inoculation. Hamsters were anesthetized with pentobarbital and inoculated with 2.6 to 7.5 × 10^3 pfu of virus suspension intranasally. Four animals from each group were sacrificed daily for 5 days,

and periodically thereafter for 1 month. Lung tissue from each animal was homogenized, and a 10% suspension was prepared and titered in RMK roller tube tissue cultures. Blood samples were collected periodically over a 28-day period, and these samples were used for measurement of serum CF antibody.

Clinical studies. Volunteers were chosen from healthy male inmates of the Maryland State House of Correction who were between 22 and 50 years of age. Each man was free of cardio-respiratory disease and allergy to the chlorotetracycline contained in the viral suspensions. These men lacked detectable serum neutralizing antibody ($\leq$1:2) for the Hong Kong virus, and had not received inactivated Hong Kong vaccine. Informed consent was obtained from each volunteer prior to participation in the study.

During each volunteer study, 7 to 8 men were isolated for a 2-week period on a private ward of the prison hospital. A history was taken and a physical examination was performed before virus challenge. Similarly, routine laboratory tests and chest X-rays were evaluated before volunteers were allowed to participate. The men were observed 3 days prior to virus inoculation to detect any ongoing respiratory ailment or systemic complaint. On the day of inoculation, each volunteer received the virus suspension being evaluated, by coarse, nasopharyngeal spray. A DeVilbus No. 15 atomizer was used for aerosolization, with 0.2-ml aliquots of virus being sprayed in the nose and throat periodically over a 1-hr interval. After a second hour had passed, the men were permitted to eat and drink, and move about in the hospital ward as usual. Each volunteer was examined independently by 2 physicians daily and vital signs were recorded four times a day.

Specimens for virus isolation were obtained each day for 10 days by mixing 4 ml of nasal washing and 8 ml of throat gargle. Veal infusion broth was for these collections. The combined nasopharyngeal samples were kept at 4° and inoculated within 1 hr into primary RMK roller tube cultures. After 2-hr incubation at 34°, the inoculated cultures were fed with 1.5 ml of medium [Eagles-199 (1:1), containing glutamine, penicillin (400 units/ml), streptomycin (400 μg/ml), chlortetracycline (25 μg/ml), and amphotericin (2 μg/ml)]. The cultures were incubated at 34°, examined for cytopathic effects CPE) and refed at 3–4 day intervals for 10 days. Medium from tubes demonstrating CPE was saved for subsequent virus identification. Cultures were routinely tested by hemadsorption using 0.1% guinea pig erythrocytes on the 10th day.

Blood samples were collected at weekly intervals, the serum was separated and stored at —20°, and later examined by the neutralization (neut.), hemagglutination inhibition (HI), and complement fixation (CF), techniques. Neutralization tests were done using BSC-1 tissue culture tubes, 16 to 32 tissue culture infectious $doses_{50}$ ($TCID_{50}$) of egg grown virus, and heat inactivated (56°, 30 min) serum. The cultures were examined by hemadsorption at 48 hr and serum titers were calculated by the Karber 50% end point method (11). HI tests were done in microtiter V bottom plates using receptor destroying enzyme (RDE) treated sera, and 0.5% chick RBC. CF antibody was measured in microtiter U bottom plates using 1.7 units of complement and overnight fixation at 4°.

Nasal secretion specimens were obtained prior to virus inoculation and at weekly intervals, by instilling 6 ml of phosphate buffered saline (PBS) into each nostril and collecting the expelled material in a sterile cup. Nasal washes were performed several times over a 2–3-day period and the wash fluids were pooled and stored at —20°. These specimens were concentrated by lyophilization and standardized to a concentration of 20 mg of IgA/100 ml (12). The resulting concentrated specimens were tested by the same method used for measurement of neutralizing antibody in serum.

Results. Growth of Hong Kong virus at 25°. Compared with the older influenza A2 strains that we have studied, the Hong Kong virus grew well in BK tissue culture on first passage at 25°. When the second BK passage virus grown at 34° was inoculated into BK roller tube cultures at an input multiplicity of 0.05 and the cultures were incubated at

TABLE II. Comparison of Infectivity, Hemagglutination Titer, and Neuraminidase Activity of Virus Suspensions.

Virus suspension	Infectivity titer		Hemagglutinin assay[b]	Neuraminidase activity[c]
	BK[a]	RMK[a]		
Low temp. adapted BK-12 (25°)	5.25	5.00	22.63	2.20
Parallel passage BK-12 (34°)	4.95	5.10	16.00	1.60
Low passage BK-4 (34°)	5.30	5.40	5.04	1.35

[a] Log_{10} $TCID_{50}$/ml of virus suspension performed at 34°.
[b] Reciprocal geometric mean titer.
[c] Units/ml of virus suspension.

25°, virus infection involved all cells by the third day as indicated by a complete pattern of hemadsorption. During subsequent passages at 25° there was no evidence that the virus grew more rapidly at this low temperature.

Properties of low temperature virus. As shown in Table II, the virus grown at 25° for 10 passages did not differ significantly from the low passage virus grown at 34° or the parallel passage virus grown at 34° with respect to titer of infectious virus or hemagglutinin. There were no significant differences in neuraminidase activity. The hemagglutinins of both the low passage (34°) virus and the low temperature grown (25°) virus were sensitive to inhibition by horse serum, and were considered inhibitor sensitive or S+. However, infectivity was not neutralized by undiluted horse serum.

After 10 passages at 25°, including 2 terminal dilutions, the Hong Kong virus exhibited partial temperature sensitivity, that is, its capacity to replicate at elevated temperatures was reduced when compared with the wild type or parallel passage 34° virus. In roller tube cultures of rhesus monkey kidney, the 25° grown virus had a titer which was about 100-fold lower at 39°, than at 34°, whereas the wild type virus (BK-4) had the same titer at both temperatures. Additional evidence for temperature sensitivity of the 25° grown virus is shown in Fig. 1. The plaque-forming efficiency of this virus was reduced 10^4-fold at 40°, whereas the low passage and parallel passage 34° grown viruses did not appear to be affected by an elevation of temperature to 40°. These results suggest that the growth of virus at low temperature had resulted in the emergence of a mutant which was temperature sensitive with a "shutoff" temperature between 39 and 40°.

Growth of virus in hamsters. The pattern of growth of 25° virus in the hamster lung did not differ from that seen with low or parallel passage virus grown at 34° (Fig. 2). Maximum viral replication in the lungs occurred within 24 hr, and thereafter the titer

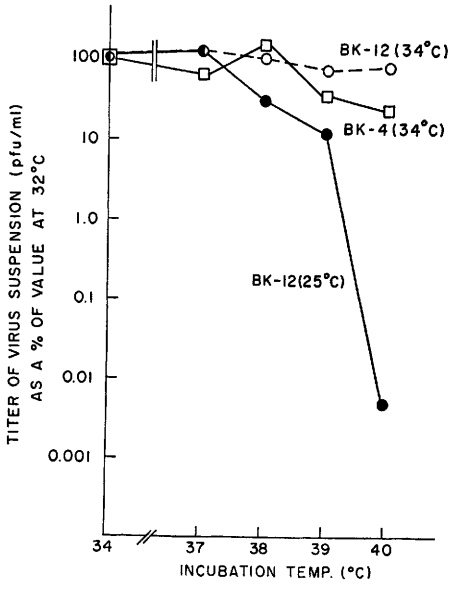

FIG. 1. Comparative plaque titers of the low passage (34°); parallel passage (34°); and low temperature-adapted (25°) A2/Hong Kong/68 virus strains incubated at different temperatures.

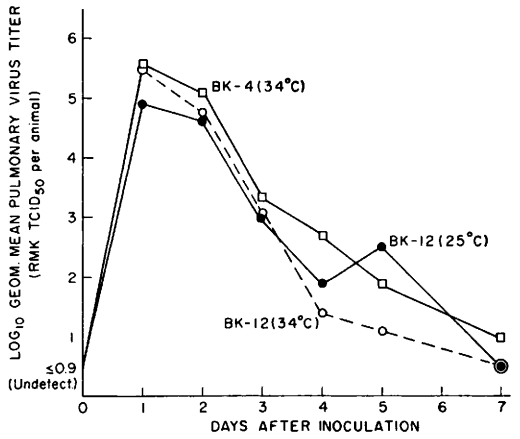

FIG. 2. Pattern of growth of the low passage (34°), parallel passage (34°), and low temperature-adpated (25°) A2/Hong Kong/68 virus strains in the hamster lung.

decreased over a 5–6-day interval. The behavior of the 25° virus was not unexpected since the shutoff temperature of this virus is 39–40°, whereas the temperature of the hamster lung is 37°. Serologic response to infection with the 3 viruses was identical with respect to time of first appearance of serum CF antibody and maximum titer of antibody which developed.

Evaluation of low passage and low temperature grown viruses in seronegative volunteers. Men who lacked detectable serum neutralizing antibody ($\leq$1:2) were selected for evaluation of the infectivity and virulence of the low temperature virus (Table III). When 10^3 $TCID_{50}$ of virus were given by nasopharyngeal spray to 7 volunteers, definite evidence of infection was not observed. Following administration of 10^5 $TCID_{50}$, one volunteer shed virus on the first day after challenge, while another man developed a serum antibody response detected by the neutralization, HI, and CF techniques. Neither volunteer exhibited any signs or symptoms of illness.

In contrast, when 10^6 $TCID_{50}$ were given, each of the 8 volunteers was infected and 5 became ill. These illnesses were characterized by fever, coryza, headache, conjunctival itching, and nasal discharge as shown in Fig. 3. Symptons began 48 hr after vlrus challenge and lasted from 1 to 4 days. Three of

TABLE III. Response of Volunteers, Lacking Detectable Serum Antibody, to Different Concentrations of Low Temperature Adapted A2/Hong Kong/68 Virus.

Virus inoculum $\log_{10}$ $TCID_{50}$[a]	Men tested	Men with influenzal illness	Men (N) with objective evidence of infection						Maximum geometric mean fold antibody rise				Men with evidence of infection
				≧4-Fold antibody response									
						Serum				Serum			
			Virus recovery	Nasal secretion	Neut.	HI	CF		Nasal secretion	Neut.	HI	CF	
	N	N											N
3.0	7	0	0	0	0	0	0		1.2	1.8	2.0	1.4	0
5.0	8	0	1	0	1	1	1		1.2	1.4	1.5	1.2	2
6.0	8	5	8	6	7	7	5		9.4	23.3	9.5	3.1	8

[a] Determined in RMK tissue culture.

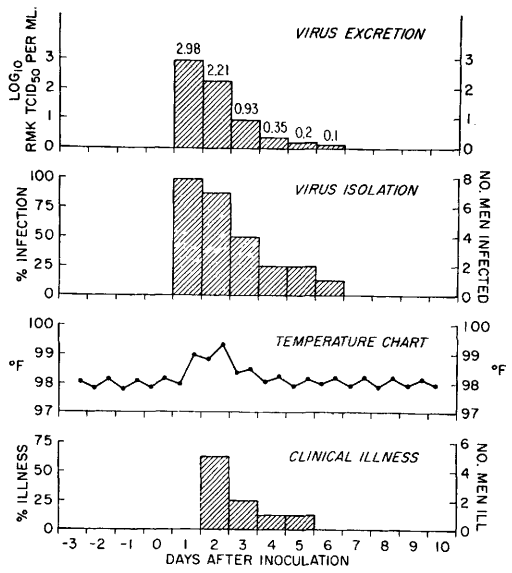

FIG. 3. Response of volunteers to 10^6 $TCID_{50}$ of low temperature-adapted A2/Hong Kong/68 virus strain.

these volunteers had temperature elevations which exceeded 100°F; the highest temperature observed was 101.2°F. Six of the men given 10^6 $TCID_{50}$ of virus developed a fourfold or greater rise in nasal secretion antibody, while 7 had a serum antibody response which was greatest when measured by the neutralization technique. The composite response of the volunteers is shown in Fig. 4. If one applied the method of Karber to the infectivity results shown in Table III, it appeared that the 50% human infectious dose for the low temperature virus was $10^{5.3}$ rhesus monkey kidney $TCID_{50}$.

These findings suggest that the cold-adapted Hong Kong virus was not attenuated. Although the virus exhibited low infectivity for susceptible individuals, when sufficient virus was given to infect all volunteers, they developed mild, but definite influenzal illness.

In order to be able to view the above observations in perspective, a similar study was performed with the low passage 34° grown virus. This virus had been grown only at 34° and had not been purified by terminal dilution. A total of 10 seronegative volunteers were given $10^{1.0}$ to $10^{5.0}$ $TCID_{50}$ of virus by nasopharyngeal spray. As shown in Table IV, infection was not produced until $10^{5.0}$ $TCID_{50}$ of virus were given. Each of the 3 volunteers given this dose of virus was infected and became ill. The estimated 50% human infectious dose was $10^{4.4}$ rhesus monkey kidney $TCID_{50}$.

Discussion. The technique of using low temperature adaptation to select an attenuated mutant of influenza virus for vaccine use was first employed in the Soviet Union in 1964 (13), but adaptation to growth at a suboptimal temperature as a means of reduc-

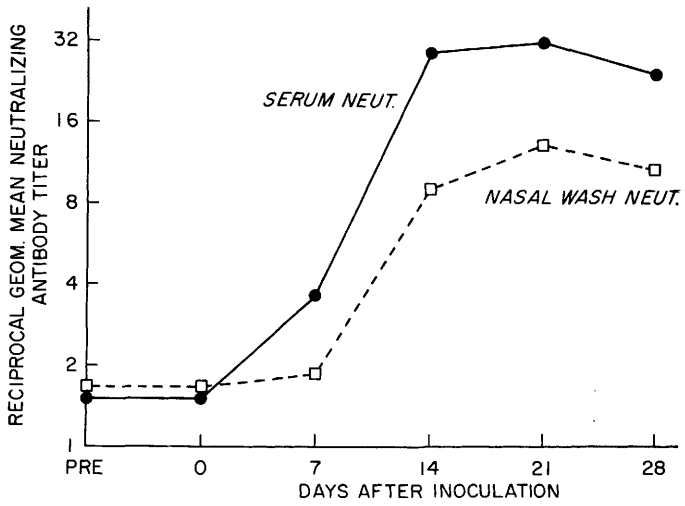

FIG. 4. Composite antibody response to volunteers given 10^6 $TCID_{50}$ of low temperature-adapted A2/Hong Kong/68 virus strain.

TABLE IV. Response of Volunteers, Lacking Detectable Serum Antibody, to Different Concentrations of Low Passage A2/Hong Kong/68 Virus.

Virus inoculum $\log_{10}$ $TCID_{50}$[a]	Men tested *N*	Men with influenzal illness *N*	Men who excreted virus *N*	Men with ≥4-fold serum neut. antibody rise *N*	Men with evidence of infection *N*
1.0	2	0	0	0	0
2.3	3	0	0	0	0
3.7	2	0	0	0	0
5.0	3	3	3	3	3

[a] Determined in RMK tissue culture.

ing the virulence of a virus for its host had been shown earlier to be feasible with type 3 poliovirus (3), and later with Japanese B encephalitis virus (4) and measles virus (5). Subsequently, Maassab showed that influenza A virus adapted to growth at 25°, either in embryonated eggs or primary chick kidney tissue culture, lost virulence for mice and ferrets when given intranasally, but produced a satisfactory serum antibody response (14). The growth of these cold-adapted strains was partially inhibited at 40°, unlike the wild-type virus which grew normally at this temperature. Because of these findings, we undertook the production and subsequent testing of a low temperature-adapted influenza strain in humans shortly after the new variant of A2 influenza was isolated in Hong Kong during July of 1968.

A cold-adapted strain of Hong Kong influenza A2 virus was produced by passage and terminal dilution at 25°, and its titer at 40° was found to be less than 0.01% of its titer at 34°, in contrast to the wild-type and parallel-passage virus strains studied, which grew about as well at both temperatures. Thus, our low temperature-adapted strain, like those studied by Maassab, had acquired a temperature-sensitive (*ts*) defect. The mutant derived in this study appeared to be "leaky" in that it was partially able to replicate at the restrictive temperature. The finding that the cold-adapted (25°) virus was not attenuated for man was surprising, in one sense, because the $rct^{-}{}_{40}$ marker (restricted growth at 40°) had been shown by Maassab and others to correlate with attenuation of influenza and other viruses for experimental animals (3–6). On the other hand, the pathogenicity of the low temperature grown Hong Kong strain for man was consistent with our experience with this virus in hamsters, where its pattern of growth in the lungs was identical with that of the unadapted, wild-type virus.

Previous work with *ts* mutants of influenza virus has shown a correlation between the height of the "shutoff" temperature and virulence (or ability to replicate) in experimental animals (15, 16). Mutants with the lowest "shutoff" temperature, *i.e.*, the lowest temperature at which restriction of growth occurred exhibited the most attenuation or restriction of growth in the lungs. The results of this study are consistent with this correlation in that the low temperature-adapted strain was a *ts* mutant with a shutoff temperature between 39 and 40°, somewhat above the normal body temperature of man. Since the replication of this virus was not inhibited at 37°, one might expect the low temperature-adapted strain to be minimally attenuated, if at all, on the basis of a restrictive effect of temperature. Although the low temperature-adapted strain appeared to be only 10% as infectious for man as the "wild type," clinical influenza developed when a sufficient quantity of the mutant was given to produce infection. If the *ts* marker is found to be a reliable index of attenuation, then selection of strains with the desired "shutoff" temperature, *i. e.*, 37°, might be made more easily by isolation of *ts* mutants with the desired degree of temperature sensitivity from a mutagenized stock of virus, than by attempting to select mutants by the indirect technique of cold adaptation. Encouraging results have

been obtained by pursuing the former course, and the results have been described recently (17).

Summary. A strain of A2/Hong Kong/68 virus was adapted to growth at 25° in calf kidney tissue culture and a virus suspension was prepared for administration to volunteers after a total of 10 passages at 25°, including 2 terminal dilution purifications. The low temperature grown virus acquired a temperature sensitive defect which restricted its growth at 40°, a temperature at which the wild-type virus grew without restriction. The low temperature-adapted virus appeared to exhibit reduced infectivity for man, but was not attenuated since illness occurred when sufficient virus was administered to infect all volunteers.

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Received July 30, 1970. P.S.E.B.M., 1971, Vol. 136.