Hybrid Formation of Influenza Virus at 25°[1] (35234)

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Attenuation of influenza virus for potential use in man as live vaccine has been achieved by adaptation of the virus to growth at 25° (1, 2). Virus was cold-adapted by gradually lowering the incubation temperature (1) or more rapidly by a plaque technique with abrupt changes in temperature (3). These "cold variants" were shown to be attenuated and antigenic for animals and in limited clinical trials for man (3, 4). However, these processes took from four to eight months to produce nonreactive but immunogenic strains. Further "speed up" in development of candidate vaccine lines seemed desirable.

It is well known that certain characteristics of influenza viruses can be transferred between different strains by genetic recombination. Kilbourne has twice demonstrated that the growth capacity in embryonated eggs at 35° of an old-established strain such as PR8 can be transferred to a new strain (5, 6). It seemed likely, therefore, that under proper conditions it should be possible to confer by genetic interchange the growth potential of a previously attenuated cold-adapted strain to a new antigenic variant. Such an accomplishment would considerably shorten the interval between virus isolation and delivery of a suitable seed for live virus vaccine production. The present report deals with the development of such attenuated "cold hybrids" in about four weeks.

Materials and Methods. Viruses. Strains of virus used in the recombination experiments were: A_0/PR/8/34 strain, referred to as "wild type", had numerous ferret passages, 593 mouse passages and 176 egg passages. The "cold variant" PR8 was derived from the "wild" strain by the procedure published elsewhere (2). Hong Kong influenza virus, strain A_2/England/878/69 was obtained as the second egg passage from the World Influenza Centre, Mill Hill, England. A/Equi-2/Miami/63 virus was obtained as the fourth egg passage from Dr. M. M. Sigel of the University of Miami.

Preparation and titration of viruses. Procedures used to prepare primary chick kidney cells and for the growth of influenza virus at various temperatures have been described previously (7). The amount of virus was estimated by determining the infectious titer for eggs using the standard method (8) or by measuring the LD_{50} when 12 g Swiss Webster white mice were inoculated intranasally. The 50% infectivity was calculated by the method of Reed and Muench (9). Virus infectivity was measured by plaque assay in chick kidney monolayers (10).

Serological tests. Hemagglutinin titrations and hemagglutination-inhibition tests (HI) were performed in plastic trays (Perspex trays as used by the World Influenza Centre) according to standard methods (10). Sera to be used in HI were routinely inactivated at 56° for 30 min. Neuraminidase inhibition (NI) tests were performed as previously described using human serum Cohn-fraction IV-4 as the enzyme substrate (1). Usually infected allantoic fluid, at the appropriate dilution, was used as the source of antigen in these tests. The specific immune sera used were prepared either in ferrets by intranasal instillation of approximately 2.0

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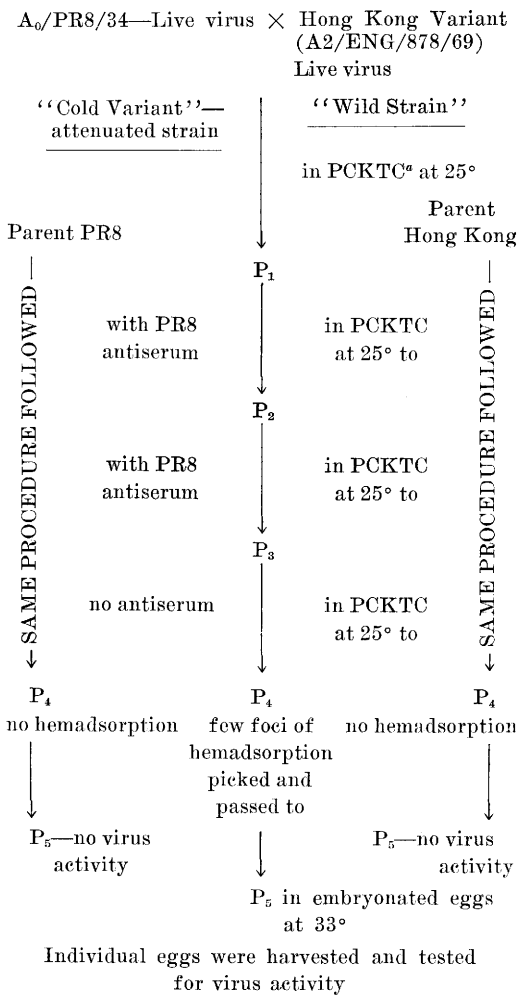
ml of 10^{-4} dilution of the strain listed, or in mice by intranasal instillation of 0.05 cm^3 of 10^{-3} dilution. The animals were bled two weeks after infection.

Results. Recombination of human influenza strains at 25°. The procedures found successful for producing antigenic hybrids which grow at 25° are summarized in the flow diagram. Primary chick kidney cells were

FLOW DIAGRAM.



[a] PCKTC—Primary Chick Kidney Tissue Culture.

infected simultaneously at 25° with $10^{5.0}$ EID_{50}/ml of two live influenza virus strains, an attenuated "cold variant" A_0/PR/8/34 and the "wild" Hong Kong strain A_2/Eng/878/69. Progeny derived were passed twice serially in tissue culture with PR8 antiserum and twice without antiserum and finally in embryonated eggs. Allantoic fluid harvest from this passage was positive by hemagglutination (HA). The presence of PR8 antiserum was necessary to prevent recovery of the infectious PR8 cold line from the initial inoculum. Each parent line treated separately in the same way failed to yield virus.

Antigenic characterization. The virus recovered from the mixed infection experiment was characterized antigenically by HI and NI tests. As shown in Table I, the virus derived at 25° exhibited predominantly the HA of the Hong Kong virus and the neuraminidase of the attenuated PR8 "cold variant." Clearly the virus derived is an antigenic hybrid. The low level of reciprocal cross reactions by HI and PR8 may be due either to the common neuraminidase or to small amounts of common PR8 HA antigen. The former seems more likely.

Biological characteristics of the recombinant. In contrast to the parent HK virus the recombinant virus has acquired the capacity to produce plaques in primary chick kidney at 25°. The growth of the hybrid virus at 25° was investigated in detail. The results of a typical experiment are presented in Fig. 1. The parent Hong Kong virus shows one cycle of growth with a slight increase of approximately two logs reached at the 4th day after infection. However, the hybrid virus and its parent "cold variant" exhibited comparable growth patterns with gradual increase in titer reaching a maximum at the 6th day. The same growth pattern is obtained when HA titer is determined. Additional properties acquired by the hybrid from its PR8 parent was temperature sensitivity defined as inability to grow at 40° (the nonpermissive temperature). The hybrid virus (hereinafter referred to as "cold hybrid", CH_I) was also capable of vigorous growth in mouse lung. The results of a typical mouse infectivity experiment, employing all three lines of virus under investigation (Table II), demonstrate

TABLE I. Antigenic Characterization of the Recombinant and Parental Viruses.

Virus strains	Ferret antiserum to PR8 HI[a]	PR8 NI[b]	A_2/England HI	A_2/England NI	$A_0 \times A_2$ HI	$A_0 \times A_2$ NI
A_0/PR8/34 ("Parent-Cold Variant")	1024	200	<10	<20	<8	760
A_2/Eng/878/69 ("Parent-Wild")	<10	<20	1024	430	512	<20
$A_0 \times A_2$-CK_4-E_1 (Recombinant)	16	80	512	<20	1024	1020

[a] HI = Hemagglutination-Inhibition titer. [b] NI = Neuraminidase-Inhibition—dilution of the serum which inhibits 50% of standard dose of enzyme activity.

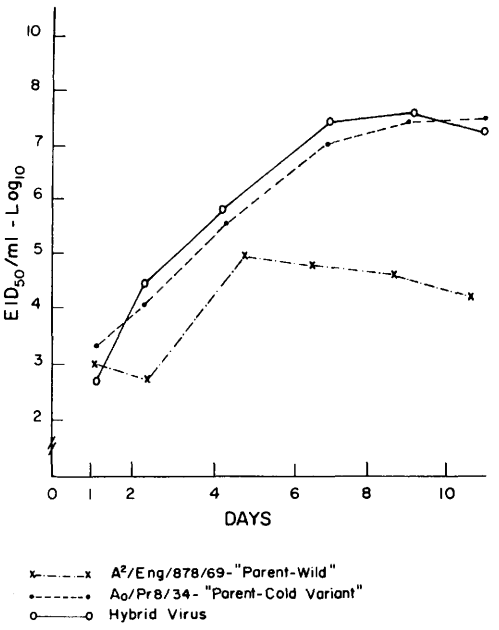


FIG. 1. Growth patterns of the hybrid virus and the parental strains in tissue culture at 25°.

that the growth capacity of the attenuated "cold variant" PR8 in mouse lung was transferred to the "cold hybrid" by recombination. There was no appreciable difference in pulmonary virus titer when mice were infected with the hybrid virus, the attenuated PR8 parent line and the original mouse adapted PR8 strains, whereas the growth of the parent HK virus was less. However, as expected, the hybrid virus and its parent "cold variant" were attenuated when compared to the original mouse adapted PR8 as shown in the last column which gives the titer of viruses in MLD_{50} at the 10th day post infection. The antigenic and biological properties of CH_1 virus described above persisted after repeated passages in embryonated eggs or in tissue culture at 25 and 33°. After five serial plaque purifications at 25° the properties of the "cold hybrid" virus also appeared unchanged.

Attenuation and immunogenicity. Previous work in our laboratory has demonstrated that cold-adapted influenza viruses with

TABLE II. Growth of the "Cold Hybrid" and the Parental Strains in Mice.

Virus strain	Virus content of mouse lung at[a] 12 hr	2 days	4 days	Mouse lethal[b] dose$_{50}$ (MLD_{50})
A_0/PR8/34 "Cold Variant"	2.5	7.0	6.7	$10^{1.3}$
A_2/Eng/878/69	2.0	4.0	3.5	0
"Cold Hybrid"	2.7	6.7	6.5	$10^{1.0}$
A_0/PR8-"Wild Mouse-Adapted"	3.0	7.7	7.5	$10^{7.0}$

[a] Titer expressed as $\log_{10}$ EID_{50}/ml and represents the content of virus from lungs of 4 animals.

[b] The titer expressed at the 10th day after infection.

TABLE III. Comparison of the Response in Ferrets to Injection with the "Cold Hybrid" CH_1 and the Parental Strains.

Virus strains	Virus dose[a] inoculated	Virus excretion	Homologous HI titer[b]	Clinical response fever-coryza
A_0/PR8/34 "Cold Variant"	$10^{5.0}$	3/4	1024	0/4[c]
A_2/Eng/878/69	$10^{4.5}$	4/4	512	4/4
"Cold Hybrid"	$10^{5.0}$	2/4	1024	0/4
A_0/PR8 "Wild" Mouse Adapted	$10^{4.0}$	4/4	1024	4/4

[a] Log_{10} EID_{50}/ml.

[b] Hemagglutination-Inhibition—ferrets bled 15 days post-infection.

[c] Numerators are number of responses, denominators are number inoculated.

stable genetic markers are avirulent for experimental animals and highly antigenic (2, 12). Pools of plaque purified "cold hybrid" and of the parental viruses were prepared in eleven day embryonated eggs and a dose ranging from $10^{4.0}$–$10^{5.0}$ EID_{50}/ml was given to ferrets intranasally. Nasopharyngeal swabs were taken daily from each of the ferrets for 5 days. Clinical response manifested by fever or coryza was recorded and each ferret was bled before and 15 days after infection. Table III shows that the derived CH_I virus and the parental attenuated live A_0/PR/8/34 infected the ferrets since high HI antibodies titer were observed; however, the cold lines elicited no clinical response in the ferrets in contrast to ferrets infected with the "wild" parental influenza viruses. From the findings, one may conclude that the "cold hybrid" virus was avirulent for ferrets while the wild types exhibited characteristic evidence of virulence. The results definitely demonstrate that attenuation was transferred by recombination.

Immunogenicity of CH_I virus was tested in mice. Groups of animals were infected with 10^{-2} dilution of the strains shown in Table IV. Two weeks later some animals were bled for antibody determination and the rest challenged with $10^3 MLD_{50}$/ml of the mouse-adapted strains. The response of mice to infection with the lines tested was excellent in terms of antibody titer and protection upon challenge with the virulent strains containing the homologous hemagglutinin. Mice infected with the "cold hybrid" virus were also partially protected against infection with mouse-adapted PR8 strain. This finding is probably due to neuraminidase shared by these viruses. The observation is very similar to that reported previously in cross-protection experiments in mice by Schulman, *et al.* (13) and in chickens by Allan, *et al.* (14).

Recombination of human and equine strains at 25°. Using the procedure described above, a second "cold hybrid" (CH_{II}) was derived by interaction between animal and human influenza viruses. Recombination was initiated by mixedly infecting primary chick

TABLE IV. Protection of Mice Immunized with the "Cold Hybrid" and the Parental Strains.

Mice infected with	HI[a] Response to PR8	HI[a] Response to A_2/Eng	HI[a] Response to CH_1	Mortality[b] Ratio after challenge with PR8 Mouse-adapted	Mortality[b] Ratio after challenge with HK Mouse-adapted
A_0/PR8/34 "Parent-cold variant"	1024	<8	16	0/10	10/10
A_2/Eng/878/69 "Parent-wild"	<8	512	1024	10/10	0/10
"Cold hybrid" CH_1	<8	1024	1024	3/10	0/10

[a] Hemagglutination-Inhibition. Mice were bled 15 days post-infection.

[b] 15 days after infection, mice were challenged with $10^3 EID_{50}$/ml dilution of the mouse-adapted viruses. The ratio represents the number of mice dead over the number inoculated.

TABLE V. Biological Characteristics of Equi-2 "Cold Hybrid" (CH_{II}).

Virus	Antigenicity		PFU[b]/ml at	Pulmonary Virus Content[c] at
	HA	NA[a]	25°	48 hr
PR8 "Parent-Cold Variant"	PR8	PR8	2×10^7	7.3
Equi-2 "Parent-Wild Strain"	Equi-2	Equi-2	No Plaques	3.0
Hybrid strain (CH_{II})	Equi-2	PR8	1.5×10^7	6.3

[a] NA—Neuraminidase.

[b] PFU—Plaque forming unit.

[c] Titer expressed as log to base$_{10}$ EID_{50}/ml and represents the content of virus from lungs of 4 animals 48 hr post-infection.

kidney cells with A/Equi-2/Miami/63 (the "wild" parent) and the attenuated "cold variant" A_0/PR/8/34. The results of biological characterization of this second "cold hybrid" are shown in Table V. The hybrid virus CH_{II}) possesses Equi-2 hemagglutinin and PR8 neuraminidase. Again, like the previous CH_I virus, this derived hybrid CH_{II} grows and forms plaques in chick kidney cells at 25° while the "wild strain" does not. The "CH_{II}" virus also exhibits vigorous growth in mouse lung. There is approximately three logs difference in titer when pulmonary virus content at 48 hr is compared between mice infected with the hybrid strain and the one infected with its Equi-2 parent. Clearly, the derived strain has acquired the capacity to grow in mouse lung from its parent, the PR8 "cold variant." Table VI shows that the hybrid line is also temperature sensitive. A pool of each line of virus listed in the table was titrated at 25, 35, and 40°. The infectious titer of the "wild strain" was comparable at 35 and 40°, and as expected the titer was much lower at the incubation temperature of 25°. The parent "cold variant" and the derived "cold hybrid" virus failed to grow at the nonpermissive temperature of 40° while the infectious titer was comparable for both lines at 35 and 25° incubation. Studies are planned to evaluate the virulence and immunogenicity of the derived Equi-2 hybrid in horses for possible use as live vaccine.

Conclusion. The present findings show that the ability of influenza virus to grow at 25°, temperature sensitivity, growth in mouse lungs and attenuation are stable characteristics which can be transferred between influenza viruses by recombination. This suggests the probability that these viral properties are genotypic and may prove useful for studies of genetic characteristics of influenza viruses. The procedure is to select through genetic interaction, at 25°, a "cold hybrid" of the current epidemic strain, by suppression of the parental attenuated "cold variant" with specific antiserum.

Various investigators have shown that certain markers of influenza viruses can be rescued or transferred by recombination between active and inactivated strains or between two live strains; this includes enhancement of viral yields (5, 6), plaque forming

TABLE VI. Effect of Different Temperatures on the Infectivity Titer of the "CH_{II}" Virus and its Parental Strains.

Virus Strain	Infectious Titer (log to base$_{10}$ EID_{50}/ml) at		
	25°	33°	40°
PR8 "Parent-Cold Variant"	8.0	8.3	2.5
Equi-2 "Parent-Wild Strain"	4.0	7.7	7.3
Hybrid Strain (CH_{II})	7.3	7.0	2.3

capabilities (15, 16), difference in host range (17) and neurovirulence (18, 19). However, in the latter case, the neurovirulent marker was unstable. The present report has furnished yet another method for studies of genetic interactions between two live influenza viruses at suboptimal temperature. In addition, transfer of attenuation to the epidemic strain by recombination may permit development of a suitable live influenza virus vaccine for use in man in a short time.

Summary. It is clear that "cold hybrids" of influenza virus can be obtained at 25° by recombination of two live influenza virus, of human and animal origins, one of which is cold-adapted. The presently described "cold hybrids" contain the hemagglutinin of the current human or equine strains and the neuraminidase of the established "cold variant". These derived lines can grow at 25° in tissue culture and embryonated eggs and are also able to form plaques at 25°. They can grow in mouse lungs and are temperature sensitive. The Hong Kong "cold hybrid" (CH_I) is attenuated for animals and highly immunogenic. Since only about four weeks were required to obtain these strains, it is evident that the procedures used offer potential practical advantages for production of live influenza vaccine strains.

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