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Behavior of Vaccine Revertants of Temperature-Sensitive Mutants of Influenza Virus in Ferret Tracheal Organ Culture

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A live attenuated influenza vaccine candidate was not genetically stable when administered to some children who lacked antibody to surface proteins of the virus. To obtain additional biological information about these revertants, the vaccine strain, the wild-type parental strain, and isolates recovered from inoculated children during a vaccine trial were evaluated in ferret tracheal organ culture for effects on the ciliated epithelium and replication at both permissive and restrictive temperatures. The studies revealed that the vaccine strain destroyed cilia and replicated to high titer at its permissive temperature (33°) but caused minimal damage and replicated to very low titer at its restrictive temperature (37°C). The wild-type parent destroyed cilia at both 33 and 37°C. Isolates which were no longer temperature sensitive (ts^+) destroyed cilia at both restrictive and permissive temperatures and grew to high titer. Isolates which retained the *ts* phenotype behaved as the vaccine strain in this system. The ts^+ virus recovered from volunteers behaved like the wild-type parent, which suggests that these viruses had not merely lost their *ts* phenotype, but had undergone reversion to wild type. Important information about the genetic stability of temperature-sensitive influenza vaccine strains recovered from volunteers can be obtained by evaluating them in ferret tracheal organ culture.

A/Hong Kong/68-*ts*-1[E], a live attenuated influenza vaccine candidate, was not genetically stable in young children who lacked antibody to the two surface proteins of the virus (1-3, 5). This provided us an opportunity to compare the behavior of revertant viruses isolated from volunteers to that of the original vaccine virus in ferret tracheal organ culture at permissive (33°C) and restrictive (37°C) temperatures. We have previously reported that A/Hong Kong/68-*ts*-1[E], a virus with a shutoff temperature for plaque formation of 38°C, destroys ciliated epithelium in this system and grows to high titer at 33°C (permissive nasal temperature), but that it does not damage cilia and grows to very low titer at 37°C (restrictive lung temperature) (4). Wild-type viruses destroy cilia and grow to high titer at both 33 and 37°C. Since it was unknown whether the ts^+ (revertant) virus had merely lost the *ts* phenotype or whether it also had lost its attenuated characteristics, it was evaluated in the ferret tracheal organ culture system.

MATERIALS AND METHODS

Viruses. The preparation and characterization of the vaccine strains and parental wild-type were previously described (7). The isolation of the revertant strains has also been described (1). The infectious dose of virus used to infect the ferret organ cultures (10^4 50% tissue culture infective doses [$TCID_{50}$]) was similar to the dose of A/Hong Kong/68-*ts*-1[E] given to the children, $10^{3.5}$ to $10^{5.5}$ $TCID_{50}$.

Preparation of tracheal organ cultures. Tracheal rings from 4- to 8-week-old ferrets were prepared, and ciliary activity was graded by techniques previously described (8). Each 1 ml of L-15 medium contained 1% L-glutamine (final concentration), 100 U of penicillin, and 20 μg of gentamicin.

Inoculation of virus. Utilizing methods previously described (4), 10^4 $TCID_{50}$ of each test virus per 0.2 ml was introduced into each of eight tubes. Four tubes were incubated at 33°C, and four tubes were incubated at 37°C. After a 2-h adsorption, each ring was washed three times, 1 ml of fresh appropriately prewarmed L-15 medium was added, and tubes were rolled at 33 and 37°C, respectively. At 24- and 48-h intervals thereafter, supernatants were harvested, and fresh medium, warmed to the appropriate temperature, was added.

Estimation of ciliary activity. Before inoculation, each ring was observed through an inverted microscope (100×) and the percentage of each ring with actively beating cilia was estimated. Rings with less than 80% of the inner circumference actively beating were discarded. Twenty-four hours after inoculation and every other weekday thereafter, the percentage of the ring containing beating cilia was recorded. At the conclusion of the experiment, each day's activity was divided into the baseline reading (day 0) and

expressed as percent ciliary activity. To prevent bias, all tubes were given a code number so the observer was unaware of which ring contained which virus.

Harvesting and titration of virus. On alternate days, the entire 1 ml of supernatant was removed, pooled with the three other companion supernatants for each group, mixed with Hanks balanced salt solution containing 0.5% albumin, and stored at −70°C. Log_{10} dilutions of each pool were inoculated into three replicate tubes of primary rhesus monkey kidney cells inoculated at 33°C and read for hemadsorption on day 6. Titers were expressed as log_{10} $TCID_{50}$ per milliliter.

RESULTS

The A/Hong Kong wild-type parent destroyed cilia at both 33 and 37°C (Fig. 1). This was accompanied by growth to high titer at both temperatures (Fig. 2). A/Hong Kong/68-*ts*-1[E], the vaccine strain given to the children, also destroyed cilia at its permissive temperature (33°C), but caused only minimal and late damage at 37°C (Fig. 1). This virus grew to high titer at 33°C but only to very low titer at 37°C (Fig. 2). This virus has a shutoff temperature for plaque formation of 38°C.

The virus isolate KM461 destroyed cilia in a manner similar to the wild-type parent of the vaccine strain whereas virus isolate OR329 behaved similarly to the vaccine strain. The KM461 strain grew equally well at 33 and 37°C (Fig. 4), whereas OR329 only grew at 33°C. In further tests in tissue culture, KM461 was a ts^+ (revertant) virus, whereas OR329 retained the *ts* phenotype (2).

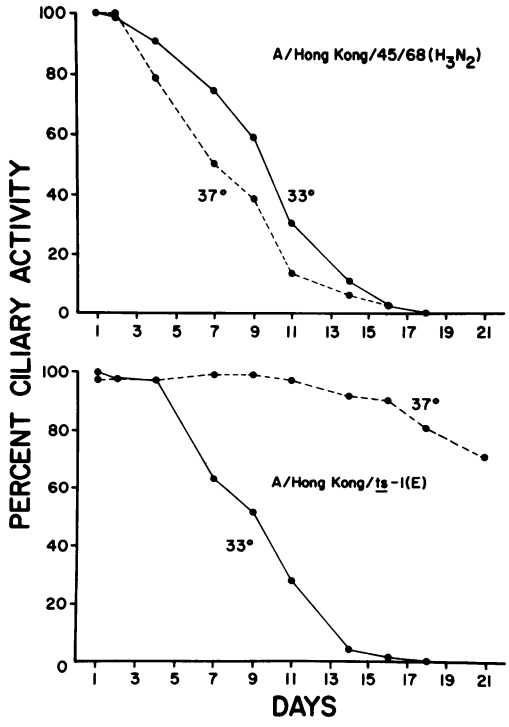

FIG. 1. *Wild-type parent, A/Hong Kong/45/68 (H3N2), destroys ciliated epithelium at both 33 and 37°C. A/Hong Kong/68-ts-1[E] vaccine strain destroys cilia at 33°C but causes only minimal damage at 37°C.*

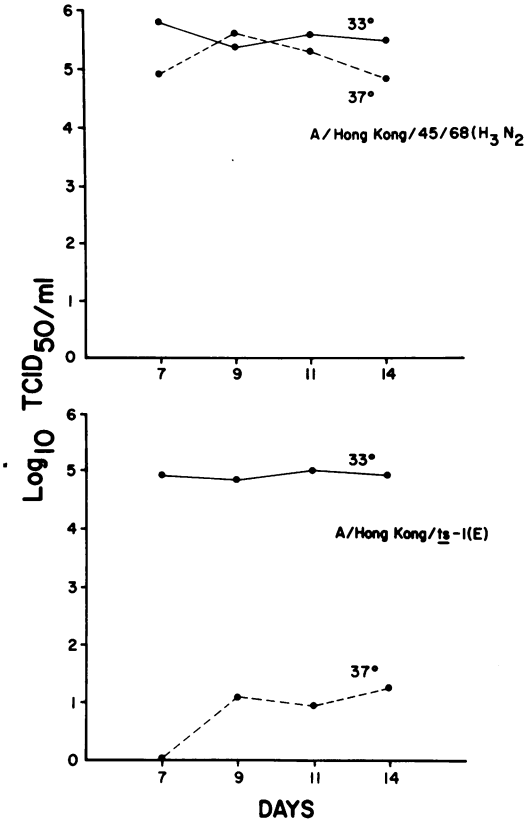

FIG. 2. *Wild-type parent grows to high titer in the ciliated epithelium at both permissive and restrictive temperatures. Vaccine strain only grows to high titer at 33°C.*

The virus strains TR463, EM259, and IS488 all destroyed cilia at both 33 and 37°C, whereas isolate OO462 destroyed cilia only at 33°C (data not shown). The TR463, EM259, and IS488 strains, all ts^+ in separate tests, grew to high titer in the ferret tracheal organ culture system at both permissive and restrictive temperatures, but the OO462 strain only grew at the permissive temperature (retained the *ts* phenotype).

Sham-inoculated controls showed minimal loss of ciliary activity for a 3-week period at 33 or 37°C, and no virus was recovered (Fig. 5).

DISCUSSION

The appearance of several revertant (ts^+) vi-

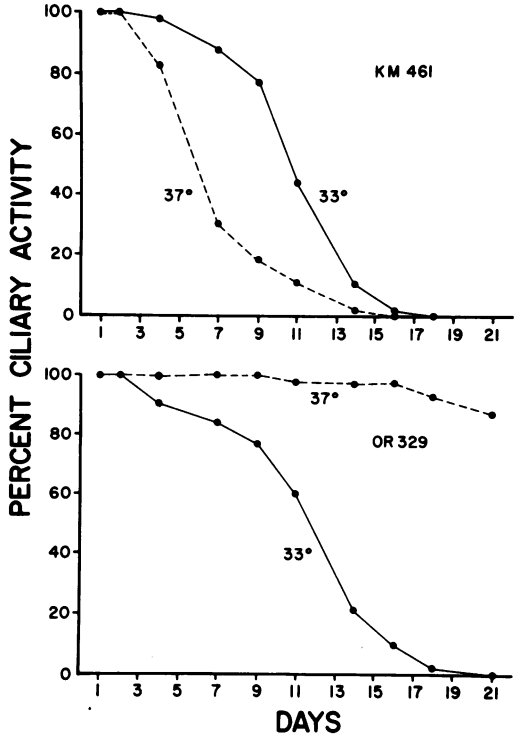

FIG. 3. *KM461, a virus isolated from a child during the vaccine trials, destroys cilia at both 33 and 37°C. This is similar to the wild-type virus. OR329, a virus isolated from another child during the trials, only destroys cilia at 33°C.*

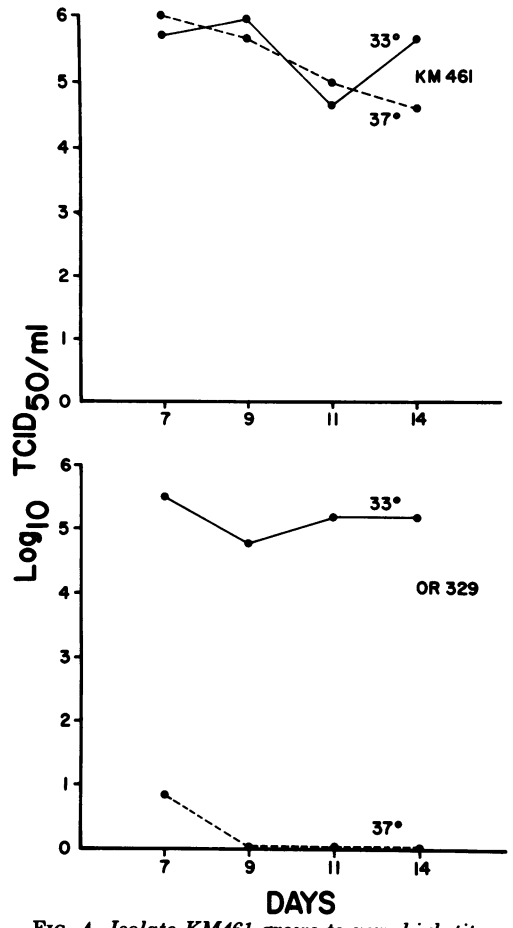

FIG. 4. *Isolate KM461 grows to very high titer at both 33 and 37°C; the OR329 isolate only grew at 33°C.*

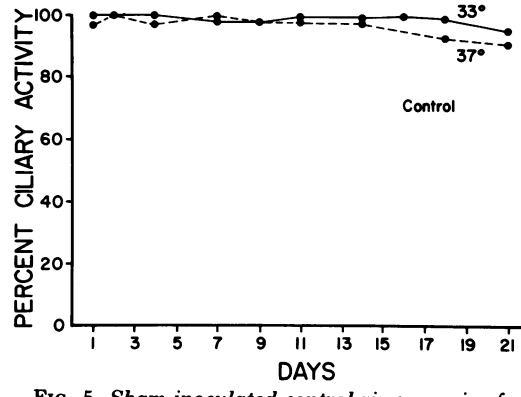

FIG. 5. *Sham-inoculated control rings survive for long periods of time at both 33 and 37°C.*

ruses from 4 of 14 doubly seronegative children who shed A/Hong Kong/68-*ts*-1[E] virus was unexpected. Revertant virus was shed on day 6 or later of virus infection and was not associated with illness (1). The absence of anti-neuraminidase antibody (adult volunteers generally have this antibody) directed to this H_3N_2 virus is one potential factor in permitting a high-titered and prolonged infection in the children which increases the likelihood of emergence of genetically altered virus. The fact that the ts^+ virus behaved in a similar manner to the wild-type parent in ferret tracheal organ culture suggests that these viruses have not undergone partial reversion but in fact have completely reverted to wild-type viruses. Although tests in ferret tracheal organ culture were not done at temperatures above 37°C (these are now in progress), the fact that the virus always caused earlier damage at 37 than at 33°C suggests the ts^+ virus is similar to wild-type viruses which cause early damage at 37°C in this system.

The ability to rapidly and predictably transfer the *ts* marker(s) of attenuation to potentially epidemic wild-type influenza virus makes this method of producing vaccines very attractive. However, the stability of the *ts* lesion remains a potential problem. Currently, new donors of the *ts* lesion are under development. It appears that

these viruses may be more stable in doubly seronegative children (6). However, since requirements for future attenuated live virus vaccines will probably include genetic stability in individuals who lack antibody to the hemagglutinin and neuraminidase, further in vitro tests such as the ferret tracheal organ culture need to be developed and evaluated.

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