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Hemagglutination inhibition antibody
influenza A virus
intranasal
local antibody
recombinant virus
serum antineuraminidase antibody
vaccine, live

Temperature-sensitive Mutants of Influenza A Virus: Response of Children to the Influenza A/Hong Kong/68-ts-1[E] (H3N2) and Influenza A/Udorn/72-ts-1[E] (H3N2) Candidate Vaccine Viruses and Significance of Immunity to Neuraminidase Antigen

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Extract

The influenza A/Hong Kong/1968-ts-1[E] (H3N2) candidate live virus vaccine strain, which had previously been shown to be safe and protective in seronegative adult volunteers, was administered intranasally to 21 children at a dose of 10^5 $TCID_{50}$. One group contained 15 children (5–11 years of age) who lacked serum antibody to the hemagglutinin ($\leq$1:8), but possessed serum antibody to the neuraminidase antigen. The second group included six children ($2^4/_{12}$–$3^1/_{12}$ years of age) who lacked serum antibody to both hemagglutinin and neuraminidase surface antigens of the influenza A virus. Twelve of the 15 children in the first group were infected, but only one child developed mild rhinitis; 6 of the 12 infected vaccinees shed virus for a short interval, while 11 of the group developed

an immunologic response. In contrast, each of the six vaccinees who lacked serum antibody for both surface antigens of the virus shed a relatively larger quantity of virus over a longer interval than those in the first group, and each child developed an immunologic response. Four of the doubly seronegative vaccinees developed a febrile response during their infection and three had rhinitis. Two additional children who lacked serum antibody to both surface antigens of influenza virus were given the influenza A/Udorn/1972-ts-1[E] recombinant virus which had the same genetic temperature-sensitive (ts) lesions as the Hong Kong virus but possessed a more contemporary hemagglutinin. This recombinant virus infected both of the children and induced transient fever in one. None of the total of 23 vaccinees developed signs or symptoms of lower respiratory tract involvement. Although the Hong Kong and Udorn ts-1[E] recombinants possessed two discrete ts lesions, evidence of genetic instability was detected during infection of two of the eight young children who lacked immunity to both surface antigens of the virus. It seems likely that a complete assessment of the virulence of vaccine candidate influenza A viruses can only be made in individuals who lack immunity to both surface antigens of the influenza A virus.

Speculation

These findings suggest the following.

1. Antineuraminidase antibody (ANAB) to previously experienced influenza strains can modify clinical response to infection with attenuated influenza virus as well as natural infection.

2. The full expression of virulence of ts recombinants or any live influenza vaccine may be manifest only in individuals lacking both hemagglutination-inhibition (HI) antibody and ANAB.

3. Evaluation of the immune and clinical response to viruses such as the ts-1[E] recombinant in young infants and children who lack both HI antibody and ANAB has value both because of their potential as vaccines and also as a model for an attenuated vaccine against the next pandemic influenza variant.

4. Standards for future ts or other live influenza vaccines must include genetic stability in individuals lacking HI antibody and ANAB and failure to induce a febrile response in such individuals.

Influenza infection and morbidity have seemed less important for infants and children than adults primarily because of the relatively greater frequency of other pediatric respiratory tract infections such as those caused by respiratory syncytial virus, parainfluenza viruses, and adenoviruses. In fact, up to 42% of infected infants and children may become ill during an influenza epidemic in an urban-suburban setting (7). Some epidemics have produced excess mortality in infants (3). Also, influenza infection is a major cause of infectious croup (6, 25). In our experience, influenza virus infection, particularly that due to the influenza A H2N2 and H3N2 strains, was related etiologically to about 5% of hospitalized pediatric respiratory disease and 14% of croup illnesses seen between 1957 and 1974. The impact of influenza A virus was particularly apparent during epidemics; the virus was associated with 70% of croup illnesses, 29% of pneumonia illnesses, and 19% of all other hospitalized respiratory tract disease during the peak month of a composite of 11 consecutive influenza A virus outbreaks (14). In addition to experiencing significant morbidity from influenza A virus infection, preschool and school age children have been implicated as a major source of spread of infection in the community (9, 15).

Despite the significant impact of influenza A virus infection in children, routine immunization in normal infants with inactivated influenza vaccines has not been recommended. Inactivated influenza vaccines have produced an unacceptable level of systemic reaction (10, 11) and have been poorly antigenic in young children (10, 11). Even with the newer purified, inactivated vaccines there remain theoretical disadvantages such as the need for parenteral administration, requirement for a large amount of influenza antigen, and the relative inability of such vaccines to stimulate formation of antibody in the respiratory tract, particularly in the infant who is immunologically inexperienced with regard to influenzal antigens. If an attenuated vaccine administered into the respiratory tract were safe and effective, this development could provide the impetus for pediatricians and families to accept routine influenza immunization for the protection of children. Such immunization might tend to interrupt the spread of influenza infection throughout the community.

One potential live influenza A virus vaccine strain is the influenza A/Hong Kong/68-ts-1[E], an H3N2 recombinant virus which is greatly restricted in its replication at 38° (17, 18). This temperature-sensitive mutant and a further recombinant derived from it, the influenza A/Udorn/72-ts-1[E] virus, were found to be safe, antigenic, stable genetically, noncommunicable, and protective in adults (17, 18). Thus, we undertook to study safety, antigenicity, and clinical response of these recombinant ts viruses in older children and then progressively in younger children.

Each of the adult vaccinees studied previously lacked serum HI antibody but possessed measurable levels of serum ANAB. Accordingly, we were particularly interested and concerned about the response of young children who might lack serum ANAB as well as serum HI antibody. Since neuraminidase immunity is effective in reducing the clinical response to infection with wild type influenza virus, it might also do so for the attenuated ts recombinant viruses (2, 5, 8, 18, 19, 23). Indeed the full expression of virulence of the ts recombinants or any live influenza virus vaccine may be manifest only in HI antibody- and ANAB-negative individuals, *i.e.*, doubly seronegative persons. Furthermore, it would be important to determine the immune response of doubly seronegative young children to viruses containing the ts-1[E] genetic lesions because of the potential usefulness of such recombinants for vaccination and as a model for the antigenicity of an attenuated vaccine directed against the next pandemic influenza variant which might possess new glycoprotein hemagglutinin and neuraminidase surface antigens.

To date we have administered the 1968 Hong Kong ts recombinant to 21 children, 2–11 years of age, who had a serum HI antibody titer of 1:8 or less. Some of the vaccinees also lacked serum ANAB. We also administered a 1972 Udorn ts recombinant containing the same ts-1[E] genetic lesions as the Hong Kong recombinant to two young children who lacked serum HI antibody and ANAB.

MATERIALS AND METHODS

VIRUS

The production and safety testing of the influenza A/Hong Kong/68-ts-1[E] (H3N2) virus employed in the present study have been described previously (17, 22). Briefly, the Hong Kong/68-ts-1[E] virus was a recombinant virus resulting from a mating of the influenza A/Great Lakes/65-ts-1 (H2N2) and the wild type influenza A/Hong Kong/68 (H3N2) viruses. The recombinant possessed the Hong Kong hemagglutinin (H3) and the temperature-sensitivity property of the 1965-ts-1 parent, *i.e.*, restriction of replication at 38°. The Hong Kong/68-ts-1[E] virus was grown in the allantoic cavity of hen's eggs free of leucosis virus (27) and titered $10^{6.0}$ $TCID_{50}$/ml in rhesus monkey kidney cell culture. The virus was administered by "nose drops" in a dose of 10^5 $TCID_{50}$/child.

The Udorn/72-ts-1[E] recombinant was prepared by mating influenza A/Hong Kong/68-ts-1[E] virus and an influenza A/Udorn/1972 (H3N2) wild type virus (antigenically similar to influenza A/England/42/72 virus) (21). The Udorn/72-ts-1[E] (clone 24) recombinant was administered intranasally in a dose of $10^{4.5}$/$TCID_{50}$.

EVALUATION OF CANDIDATE VACCINES IN CHILDREN

The recombinant vaccines were evaluated in children who had a serum HI antibody titer of 1:8 or less. Two children were admitted at a time to the Clinical Research Center of Children's Hospital, Washington, D.C., and were observed closely for 7–12 days after

virus administration. They shared a hospital room and special precautions were taken to prevent extraneous infection. In particular, parents and hospital personnel entering the room were required to wear masks. For each child, written informed consent was obtained from a parent or guardian after the aims and procedures of the study had been explained. Older children were evaluated first, then young children, and finally younger children without serum ANAB.

The children were examined at least twice daily and various specimens obtained as follows: nasal and throat swabs daily; anal swabs twice weekly; nasal secretions before vaccine administration and 1, 2, 3, 5, 10, and 20 weeks thereafter; serum samples before administration and 3, 5, 10, and 20 weeks thereafter.

Antibody studies have been performed to date only on nasal secretions obtained before vaccination and at 3, 5, and 20 weeks thereafter and on serum specimens before vaccination and at 3 or 5 weeks and 20 weeks for HI antibody and 3 weeks for ANAB.

VIRUS ISOLATION AND TEST FOR GENETIC STABILITY

A combined nose and throat swab specimen was inoculated into primary rhesus monkey kidney (RMK) roller tube cultures which were incubated at 33° and tested for hemadsorption with 0.4% guinea pig erythrocytes as described previously (24). All positive nasal-throat swab specimens were subsequently titered in parallel in a single test on RMK tube cultures and the titers expressed as $TCID_{50}$ per/ml of swab fluid. In addition, daily specimens from children who received the Udorn ts-1[E] recombinant were titrated for virus content without prior freezing.

The ts recombinant vaccine viruses did not produce plaques in monolayer cultures of RMK at 39°, which was considered a restrictive temperature. To determine the genetic stability of the Hong Kong/ts-1[E] and Udorn/ts-1[E] viruses after replication in children, each of the isolates recovered in RMK culture was tested for its ability to form plaques on RMK monolayer cultures at 33° and 39° (17). Those isolates which produced plaques at 39° were considered to contain genetically altered (ts+) virus.

HI, NEUTRALIZING, AND ANAB DETERMINATIONS

For the Hong Kong/68-ts-1[E] studies, the HI antibody titer was determined by standard microtiter assay using 4 antigen units of a recombinant virus containing the hemagglutinin of the influenza A/Aichi/68 (H3N2) virus and an irrelevant neuraminidase, N $equi_1$. For the Udorn/72-ts-1[E] studies, a recombinant virus containing the hemagglutinin of the influenza A/England/42/72 (H3N2) virus and the N $equi_1$ neuraminidase was used. The influenza A/Hong Kong/68 and influenza A/Aichi/68 viruses were antigenically equivalent strains; similarly, influenza A/England/42/72 and A/Udorn/307/72 viruses were equivalent. All serum samples were tested in parallel in a single test.

Since it has recently been reported that the standard neutralization test performed in roller tube cultures measures both HI antibody and ANAB (13), the technique employed in the present study utilized the H3 (Aichi) N $equi_1$ or the H3 (England/72) N $equi_1$ recombinant virus as antigen and therefore measured only antihemagglutinin antibody. The seed viruses, provided by Dr. Marion Coleman, were adapted to growth in RMK culture by serial passage at limit dilution to achieve a test antigen suspension that contained approximately 10^6 infectious units for each hemagglutinin unit. The test was performed in RMK tube cultures using 16–32 antigen units as described previously and titers were adjusted to 10 mg/100 ml immunoglobulin A (24). All nasal wash specimens were assayed in a single test.

The serum ANAB titer was determined by neuraminidase-inhibition assay using the H $equi_1$ N_2 (Aichi/68) or the H $equi_1$ N_2 (England/72) recombinant virus antigen as described previously (1). These viruses were also provided by Dr. Coleman. Virus titers were expressed as that dilution of serum which caused a 50% inhibition of enzyme activity. A rise in titer of 1.5 log_2 was considered significant in our assay system.

RESULTS

CLINICAL RESPONSE

The Hong Kong/68-ts-1[E] recombinant virus was administered to 21 children, 2–11 years of age (Table 1). Eighteen were infected as determined by any or all of the following criteria: virus recovery, serum HI antibody rise, serum ANAB rise, or nasal secretion antibody rise. Each of the six youngest vaccinees who lacked serum ANAB became infected.

The children were remarkably free of lower respiratory tract symptoms during infection. Cough, chills, myalgia, sweating, sneezing, or nasal obstruction were not observed. Mild, transient, nonpurulent rhinorrhea of 2 or 3 days duration was observed in 3 of 6 children who lacked serum ANAB and 1 of 12 who had serum ANAB. Four of the six vaccinees who lacked pre-existing serum ANAB developed a transient temperature elevation to 38.2° or greater during virus shedding; the highest temperature was 39°. In contrast none of the children with ANAB became febrile. The vaccinees were free of systemic reactions except for the four children who had transient fever.

Although there were no placebo controls in these studies to monitor the possibility that other viruses or bacteria might be responsible for the fever and rhinitis, there was careful viral and bacterial monitoring. No other agents were found to explain these symptoms.

VIRUS SHEDDING BY VACCINEES

Virus shedding was detected in 12 of the 21 vaccinees. The six children who lacked serum ANAB prior to administration of vaccine shed more virus for a longer duration than those who possessed serum ANAB (Table 1). There was no relationship

Table 1. *Response of seronegative children (serum hemagglutination-inhibiting antibody ≤1:8) to intranasal administration of $10^{5.0}$ $TCID_{50}$ of influenza A/Hong Kong/68-ts-1[E] vaccine virus (H3N2)*[1]

Vaccinees with pre-existing serum ANAB	Age, yr	No. in group	No. infected[2]	No. who shed virus	Average no. of days virus shed[3]	Geometric mean[3] peak titer of virus shed (log_{10})	Illness: No. who had rhinitis	Illness: No. who had fever (range of peak temp, °C)	Illness: Total with illness
No	2½–3½	6	6	6	4.5	2.1	3	4 (38.2–39.0)	6
Yes	5½–11	15	12	6	1.2	0.7	1	0	1

[4] Statistically significant difference between values at the ends of bracket (Student's *t*-test, P 0.01).

[5] Statistically significant difference between values at the ends of bracket (Fischer exact test, P 0.005).

[1] ANAB: antineuraminidase antibody.

[2] Recovery of virus and/or a fourfold or greater rise in serum and/or nasal secretion antibody.

[3] Calculated only for infected vaccinees.

Table 2. *Response of seronegative children (serum hemagglutination-inhibition antibody (HI) ≤1:8) to intranasal administration of 10^5 $TCID_{50}$ of influenza A/Hong Kong/68-ts-1[E] vaccine virus (H3N2)*[1]

Vaccinees with pre-existing serum ANAB	No. in group	Serum HI antibody[2]: Reciprocal (geometric mean log_2) antibody titer at indicated week after virus administration: 0	3–5	20	Serum HI antibody[2]: No. with 4-fold or greater rise	Serum ANAB[3]: Reciprocal (geometric mean log_2) antibody titer at indicated week after virus administration: 0	3	Serum ANAB[3]: No. with 1.5 log_2 or greater rise	Nasal wash neutralizing antibody[4]: Reciprocal (geometric mean log_2) antibody titer at indicated week after virus administration: 0	3	5	20	Nasal wash neutralizing antibody[4]: No. with 4-fold or greater rise
No	6	1.1	5.0	3.8	6	≤1	2.9	5	0.49	3.2	1.2	1.4	3
Yes	15	2.0	3.6	3.4	10	8.8	10.0	5	1.1	1.8	2.7	1.2	7

[1] ANAB: antineuraminidase antibody.
[2] HI antibody titers determined using 4 antigen units of H3 (Hong Kong) N $equi_1$ virus.
[3] ANAB Titers determined using H $equi_1$ N2 (Hong Kong) virus as antigen.
[4] Neutralizing antibody titers determined using 16 antigen units of H3 (Hong Kong) N $equi_1$ virus.

between virus shedding and the level of serum ANAB in those with pre-existing ANAB. With one exception, the Hong Kong/68-ts-1[E] virus maintained its ts phenotype during replication in children. Of 42 isolates obtained, 41 maintained ts phenotype. One isolate obtained from a vaccinee on *day 7* produced plaques at 39° which contained virus with the wild type phenotype (ts+), but such ts+ virus made up only a very small proportion of the total virus population in the isolate (a ratio of 100 ts:1 ts+). Significantly, no ts+ virus was detected in the isolates obtained on *day 8* and *day 9* from this vaccinee. No ts+ virus was detected by direct plaque assay of the original *day 7* swab specimen from this vaccinee.

HI ANTIBODY, ANAB, AND NASAL NEUTRALIZING ANTIBODY RESPONSE

Sixteen of the 21 vaccinees had a fourfold or greater rise in serum HI antibody and this antibody persisted for at least 20 weeks postvaccination ($P = 0.01$, using Student's t-test on 0- and 20-week serum specimens) (Table 2). Ten of the 21 vaccinees developed a significant rise in ANAB titer. Five of the six children who lacked serum ANAB (<1:2) at the time of virus administration had a significant rise in ANAB titer.

Ten of 21 vaccinees had a fourfold or greater rise in nasal wash neutralizing antibody that persisted for at least 5 weeks after vaccination. The difference in antibody titers between the 0- and 3-week and 0- and 5-week serum specimens was significant ($P < 0.05$, Student's t-test). However, in contrast to the serum HI antibody pattern, there was not a significant elevation in nasal wash antibody level at 20 weeks.

RESPONSE TO UDORN/1972 ts-1[E] RECOMBINANT

The two children (age 2 9/12 and 3 years) who received $10^{4.5}$ $TCID_{50}$ of the Udorn/72-ts-1[E] virus lacked serum HI antibody and ANAB at the time of virus administration. They shed virus from the 2nd to the 10th day after inoculation with peak titers of $10^{3.7}$ $TCID_{50}$/ml of swab fluid. Revertant virus (ts+) was detected in an isolate of one vaccinee on *day 5* but was not detected on previous or subsequent days. This virus represented a minority component (<1 ts+:1,000 ts) in the original swab specimen. Both children had serum HI antibody and serum ANAB responses comparable with those seen in children receiving the Hong Kong/68-ts-1[E] virus. One of the vaccinees had a febrile response (maxium fever 39°) which lasted 2 days and both had watery rhinorrhea for 7–10 days, but neither vaccinee exhibited any signs or symptoms of lower respiratory tract disease. Thus it appeared that the Udorn recombinant behaved similarly to the Hong Kong-ts-1[E] recombinant in young children who had not undergone influenza A virus infection previously.

DISCUSSION

This study indicated that children 5 years and older who lacked serum HI antibody, but who had prior immunologic experience with influenza A virus as indicated by possession of serum ANAB, behaved similarly to immunologically comparable adults after intranasal administration of the influenza A/Hong Kong/68-ts-1[E] candidate vaccine virus. The children's response was characterized by: (*1*) the occurrence in a minority of vaccinees of mild signs and symptoms affecting the upper respiratory tract; (*2*) virus shedding of short duration, limited to the first 5 days after vaccine administration; (*3*) low level of virus replication in the upper respiratory tract; and (*4*) stimulation of serum HI antibody, serum ANAB, and nasal wash neutralizing antibody. A single dose of 10^5–10^6 $TCID_{50}$ of the ts-1[E] vaccine infected approximately 90% of adults or children who had a serum HI antibody titer of ≤1:8 (17, 18). These results suggest that the Hong Kong/68-ts-1[E] virus has an acceptable level of attenuation and is antigenic for individuals 5 years of age and older. Importantly, such children did not manifest an exaggerated response to the vaccine virus.

In contrast to the children who possessed serum ANAB at the time of virus administration, those children who lacked both serum ANAB and HI antibody had a more extensive clinical and virologic response. Four of the six doubly seronegative children had a temperature elevation (38.2–39°) and this group as a whole shed more virus for a longer period than children who had pre-existing serum ANAB. This suggests that antineuraminidase immunity naturally acquired from previous infection provided some protection against illness produced by the candidate vaccine viruses. This suggestion is offered with the reservations that there was an age difference in the two groups and that the effects of antihemagglutinin immunity below the level of detection of the HI test could not be assessed. However, it seems likely that a complete assessment of the virulence of vaccine candidate influenza A viruses can be made only in individuals who lack immunity to both surface antigens of the influenza A virus. Only when doubly seronegative children were evaluated did it become apparent that the ts-1[E] recombinant viruses retained some virulence. Nonetheless, we were encouraged that the level of virulence of the Hong Kong/68 and Udorn/72 ts-1[E] viruses was low in such children, *i.e.*, no signs of lower respiratory tract involvement were evident. Systemic febrile responses similar to those seen in the doubly seronegative children would be anticipated if a ts-1[E] recombinant virus with the appropriate new surface antigens were administered at the time of the next influenza pandemic to individuals of any age who lacked immunity to both hemagglutinin and neuraminidase antigens of the new virus.

Importantly, the live attenuated ts viruses were antigenic in all children who lacked serum HI antibody and ANAB. The HI

antibody persisted for at least 5 months after virus administration. Seven of the eight doubly seronegative children developed ANAB. This was somewhat unexpected in light of previous studies which suggested that an ANAB response to primary infection with influenza A virus (H2N2) was unusual (4, 12). This apparent difference most likely reflects the increased sensitivity of the neuraminidase-inhibition assay used in the present study (1).

The present study suggested that the Hong Kong/68-ts-1[E] virus is attenuated and is immunogenic in both immunologically experienced and inexperienced children. Similar results in the two children who received the more contemporary Udorn ts-1[E] recombinant suggested that findings with the Hong Kong recombinant can be extrapolated to other recombinants prepared in the same manner and containing the ts-1[E] temperature-sensitive lesions.

The ts-1[E] recombinants were shown recently to contain two discrete ts lesions which were located on different RNA pieces of the segmented genome (20, 21). Despite the existence of ts lesions on two separate RNA segments of the viral genome, the recombinants exhibited evidence of genetic instability during infection of 2 of 8 young doubly seronegative children in the present study and 4 of 18 similar children in another study (26). In contrast, the recombinants were stable genetically when given to a considerably larger number of adults and older children who lacked detectable antibody to HA antigen, but possessed measurable neuraminidase immunity (17, 18). It appeared that emergence of ts+ revertants occurred only in the absence of immunity to both hemagglutinin and neuraminidase antigens; presumably this favored an unrestricted type of infection. These revertants represented only a very small proportion of the virus produced during infection of doubly seronegative children and the emergence of revertant virus under these conditions was not associated with lower respiratory tract disease.

In view of the occurrence of reversion and low grade fever, studies with the ts-1[E] recombinants in children have been discontinued. Although the Hong Kong ts-1[E] virus did not prove to be the ideal donor of ts lesions for a live influenza vaccine it did prove helpful in establishing new standards to be required for future ts influenza vaccine viruses—namely genetic stability in doubly seronegative individuals and failure to induce a febrile response in such persons. Other ts mutants which contain two independent ts lesions in regions of the viral genome which do not code for hemagglutinin and neuraminidase antigens are currently under study and several appear to be more stable genetically than the ts-1[E] virus. These viruses will be evaluated in adult volunteers shortly.

SUMMARY

One of two slightly different influenza A/ts-1[E] recombinant candidate live vaccines was given intranasally to each of 23 young children. Twelve of 15 children who had no serum HI antibody but who did have serum ANAB at the time of administration became infected and 1 had mild rhinitis. All eight who lacked both types of antibody became infected and they shed virus in higher titer and for longer than the former group; five had rhinorrhea and five had mild fever. These findings suggest that serum ANAB plays a part in modulating influenza virus infection and that the full expression of virulence of these or other attenuated influenza vaccines may be manifest only in individuals lacking both HI antibody and ANAB. These particular candidate vaccine strains appear to be attenuated for older children (who have some prior experience with influenza A as demonstrated by serum ANAB), but the occurrence of fever in over half who had no prior experience indicates that they would not be acceptable for a vaccine in wide-spread use.

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