JOURNAL OF CLINICAL MICROBIOLOGY, May 1989, p. 909–914
0095-1137/89/050909-06$02.00/0

Vol. 27, No. 5

Evaluation of Cold-Recombinant Influenza A/Korea (CR-59) Virus Vaccine in Infants

EDWIN L. ANDERSON,[1]* ROBERT B. BELSHE,[1] BETTY BURK,[1] JULIE BARTRAM,[1] AND H. F. MAASSAB[2]

Center for Vaccine Development, Departments of Medicine and Pediatrics, Marshall University School of Medicine, and Research Service, Huntington Veterans Administration Medical Center, Huntington, West Virginia 25755-9410,[1] and Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48109[2]

Received 14 November 1988/Accepted 7 February 1989

Twenty-four infants 5 to 13 months of age were intranasally vaccinated with a live cold-recombinant influenza A/Korea (CR-59, H3N2) virus vaccine. Nineteen infants served as controls. The inocula ranged from $10^{3.2}$ to $10^{6.2}$ 50% tissue culture infective doses ($TCID_{50}$) per infant. Zero of six, one of four, seven of ten, and four of four infants receiving $10^{3.2}$, $10^{4.2}$, $10^{5.2}$, and $10^{6.2}$ $TCID_{50}$, respectively, were infected by the intranasal vaccine. The amount of virus required to infect 50% of infants was calculated to be $10^{4.6}$ $TCID_{50}$. The occurrence of fever, respiratory illness, and otitis media was common among both controls and vaccinees in the postinoculation period. Maternal antibody was present in low titers in some infants and did not inhibit replication of the vaccine virus.

Influenza A virus infection often causes large outbreaks of febrile illness among infants and young children and can precede community outbreaks of influenza in adults (7, 9, 26). During influenza A epidemics, up to three-quarters of the patients seen by pediatricians for outpatient care may have influenza A virus infections (26). Influenza A virus infections in children are often complicated by otitis media (9, 26, 27). A vaccine effective in preventing illness and decreasing dissemination of infection among infants and young children would reduce the attack rate of influenza in the general population and may reduce associated illnesses, such as otitis media, in the age group at greatest risk for middle ear infection (9, 10, 12, 16, 21).

Cold-adapted, live, attenuated reassortant vaccines have been derived from the influenza A/Ann Arbor/6/60 virus master strain that donates six RNA segments coding for internal proteins and wild-type influenza viruses that contribute RNA segments encoding for hemagglutinin and neuraminidase (the 6/2 reassortant) (5, 13–15). Studies with adults and children indicate that these intranasally administered attenuated vaccines are safe and protect against experimental challenge (1–4, 8, 17, 18, 25). However, cross-reacting antibodies to influenza virus from previous naturally occurring infections may inhibit replication of live attenuated vaccines in the respiratory tract. These live attenuated vaccines have not been evaluated in children less than 1 year of age. However, this is the age group in whom the intranasal attenuated vaccines may prove to be most useful. Administration of these vaccines to infants and young children would avoid the problem of inhibition of replication of vaccine virus by preexisting antibodies from previous influenza virus infections (1, 2, 18, 25). Furthermore, influenza virus causes significant morbidity in infants (26). The purpose of this study was to evaluate the safety and antigenicity of CR-59 influenza A/Korea (H3N2) virus vaccine in infants and to determine whether maternal antibody against related influenza A virus strains would inhibit the infectivity of the vaccine. We also determined the amount of vaccine virus required to infect 50% of infants following intranasal vaccination.

* Corresponding author.

MATERIALS AND METHODS

Clinical studies. Families with infants 5 to 15 months of age were recruited from pediatric practices in the community. The infants were examined by a pediatrician. After screening tests of complete blood count, liver function tests, urinalysis, tuberculosis skin test, and serum antibody to influenza A virus, the mothers were asked to volunteer their healthy infants to receive cold-adapted H3N2 vaccine or to serve as unvaccinated controls. Infants whose mothers were pregnant were excluded from the study.

The infants were examined for signs of respiratory illness 3 days before vaccination, and nose and throat swabs were taken for isolation of viruses. Twenty-four healthy children were inoculated intranasally (0.25 ml per nostril) with $10^{6.2}$, $10^{5.2}$, $10^{4.2}$, or $10^{3.2}$ 50% tissue culture infective doses ($TCID_{50}$) of influenza A/Korea CR-59 virus vaccine. Nineteen infants served as unvaccinated controls. Nasopharyngeal secretions for virus isolation were obtained just before inoculation and daily thereafter for 11 days from vaccinees and control children. The infants had their temperatures taken daily by study personnel and were examined for signs of respiratory illness. Acute otitis media was diagnosed when an infant had a compatible clinical history and the tympanic membrane was red and swollen and mobility was decreased on pneumatic otoscopy.

Sera for antibody titer determinations were obtained from vaccinees before and at 4 weeks, 6 months, and 1 year after vaccination. In many instances, cord sera were available for study.

If a vaccinee or family members developed signs of illness, nasal secretions were inoculated onto other tissue culture cell lines (MRC-5 and HEp-2) to attempt to recover viruses in addition to vaccine virus, and throat cultures for beta-hemolytic streptococci were done.

Vaccine. The vaccine, influenza A/Korea/1/82 (CR-59) virus clone 19-1, lot E-204, was a recombinant having the six internal genes of cold-adapted influenza A/Ann Arbor/6/60 (H2N2) virus and the hemagglutinin and neuraminidase genes from influenza A/Korea/1/82 (H3N2) virus (15). Vaccinees received 0.5 ml intranasally of a 1/10 ($10^{6.2}$ $TCID_{50}$ per dose), 1/100 ($10^{5.2}$ $TCID_{50}$ per dose), 1/1,000 ($10^{4.2}$ $TCID_{50}$ per dose), or 1/10,000 ($10^{3.2}$ $TCID_{50}$ per dose)

dilution of vaccine. Undiluted vaccine contained $10^{7.5}$ $TCID_{50}$ of virus per ml.

Laboratory studies. The total quantities of virus in throat swab specimens were determined by culturing at 34°C. Throat and nasal swab specimens were placed in veal infusion broth, transported on wet ice to the laboratory, and promptly inoculated into tissue culture in serial 10-fold dilutions from no dilution to a 1:10,000 dilution. In addition, undiluted throat swab specimens were inoculated onto tissue cultures and maintained at 39°C for assessment of the presence of revertant virus, i.e., virus that regained the ability to grow at 39°C.

Hemagglutination inhibition (HAI) antibodies in serum were assayed by the use of chicken erythrocytes and reagents supplied by the World Health Organization Influenza Center, Atlanta, Ga. (23). An enzyme-linked immunosorbent assay (ELISA) for antibody to H3 hemagglutinin was performed by the procedure of Murphy et al. (19).

Genetic stability studies on vaccine virus recovered from infants. Preparation, maintenance, and infection of primary chick kidney (PCK) cells were done by established procedures (13, 14). The plaque assays for evaluation of the phenotypes were performed in PCK cells by infecting confluent monolayers in 25-cm^2 plastic flasks with 1.0 ml of virus diluted in Eagle minimum essential medium. Virus was adsorbed for 1 h at room temperature with continuous, gentle rocking, after which the virus inoculum was aspirated off and 5 ml of overlay medium consisting of 0.8% purified agar (BBL Microbiology Systems, Cockeysville, Md.) in modified 199 medium was added. After incubation for 48 h at 33 and 39°C or for 5 days at 25°C, the monolayers were stained by adding 4 ml of 0.8% agar to the modified medium 199 containing 0.01% neutral red. Plaques were read on days 5, 6, and 7 for the cultures incubated at 33 and 39°C and on days 6 to 12 for the cultures incubated at 25°C.

Gene constellations of the vaccine virus and isolates of virus that were recovered from respiratory secretions were determined as described previously (5, 20). Briefly, the virus was grown for 2 days in PCK cells with [^{3}H]uridine to label the viral RNA. The harvest was purified and lysed, and the viral RNA was electrophoresed on two 3.0% polyacrylamide–0.6% agarose mixed gels (230 V, 18 h, 30 and 37°C). The viral RNA was also run on a 1.5% agarose gel (60 V, 18 h, 37°C). Both types of gels were run in 1× TBE (0.09 M Trizma base, 0.09 M boric acid, 1 mM disodium EDTA) with 0.1% sodium dodecyl sulfate. After the run, the mixed gels were soaked on 0.75% M sodium salicylate containing 0.1% hexadecyltrimethylammonium bromide for 45 min. The agarose gel was soaked in En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) for 45 min and then in H_2O for 30 min. Both types of gels were then dried and exposed to X-ray film at −70°C for 24 to 48 h.

Viruses recovered from infants were also examined for the attenuation phenotype by evaluation in ferrets. Infection of ferrets was done by a procedure published earlier (15). Ferrets were inoculated intranasally and examined for coryza, rhinitis, and fever twice a day for the duration of the experiment. Nasopharyngeal swabs were taken daily, and on days 3 and 8 ferrets were sacrificed and 20% suspensions of turbinate and lung tissues were made in nutrient broth. The viruses in these samples were titrated in embryonated eggs.

RESULTS

Infection of children with CR-59. All four infants who received $10^{6.2}$ $TCID_{50}$ of vaccine virus shed vaccine virus in respiratory secretions. Seven of ten infants who received $10^{5.2}$ $TCID_{50}$, one of four who received $10^{4.2}$ $TCID_{50}$, and none of six who received $10^{3.2}$ $TCID_{50}$ shed vaccine virus (Table 1). All four infants who received $10^{6.2}$ $TCID_{50}$ of vaccine virus manifested a fourfold antibody titer rise as measured by HAI. Seven of ten who received $10^{5.2}$ $TCID_{50}$ of virus showed a fourfold antibody titer rise by both HAI and ELISA. One of the four who received $10^{4.2}$ $TCID_{50}$ of virus had a fourfold HAI and ELISA antibody titer rise. No antibody titer rises occurred among infants given the lowest dose of virus. In each case, the antibody increases occurred in children who shed vaccine virus. There were no antibody rises among infants who did not shed the vaccine virus. The magnitude of the antibody response was not different among those given a higher or lower dose of vaccine (Table 1). As indicated by both shedding of vaccine virus and the fourfold antibody antibody titer increase, the infectivity of this vaccine for these infants was 100% at a dose of $10^{6.2}$ $TCID_{50}$, 70% at $10^{5.2}$ $TCID_{50}$, 25% at $10^{4.2}$ $TCID_{50}$, and 0% at $10^{3.2}$ $TCID_{50}$. The amount of virus required to infect 50% of the infants was $10^{4.6}$ $TCID_{50}$ as calculated by the Reed-Muench method.

TABLE 1. Number of infants infected with vaccine virus (CR-59) as indicated by shedding of virus, ELISA, or HAI antibody response

Dose (log_{10} $TCID_{50}$)	No. inoculated	Mean age (range, mo)	No. shedding virus	No. with a ≥4-fold antibody response		No. (%) infected
				HAI	ELISA	
6.2	4	7.25 (5–11)	4	4 (27)[a]	3	4 (100)
5.2	10	9.1 (5–11)	7	7 (39)	7	7 (70)
4.2	4	8.0 (5–12)	1	1 (64)	1	1 (25)
3.2	6	10.16 (5–13)	0	0	0	0 (0)

[a] Reciprocal geometric mean convalescent antibody titer.

Among the infants who were infected by the intranasal vaccine, the amount of virus shed was not dependent upon the titer of the inoculum (Fig. 1). The duration of virus shedding was greater than 7 days in six infants and greater than 10 days in one infant.

The HAI antibody test was insensitive for detecting maternal antibody. The reciprocal geometric mean HAI antibody titers in the cord sera for the infants who received $10^{6.2}$, $10^{5.2}$, $10^{4.2}$, and $10^{3.2}$ $TCID_{50}$ of vaccine virus were 5.6, 6.5, <4, and <4, respectively. Maternal antibody was at a low level by the vaccination date. At 4 to 13 months of age, the age of vaccination, nearly all of the infants had HAI antibody titers of <4 or 4. ELISA was more sensitive than HAI for identifying maternal antibody at birth and at the time of vaccination (Fig. 2). ELISA antibody to influenza A/Korea virus hemagglutinin was present in all cord sera. The mean ELISA titer was 3,881 at birth (Fig. 2). By the time of vaccination at 4 to 13 months of age, a significant loss of maternal antibody had occurred; antibody titers were 10-fold lower, with a mean ELISA titer of 341. At 1 month postvaccination, vaccinees who were infected by vaccine virus had a mean ELISA titer of 1,000. The mean antibody titer continued to increase beyond 1 month postvaccination. At 6 months and 1 year after vaccination, the mean ELISA titers were 6,309 and 8,483, respectively, among infected vaccinees (Fig. 2).

Clinical response to CR-59. Of 10 infants given $10^{5.2}$ $TCID_{50}$ of virus, 2 had fevers greater than 38.4°C but only 1 of these children was infected with the vaccine virus as

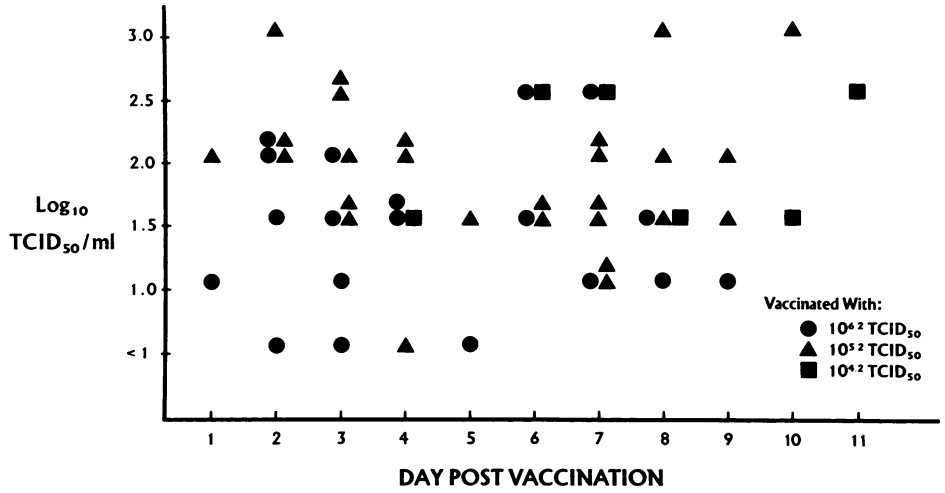

FIG. 1. Quantity and duration of vaccine virus shed in upper-airway secretions among infants given $10^{6.2}$, $10^{5.2}$, or $10^{4.2}$ $TCID_{50}$ of live attenuated influenza A/Korea/1/82 (CR-59) virus vaccine. The titers are expressed as $\log_{10}$ $TCID_{50}$. The points between <1 and 1.0 indicate that virus was recovered in only one of several tissue cultures inoculated with a throat swab specimen.

determined by viral shedding and/or antibody titer rise; the other infant was not infected with the vaccine virus. No temperature elevations occurred among the four infants who received either $10^{4.2}$ or $10^{6.2}$ $TCID_{50}$ of vaccine virus (Table 2). Temperature elevations occurred throughout the postvaccination period, and there was no clustering of fevers. None of the three infants vaccinated with $10^{3.2}$ $TCID_{50}$ who experienced fever was infected with the vaccine. One control infant had a fever greater than 101°F (38.8°C). Nine infant vaccinees had rhinorrhea, but only three were infected with vaccine virus; six control infants had rhinorrhea.

Otitis media occurred among seven of the vaccinees, only two of whom were infected by the vaccine virus. Three infants in the control group had otitis media. Intercurrent infection with nonpolio enterovirus (isolated from respiratory secretions) was associated with otitis media in one vaccinee, and intercurrent respiratory syncytial virus infection was associated with otitis media in a second vaccinee. Intercurrent respiratory virus infection was not found in any of the other vaccinees with otitis media.

One of the infants given intranasal vaccine had lower respiratory tract illness characterized by coughing and wheezing which began on day 1 postvaccination. This infant neither shed vaccine virus nor had an antibody titer rise (Table 2). Two control infants had lower respiratory tract infections with wheezing as the initial clinical finding. Both

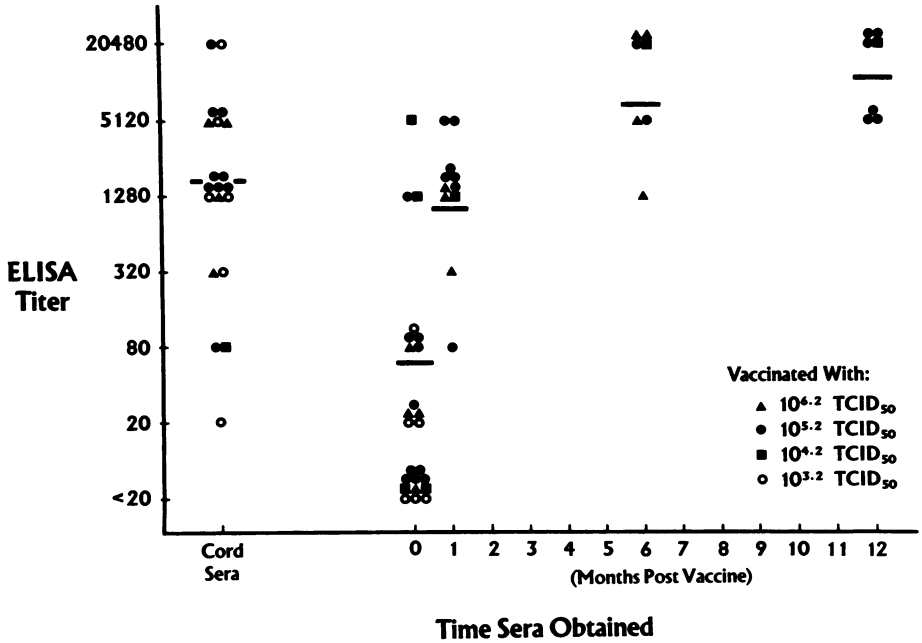

FIG. 2. ELISA antibody titer to influenza A/Korea/1/82 virus hemagglutinin in cord sera and prevaccine sera with respect to the vaccine dose. Antibody responses to vaccination at 1, 6, and 12 months postinoculation are shown for infants infected with vaccine virus. None of the infants who received $10^{3.2}$ $TCID_{50}$ became infected with vaccine virus. Serum was not obtained from all infants at each postvaccination date.

TABLE 2. Clinical responses to CR-59[a]

Dose (log_{10} $TCID_{50}$)	No. tested	No. of infants with:				
		T > 38.4°C	Rhinorrhea	Cough	Otitis media	LRI
6.2	4	0	2	0	1	0
5.2	10	2[b]	2	1	3[b]	0
4.2	4	0	2	1	2[c]	0
3.2	6	3[c]	3[c]	1	1[c]	1
0 (control)	19	1	6	3	3	2

[a] Occurrence of rectal temperatures (T) of >38.4°C, rhinorrhea, cough, otitis media, or signs of lower respiratory illness (LRI) among 24 vaccinees within 11 days of vaccination with the indicated dose of CR-59 compared with control children under observation at the same time.
[b] Only one vaccinee was infected by vaccine virus.
[c] None of these vaccinees was infected by vaccine virus.

infants had otitis media, but only one infant had lower respiratory tract disease and otitis media concurrently. Herpes simplex virus was cultured from the upper airway of one of these infants, and a nonpolio enterovirus was cultured from respiratory secretions of the other infant.

Genetic stability of vaccine virus removed from infants. Twenty-one isolates from the trials were examined for genetic stability as determined by retention of the cold adaptation, temperature sensitivity, and attenuation phenotypes and the 6/2 gene profile (Table 3). Two isolates from nasal swabs (clones 16592 and 16620) were intermediate in temperature sensitivity upon amplification in embryonated eggs. The infectivity titer at 39°C when compared with that at 33°C showed a 10^2 to 10^3 reduction when compared with the rest of the isolates, for which no observable infectivity ($>10^3$ reduction) at 39°C was recorded. However, upon further analysis in ferrets, these two isolates retained the attenuation phenotype and were cold adapted and temperature sensitive (Table 3).

DISCUSSION

The infectivity of this vaccine was directly proportional to the vaccine dose given; the infective dose for 50% of the infants was $10^{4.6}$ $TCID_{50}$. This is similar to the 50% infective doses of the other cold-adapted influenza A virus vaccines that have been evaluated in children 1 to 5 years old but 100-fold lower than that observed for adults (1, 8, 17, 24). The cold-adapted influenza A virus vaccines which have been evaluated previously in seronegative children include influenza A/California 10/78 (H1N1) virus. The dose of cold-recombinant influenza A/California virus necessary to infect 50% of children was approximately $10^{3.5}$ $TCID_{50}$ (14). In adults screened for low levels of nasal wash antibody or low antibody levels in serum, the amount of cold-adapted vaccine virus required to infect 50% of the volunteers was between $10^{5.5}$ and $10^{6.5}$ $TCID_{50}$ with influenza A/Washington/897/80 (H3N2) virus and between $10^{5.8}$ and $10^{6.8}$ $TCID_{50}$ for influenza A California 10/78 (H1N1) virus (3, 17, 22, 24).

In this study, adverse effects were not related to vaccine dose. Otitis media was the most frequent significant adverse effect observed among the vaccinees and controls in this

TABLE 3. Evaluation of CR-50 isolates obtained from four infant vaccinees for temperature sensitivity and cold adaptation phenotypes

Vaccinee no.	Day of shedding	PFU/ml (isolate in RMK)[a]			PFU/ml (nasal swab specimen)[b]			Clone picked	PFU/ml[c]		
		25°C	33°C	39°C	25°C	33°C	39°C		25°C	33°C	39°C
004	2	3×10^6	5×10^6	$<10^3$	NT	NT	NT				
004	3	3×10^5	4×10^5	$<10^3$	NT	NT	NT				
004	6	2×10^6	8×10^6	$<10^3$	1×10^1	3×10^1[d]	$<10^1$	16592[d]	3×10^6	2×10^7	1×10^4[e]
004	8	NT	NT	NT	2×10^1	4×10^1[d]	$<10^1$	16620[d]	3×10^6	6×10^7	3×10^3[e]
002	2	5×10^5	4×10^6	$<10^3$	NT	NT	NT				
002	3	1×10^7	4×10^7	$<10^3$	3×10^1	5×10^1[d]	$<10^1$	16496[d]	1×10^8	2×10^8	$<10^4$
002	4	1×10^5	7×10^5	$<10^3$	NT	NT	NT				
002	7	5×10^5	3×10^6	$<10^3$	2×10^2	3×10^3[d]	$<10^1$	16563[d]	2×10^6	1×10^8	$<10^4$
003	2	1×10^5	2×10^6	$<10^3$	2×10^2	3×10^2[d]	$<10^1$	16479[d]	1×10^8	2×10^8	$<10^4$
003	3	4×10^6	3×10^7	$<10^3$	2×10^2	4×10^2[d]	$<10^1$	16495[d]	7×10^7	1×10^8	$<10^4$
003	4	5×10^6	2×10^6	$<10^3$	NT	NT	NT				
003	5	1×10^6	3×10^6	$<10^3$	3×10^1	2×10^1[f]	$<10^1$				
003	7	4×10^5	5×10^5	$<10^3$	2×10^1	3×10^1[f]	$<10^1$				
008	3	NT	NT	NT	2×10^1	3×10^1[d]	$<10^1$	17004[d]	9×10^6	6×10^7	$<10^3$
008	4	2×10^5	1×10^6	$<10^3$	NT	NT	NT				
008	6	9×10^5	3×10^6	$<10^3$	9×10^1	3×10^2[d]	$<10^1$	17048[d]	1×10^8	6×10^7	$<10^4$
008	7	2×10^5	4×10^5	$<10^3$	2×10^1	3×10^2[d]	$<10^1$	17070[d]	4×10^7	1×10^8	$<10^4$
008	8	2×10^5	3×10^6	$<10^3$	NS	NS	NS				
008	10	4×10^4	6×10^6	$<10^3$	NT	NT	NT				
008	11	3×10^5	3×10^5	$<10^3$	2×10^1	9×10^1[d]	$<10^1$	17125[d]	3×10^6	2×10^7	$<10^4$
CR-59[g]									2×10^7	4×10^7	$<10^3$
A/Korea wild type[g]									$<10^3$	3×10^7	8×10^6

[a] Efficiency of plaque formation at various temperatures in PFU of virus isolated from infected infants per milliliter. Virus was isolated in rhesus monkey kidney (RMK) tissue culture at 33°C, and the plaque assay was done at the indicated temperature in PCK cells. NT, No titer in PCK cells.
[b] PFU per milliliter in PCK tissue culture of the original nasal swab specimen obtained from infant vaccinees. NS, no nasal swab available.
[c] PFU per milliliter in PCK tissue culture of egg-passaged virus obtained from the indicated clone at the indicated temperature.
[d] The clone was picked from 33°C PCK plaques from a nasal swab specimen, amplified in eggs, and titrated for the cold adaptation and temperature sensitivity phenotypes and gene profile.
[e] These two clones isolated from the 39°C cultures were amplified in eggs and tested for the temperature sensitivity and cold adaptation phenotypes. They were also evaluated in ferrets and for genotype. Both clones had 6/2 gene profiles analogous to the vaccine. No reactogenicity in ferrets was demonstrated. Both clones were temperature sensitive and cold adapted.
[f] The clones picked were negative in embryonated eggs.
[g] Control vaccine virus, control influenza A/Korea wild-type virus.

study and is of particular concern among infants. The most common complication of upper respiratory tract infection in children is otitis media, and the peak incidence occurs in the 6- to 12-month age group (9, 16, 26). As many as 30% of infants have had at least one episode of otitis media by 1 year of age, and by 2 years of age over 60% of children have experienced one or more middle ear infections (10, 12, 21). Children who experience an initial attack of otitis media in year 1 of life are at increased risk of recurrence (16). Influenza A has been associated with otitis media, and control of this epidemic illness may reduce the incidence of otitis media among young children (9, 26, 27). The possible association of vaccine virus with otitis media was a concern. In this study, only two of the episodes of otitis media occurred in infants infected with the vaccine virus. However, 5 of 12 vaccinees who were not infected by vaccine virus and 3 of 19 control children developed otitis media within 11 days of entry into the study (2 of 12 versus 5 of 12 [$P = 0.15$] or 2 of 12 versus 3 of 19, [$P = 0.38$; Fisher exact test]). During other studies evaluating cold-adapted influenza A virus vaccines in children 1 to 5 years old, we have observed that intercurrent respiratory disease occurred significantly more often among vaccinees who were not infected by intranasally administered vaccine virus (1). We suggested that the intercurrent illness may have interfered with the infectivity of the intranasal vaccines. Perhaps the otitis media observed in this study reflected the common intercurrent respiratory illnesses in this age group. The cause of viral respiratory infection associated with otitis media cannot always be determined. Henderson et al. (9) were able to identify the etiology of viral respiratory infection in only 39% of initial episodes of otitis media. Other investigators have not detected a significant increase in otitis media among children vaccinated with these live intranasally administered vaccines (1, 2, 24). However, other studies involved older children, and the risk of otitis media among younger vaccinees must be determined.

Antibody concentrations achieved in serum after administration of CR-59 vaccine were, for the most part, in the range that correlated with protection against clinical influenza illness, and the antibody titers remained stable for a year (1, 2, 11). There was a delay in achieving maximum antibody titers among the children who were infected by vaccine virus. This further increase in antibody titers between 1 and 6 months postvaccination was not likely due to intercurrent influenza A virus infection. Although intercurrent natural infection with influenza A virus may have boosted the antibody titers, this is unlikely. Infants who were vaccinated with CR-59 but did not become infected with vaccine virus had antibody titers that remained low for the 1 year of follow-up; the geometric mean ELISA titers of vaccinated infants who were not infected by CR-59 at 1 month, 6 months, and 1 year postvaccination were 158, 54, and 69, respectively.

Even though we vaccinated some infants who were less than 6 months old, the maternal antibody level was low by the vaccination date and these low maternal antibody levels did not seem to influence the infants' susceptibilities to vaccine virus. This is in contrast to findings after parenteral immunization against measles virus (6). Further studies with this and other cold-adapted influenza virus vaccines should be conducted in infants, since this population is at risk for significant disease and is susceptible to infection with these live attenuated vaccines.

ACKNOWLEDGMENTS

This work was supported by Public Health Service contract N01-AI-52575 from the National Institute of Allergy and Infectious Diseases.

We thank the infants' parents for their cooperation and Frances Newman, Carol Berry, Sally Wells, and Arlene Napier for laboratory and clinical assistance and study coordination.

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