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Secretory and Systemic Immunological Response in Children Infected with Live Attenuated Influenza A Virus Vaccines

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An enzyme-linked immunosorbent assay was used to measure isotype-specific antibody to purified hemagglutinin (HA) of influenza A virus, using serum and nasal-wash specimens from young children undergoing primary infection with live cold-adapted influenza A/Alaska/77 (H3N2) or A/Hong Kong/77 (H1N1) candidate vaccine virus. The serum antibody response followed the pattern expected for a primary viral infection. Each of 17 vaccinated children had a serum immunoglobulin G (IgG) HA antibody response, 16 had an IgM antibody response, and 13 had an IgA antibody response. Nasal-wash HA antibody was detected in the IgA, IgM, and IgG isotypes. Of the 17 vaccinated children, 14 had an IgA response, 13 had an IgM response, and 9 had an IgG response. Most of the IgA and IgM HA antibody was actively secreted locally, whereas only some of the IgG HA antibody could be shown to be actively secreted into the respiratory tract. There was a good correlation between the level of nasal-wash antibodies measured by the HA-specific IgA enzyme-linked immunosorbent assay and by a plaque neutralization assay. These data indicate that intranasal vaccination of susceptible children with live, attenuated, cold-adapted influenza A viruses efficiently stimulates both systemic and local antibody responses.

Although currently licensed inactivated influenza virus vaccines are efficacious, they do not provide complete protection (17), and recently, the effectiveness of their annual administration has been questioned (9). For these reasons, there is renewed interest in the development of a live attenuated vaccine that would mimic natural infection in its broader and more durable immunity. One method for the rapid attenuation of new influenza A viruses involves the use of the A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) virus as a donor of its attenuating genes to the new variants of influenza A virus (13). Candidate live vaccine viruses are reassortant viruses that possess the six internal genes of the *ca* donor virus and the surface antigens, i.e., the hemagglutinin (HA) and neuraminidase glycoproteins, of the new epidemic wild-type virus (4). Two such *ca* reassortants, the A/Alaska/6/77 (H3N2) and A/Hong Kong/123/77 (H1N1) *ca* viruses, have been shown to be attenuated and restricted in replication in animals and humans (5, 12, 20, 22, 33). The ability of these viruses to induce a local, secretory antibody response in the respiratory tract has not been assessed previously. In the present study, an enzyme-linked immunosorbent assay (ELISA) was used to measure isotype-specific antibody to the purified HA of influenza A virus, using serum and nasal-wash specimens from young children infected with the A/Alaska/77 (H3N2) or A/Hong Kong/77 (H1N1) *ca* reassortant virus (21).

MATERIALS AND METHODS

Clinical studies. Children aged 1.5 to 4.5 years were inoculated intranasally with $10^{6.2}$ 50% tissue culture infective doses of A/Alaska/6/77 (H3N2) CR-29 clone 2 or $10^{6.6}$ 50% tissue culture infective doses of A/Hong Kong/123/77 (H1N1) CR-35 reassortant virus. The production and safety testing of the *ca* reassortants and their evaluation in adults and children have been described (12, 20, 22). Of 10 vaccinated children who were infected with the A/Alaska/77 (H3N2) *ca* virus, 7 had previously been naturally infected with an influenza A virus belonging to the H1N1 subtype, since they possessed hemagglutination-inhibition antibody in their preinoculation serum specimens. Similarly, two of seven infected children who received the A/Hong Kong/77 (H1N1) vaccine had previously been infected with an H3N2 influenza A virus. The serum and nasal-wash specimens of five unvaccinated control children and five uninfected vaccinated children were also analyzed. The results of the clinical and virological evaluation of these children will be the subject of separate reports (12; P. Wright et al., manuscript in preparation).

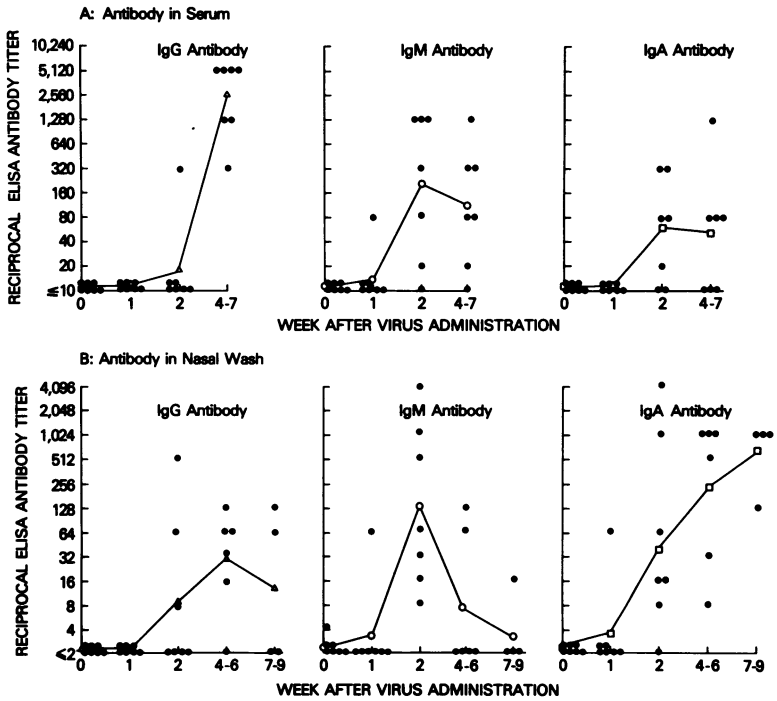

FIG. 1. HA antibody response of children to A/Hong Kong/77 (H1N1) cold-adapted reassortant virus. Antibody present in the serum or nasal-wash specimens of children undergoing primary infection with A/Hong Kong/77 (H1N1) *ca* virus was determined by ELISA. The line connects the geometric mean titers at the indicated time period.

ELISA. An ELISA for the detection of immunoglobulin A (IgA), IgG, or IgM antibody to purified HA, using specific rabbit anti-human immunoglobulin sera, was previously described (21). Briefly, 96-well, flat-bottomed plates were used with a ladder of reagents from the solid phase up consisting of (i) purified HA; (ii) human serum or nasal wash; (iii) rabbit anti-human IgA, IgG, or IgM; (iv) goat anti-rabbit IgG serum conjugated with alkaline phosphatase; and (v) substrate. The ELISA titer was calculated by the conventional positive-over-negative method, in which the endpoint was the highest dilution that gave a positive-over-negative ratio of equal to or greater than 2. To derive this ratio, the optical density of an antigen-containing well (positive) was divided by the optical density of the respective control well lacking antigen (negative). Serum and nasal-wash specimens were tested for ELISA HA antibody, using only the HA of the vaccine virus administered.

The ELISA nasal-wash antibody titers were not corrected to an IgA concentration of 20 mg/ml, as was done previously (19), because a proportion of the specimens did not have IgA detectable by the standard radial immunodiffusion assay. The nasal-wash value is thus not a corrected titer but represents the titration endpoint for antibody present in 100 μl of a nasal-wash specimen concentrated approximately 10-fold with aquacide (Calbiochem, La Jolla, Calif.).

A nasal-wash ELISA response was defined as a post-inoculation specimen ELISA titer of greater than or equal to 1:8. This value was chosen since none of the vaccinated or contact control children had a preinoculation nasal-wash specimen titer that exceeded 1:4. A serum ELISA antibody response was defined as a fourfold or greater rise in titer between pre- and post-inoculation specimens.

Concentration of total IgG, IgA, and IgM in selected serum and nasal-wash specimens was kindly determined by radioimmunoassay by Thomas A. Waldman, National Cancer Institute, Bethesda, Md., as previously described (35).

To preferentially adsorb IgG from serum specimens, 0.75 ml of packed *Staphylococcus aureus* protein A bound to sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was added to 1.5 ml of a 1:50 dilution of serum, and the IgG-*S. aureus* protein A complex was removed by centrifugation after 1 h of incubation at room temperature.

Neutralization assay. Nasal-wash neutralization titers were determined by 50% plaque reduction neutralization in a continuous canine kidney cell line (MDCK), using approximately 50 PFU of vaccine virus with 1 μg of TPCK trypsin (Worthington Diagnostics, Freehold, N.J.) per ml in the agarose overlay.

RESULTS

Serum and secretory ELISA antibody. The serum and secretory IgG, IgM, and IgA HA antibody levels of young children who were infected with a *ca* reassortant virus are shown in Fig. 1 and 2. None of the vaccinated children

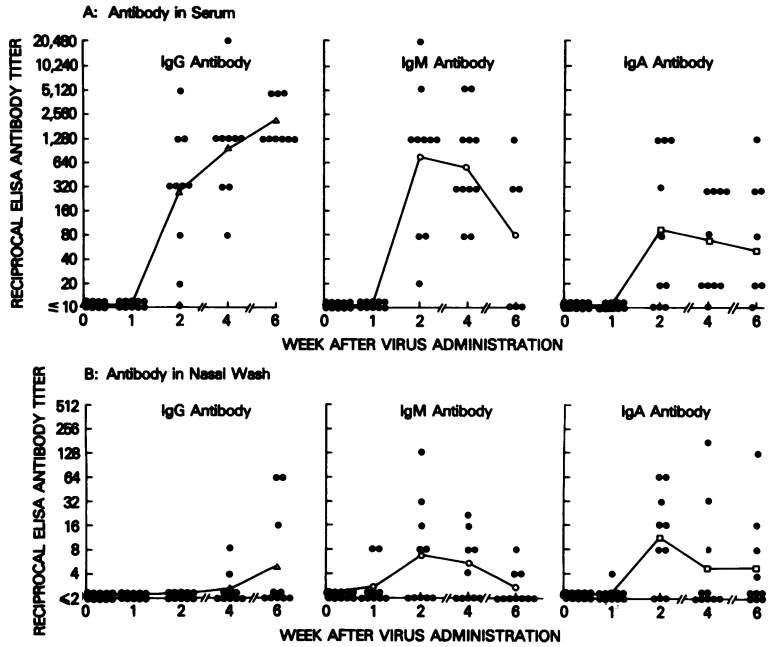

FIG. 2. HA antibody response of children to A/Alaska/6/77 (H3N2) cold-adapted reassortant virus. Antibody present in serum or nasal-wash specimens of children undergoing primary infection with A/Alaska/77 (H3N2) *ca* virus was determined by ELISA. The line connects the geometric mean titers at the indicated time period.

had preinoculation serum or nasal-wash ELISA HA antibody to the virus administered. Each of the 17 vaccinated children developed a serum IgG response, and 16 developed an IgM response. The serum ELISA IgG antibody peaked at around 4 to 6 weeks, whereas the IgM antibody peaked at 2 weeks and declined by 4 to 7 weeks. Of the 17 vaccinated children, 13 had a serum IgA HA antibody response which peaked at 2 weeks and remained at about the same or a slightly lower level for up to 6 to 7 weeks after infection. Five contact control children who did not receive vaccine and five vaccinated children who were not infected by vaccine did not develop detectable antibody in their serum or nasal-wash specimens.

It is possible that high levels of IgG antibody could have interfered with the detection of IgA or IgM HA antibody by binding to all of the available antigen on the assay plate. To investigate this possibility, IgG present in three serum specimens that contained high titers of both IgG and IgM HA antibody was adsorbed with *S. aureus* protein A, and the concentration of IgG, IgM, and ELISA IgG and IgM HA antibody was determined on absorbed and unabsorbed serum specimens. Approximately 99% of IgG and 35% of IgM were adsorbed from the protein A-treated serum specimens. The ELISA IgG and IgM HA antibody titers were decreased in proportion to the amount of immunoglobulin removed. If IgG antibody had been interfering with detection of IgM HA antibody, the ELISA IgM titer should have increased as the IgG antibody was removed. As this was not observed, it was concluded that the level of IgG HA antibody produced by the vaccinated children did not interfere with the detection of HA antibody in the other isotypes tested.

IgG, IgM, and IgA HA antibodies were also measured in nasal-wash specimens (Fig. 1 and 2). Of the 17 vaccinated children, 14 developed nasal-wash IgA HA antibody after vaccination; 13 children developed IgM HA antibody, whereas 9 children developed IgG HA antibody. Of the 14 vaccinated children who developed nasal-wash IgA HA antibody, 13 developed IgA HA antibody in their sera. Conversely, of the three vaccinated children who failed to produce detectable nasal-wash IgA HA antibody, none produced detectable serum IgA HA antibody. Among A/Hong Kong/77 (H1N1)-vaccinated children, titers of IgA HA antibody were higher than IgG or IgM titers in the nasal-wash specimens. Interestingly, most vaccinated children developed IgM HA nasal-wash antibodies. There was a discordance in individual nasal-wash ELISA HA antibody responses in the IgA and IgM isotypes, suggesting that the high frequency of detection of IgM antibody was not

TABLE 1. Active secretion into the respiratory tract of IgA, IgM, and IgG antibody to influenza HA

Volunteer	[(Nasal-wash titer)/(concn of immunoglobulin in nasal wash)]/[(serum titer)/(concn of immunoglobulin in serum)][a]		
	IgA	IgM	IgG
1	261	—[b]	12
2	209	88	636
3	76	—	0.91
4	53	86	1.3
5	25	25	3.1
6	20	27	—
7	7.0	—	2.9
8	6.9	—	1.5
9	1.1	3.3	0.4
10	0.9	28	—
11	0.6	—	0.5

[a] The specific activity of antibody in serum or nasal-wash specimens collected at the same time was estimated by calculation of the ratio of ELISA antibody titer (isotype specific) to the concentration of the immunoglobulin in that specimen. If antibody is actively secreted into the respiratory tract, then the specific antibody activity in the nasal wash should exceed that in the serum. When the ratio indicated in the table exceeds 1, it suggests that antibody is actively secreted. However, since the ELISA titers are estimates based on fourfold dilutions, values between 1 and 4 cannot be unambiguously interpreted. Ratios greater than 4 are, therefore, suggestive that the antibody is actively secreted.

[b] —, Specimens did not contain detectable nasal-wash antibody.

due to a cross-reacting immunoglobulin in the rabbit anti-human IgA antibody. Previous infection with heterosubtypic influenza A virus did not appear to influence the serum or nasal-wash response to vaccine virus HA nor the quantity of vaccine virus shed (data not shown).

To determine whether the nasal-wash IgA, IgG, or IgM HA antibody was actively secreted, the specific activity of antibody in a nasal-wash specimen was compared with that in a serum specimen collected at the same time. The specific activity of the antibody is the ratio of ELISA antibody titer (isotype specific) to the concentration of that immunoglobulin in the specimen. If nasal-wash HA antibodies were actively secreted, then the specific activity in nasal-wash specimens should be greater than that in serum. Our analysis (Table 1) indicated that most IgA and IgM HA nasal-wash antibodies were actively secreted. Secretion of IgG HA antibodies was observed less often.

Relationship between nasal-wash neutralizing and IgA ELISA antibody. Neutralizing antibody titer correlated with the IgA ELISA antibody titer in nasal-wash specimens from A/Hong Kong/77 (H1N1)- and A/Alaska/77 (H3N2)-vaccinated children (Fig. 3). In both groups of vaccinated children, there was a significant correlation between IgA ELISA and neutralizing antibody titers. However, the slopes of the two lines were significantly different (t test, $P < 0.01$), with that for the A/Hong Kong/77 (H1N1)-vaccinated children being greater than that for the A/Alaska/77 (H3N2)-vaccinated children. This observation indicates that the IgA ELISA is more sensitive than the neutralization test for the detection of antibody to H1 virus HA. Nine of the A/Alaska/77 (H3N2) nasal-wash specimens had neutralizing titers of 1:4 or greater in the absence of ELISA IgA or IgM antibody. Five were either preinoculation or 1-week specimens, suggesting that the nasal-wash neutralization test may yield false-positive results. Such false-positive values would decrease the slope of the regression line shown in Fig. 3, which is a measure of the relative sensitivities of the two test procedures.

DISCUSSION

The present study characterizes the primary immune response of young children to infection with two similarly attenuated influenza A vaccines. The immune response that was measured was most likely a primary response, since the children lacked preinoculation antibody to the HA of the infecting virus and were fully susceptible to infection. In addition, these children had been followed in a vaccine clinic from birth to the time of vaccine virus administration and at no time had virological or serological evidence of infection with a virus antigenically related to the vaccine virus administered. The serum antibody response was similar to that previously described for other primary viral infections (16, 25, 32). IgM HA antibody, which was detected in 16 of 17 vaccinated children, attained peak levels at 2 weeks and then declined. IgG HA antibody, which peaked at 4 to 7 weeks, was detected in each of the 17 vaccinated children. A serum IgA HA antibody response was detected less consistently (13 of 17 vaccinated children) and was of a lower magnitude than the IgG and IgM responses.

Nasal-wash HA antibody was detected in the IgA, IgM, and IgG isotypes. Of the 17 vaccinated children, 14 had an IgA response, 13 had an IgM response, and 9 had an IgG response. A serum IgA HA antibody response was associated with a nasal-wash IgA antibody response in 13 of 14 vaccinated children. These data suggest that, in primary influenza A virus infections, a serum IgA HA antibody response reflects a concomitant local antibody response, perhaps due to the escape of locally produced IgA antibody into the systemic circulation. In general,

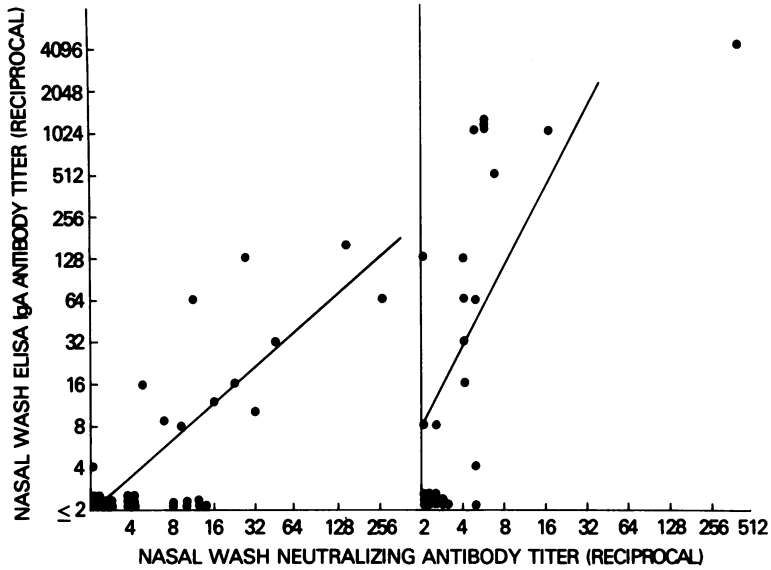

FIG. 3. Correlation of neutralization and IgA ELISA antibody titers in nasal-wash specimens obtained from children undergoing infection with a *ca* reassortant virus. The slopes of the lines (± standard error) for (A) A/Alaska/77 (H3N2)- and (B) A/Hong Kong/77 (H1N1)-vaccinated children was 1.27 ± 0.03 and 2.64 ± 1.05, respectively. The slopes and the intercepts of the lines were determined by regression analysis.

previous studies of viral infections of mucosal surfaces have indicated that local antiviral antibody is predominantly of the IgA isotype (14, 15, 24, 25, 30, 36). Recently, local antiviral antibody in the IgM and IgG isotypes has been detected during primary viral infection of the respiratory or gastrointestinal tract (16, 32). In the present study, 13 of 14 of the vaccinated children who had a nasal-wash IgA HA antibody response also had an IgM HA response. The peak titer of nasal-wash IgM antibody was reached at 2 weeks after virus administration.

Evidence from the present study indicated that most IgA and IgM HA antibody was actively secreted locally. This was not surprising since previous studies in humans indicated that IgM antibody is found in intestinal columnar epithelial cells in a distribution like that of IgA (2) and also contains secretory component (1). Furthermore, in patients with IgA immunodeficiency, IgM-producing cells increase in number (29), and specific antiviral antibody is found in the IgG and IgM isotypes (23). In earlier studies, some of the IgG present in nasal-wash specimens was shown to be produced locally (3). The present results were consistent with each of these prior observations.

In the present study, we observed a correlation between the level of antibodies measured by HA-specific IgA ELISA and by neutralization assay. Such a correlation has been demonstrated previously for H3N2 virus infection in adults (21). However, during a study of parainfluenza and respiratory syncytial virus infections in children, a dissociation was noted between nasal-wash neutralizing activity and specific IgA antibody determined by immunofluorescence (15, 36). A likely explanation for this difference is that the IgA ELISA used in the present study detects only antibody to the influenza A virus HA, which is the surface glycoprotein known to induce neutralizing antibody (7, 11). On the other hand, immunofluorescence detects antibodies to internal virus antigens that do not induce neutralizing antibodies as well as antibodies to viral surface antigens (15, 36). In addition, it is possible that some of the neutralizing activity present in nasal secretions may be mediated not by specific immunoglobulins but, instead, by nonspecific neutralizing factors (6, 10). Such nonspecific neutralizing factors could account for the neutralizing activity in some nasal-wash specimens that were obtained in the present study before virus administration.

The presence of specific secretory IgA antibody has been correlated with resistance to certain viral infections of humans (18, 19, 24, 26, 31, 34). In other infections, such as those caused by adenovirus, serum antibody correlates with resistance to illness but not infection (8). The relative contribution of local and systemic immunity to influenza virus infection has not been clearly defined in humans. Local antibody in the absence of detectable serum antibody has been

associated with resistance to infection and illness (19). A contribution of serum antibody alone has been inferred from studies of the correlation of resistance to illness caused by influenza A virus with the level of maternally transferred antibody in neonates (27). Perhaps in humans antibody present in either the local or systemic compartment can contribute to resistance to illness caused by influenza virus. In experimental murine influenza virus infection, there is both a systemic and a local contribution to immunity (28). For these reasons, it was important to determine whether candidate live, intranasally administered virus vaccines induced antibody in both compartments, and this was indeed shown to be the case. The role that local antibody induced by vaccine virus has in resistance to subsequent infection with wild-type virus is currently being studied.

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