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Development of Subtype-Specific and Heterosubtypic Antibodies to the Influenza A Virus Hemagglutinin after Primary Infection in Children

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Children undergoing primary infection with an H1N1 or H3N2 influenza A virus developed subtype-specific hemagglutination inhibition antibodies and enzyme-linked immunosorbent assay antibodies to purified hemagglutinin (HA) of the infecting virus subtype. They also developed lower titered ELISA antibodies to the noninfecting H1 or H3 HA and to H8 (an avian strain) HA. Thus, after primary infection with an influenza A virus, children develop enzyme-linked immunosorbent assay, but not hemagglutination inhibition, antibodies reactive with heterosubtypic HAs. These heterosubtypic antibodies could influence the response to infection with other wild-type or attenuated vaccine strains of influenza A virus.

Adults infected with influenza A viruses of the H3N2 or H1N1 subtypes develop hemagglutinin (HA)-specific antibodies as measured by an enzyme-linked immunosorbent assay (ELISA) (3). Young adults who by epidemiological considerations should never have experienced natural infection with an influenza A virus bearing the H1 HA have ELISA antibody to the H1 HA (3). In contrast, children of about 1 year of age never known to have been infected with an influenza A virus lack antibody to any HA tested (8). This suggests that adults have acquired H1 HA antibody as a result of infection with H2N2 or H3N2 influenza A virus or both. We sought to determine whether primary infection with influenza A virus could lead to such a heterosubtypic HA antibody response.

Children undergoing naturally occurring primary infection with influenza A virus were tested for antibody responses to homologous HA and to two other influenza A virus HAs, one heterologous human HA (H1 or H3) and one avian HA (H8). These children had no evidence of prior infection with viruses bearing the heterosubtypic human HA and would not have been exposed to the HA of the avian virus.

Seventeen children from 1 to 4 years of age were followed from birth as part of a vaccine development program which involved intensive serologic and virologic surveillance of each respiratory tract illness. Ten children had documented primary influenza A virus infection with an H3N2 subtype and seven had a primary H1N1 influenza A virus infection. The pre- and postinfection sera from these children were tested for antibody by the hemagglutination inhibition (HAI) assay and ELISA. This was the first infection with an influenza A virus for the children since they lacked HA antibody by both HAI and ELISA in their preinfection sera but developed such antibody after an influenzal illness. Paired sera from four additional children who had a primary infection with an influenza B virus were also studied.

HAI antibody was measured by the standard microtiter method (4), using egg-grown A/Texas/1/77 (H3N2), A/Bangkok/1/79 (H3N2), A/USSR/92/77 (H1N1), and A/Brazil/11/78 (H1N1) influenza viruses. Purified HA antigens for the ELISA were prepared from A/USSR/92/77 (H1N1), A/Bangkok/1/79 (H3N2), A/Mallard/Alberta/704/78 (H8N4), and B/Singapore/222/79 influenza viruses as previously described (10). Briefly, virus present in allantoic fluid was pelleted by ultracentrifugation and further purified by banding the virus twice on 20 to 60% continuous sucrose density gradients. After disruption with 1% Nonidet P-40, viral proteins were fractionated on a 5 to 25% sucrose gradient. The comigrating HA and neuraminidase were then separated by HA affinity chromatography. These HA H1, H3, and H8 preparations contained 27, 58, and 33 μg of protein per ml, respectively.

The influenza A HA preparations were examined for the presence of other influenza A virus proteins which might react with antibodies present in the sera of the children. Each HA preparation was free of neuraminidase activity as shown by its total inability to cleave 2′-(4-methylumbelliferyl)-D-*N*-acetyl-neuraminic acid (14). In contrast, 0.1 μg of whole viral protein per ml (10 ng per well) had readily detectable neuraminidase activity. Contaminating matrix protein or nucleoprotein was not found by ELISA with monoclonal antibodies reactive with these proteins (11, 12). This was so even when ELISA plates were coated with 5 μg of the HA preparation proteins per well, a 50-fold excess over that used in the HA-specific ELISA. In contrast, when whole virus was used as the coating antigen, the monoclonal antibodies reacted strongly with 10 ng of whole viral protein per ml (1 ng per well) (of which less than 30% would be expected to be matrix protein or nucleoprotein). Polyacrylamide gel electrophoresis indicated that in each HA preparation more than 95% of the protein was HA and that no other viral proteins were identified (10). Silver staining demonstrated prominant HA bands and minor low-molecular-weight smeared bands thought to be degraded polypeptides. No neuraminidase, matrix protein, nucleoprotein, or polymerase proteins were identified. Therefore, it was likely

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TABLE 1. HA-specific serum antibody titers after primary influenza A or B virus infection

Infecting virus	Reciprocal geometric mean titer[a]									
	HAI antibody				HA-specific ELISA IgG antibody					
	H1		H3		H1		H3		H8	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Influenza A H1N1 (n = 7)	≤8	40 (7)[b]	≤8	≤8 (0)	10	3,120 (7)[b]	14	119 (5)[c]	12	160 (5)[c]
Influenza A H3N2 (n = 10)	≤8	≤8 (0)	≤8	147 (10)[b]	20	80 (4)[c]	19	2,560 (10)[b]	22	299 (8)[b]
Influenza B (n = 4)	NT[d]	NT	NT	NT	10	10 (0)	10	14 (0)	10	10 (0)

[a] Geometric mean ELISA antibody titers were calculated from serial fourfold dilutions beginning at 1:40; those unreactive at 1:40 were assigned titers of 1:10 for computational purposes. Numbers in parentheses show number of children with a greater than or equal to fourfold increase in geometric mean titer. Pre, Preinfection; post, postinfection.

[b] The pre- and postgeometric mean titers differed significantly ($P < 0.01$).

[c] The pre- and postgeometric mean titers differed significantly ($P < 0.05$).

[d] NT, Not tested. Note: Influenza B virus was isolated from each of the four children and a homologous ELISA antibody rise was detected.

that antibody detected by ELISA in the sera of these children was HA specific.

The ELISA used to detect antibody to HA was performed as described previously (3, 9), using the following ladder of reagents from the solid phase up: (i) purified HA; (ii) human serum; (iii) rabbit anti-human immunoglobulin A (IgA), IgM, or IgG; (iv) goat anti-rabbit IgG serum conjugated to alkaline phosphatase; and (v) *para*-nitrophenylphosphate substrate. The end point was taken as the highest dilution of antibody producing an absorbance of at least 0.1, providing that the reading in the antigen-containing well was at least twice that in the comparable antigen-free control well. A single lot of polystyrene plates (Immulon I; lot no. 12-9-1; Dynatech Laboratories, Inc., Alexandria, Va.) was used throughout the study.

The serum HAI and ELISA HA antibody responses of children undergoing primary infection are presented in Table 1. Each child had a fourfold or greater increase in IgG ELISA antibody to the HA of the homologous infecting virus subtype. Antibodies to heterologous HA antigens were also detected. Of the children tested, 5 of 7 infected with H1N1 virus and 8 of 10 infected with H3N2 virus developed an increase in IgG ELISA antibody titer to the avian H8 HA. Similarly, some children in each group had an increase in IgG ELISA antibody titer to the heterologous human H1 or H3 HA subtype. Of 7 H1N1-infected children, 5 had an increase in IgG ELISA antibody to H3 HA, and 4 of 10 H3N2-infected children had an increase in IgG ELISA antibody to H1 HA.

A total of 13 children (6 of 7 H1N1 infected and 7 of 10 H3N2 infected) also developed IgA ELISA antibody to the homologous HA. Postinfection homologous HA IgA antibody titers were much lower than IgG titers, and none of the 17 children had a detectable increase in IgA antibody to any of the heterosubtypic HAs (data not shown). These sera were not collected at a time optimal for detection of IgM seroconversion or maximal titer. Indeed, only 5 of 17 children had an increase in IgM antibody to the homologous HA subtype, and none of the children developed IgM antibody to any of the heterosubtypic HAs.

None of four children infected with influenza B virus developed influenza A HA ELISA antibody, and none of the children infected with influenza A virus had a rise in influenza B HA antibody titer. In the H3N2-infected group, three children had significant but unchanging titers of ELISA antibody to influenza B virus, suggesting prior infection with an influenza B virus.

These results demonstrate that children with primary influenza A virus infection develop a low titer of serum antibodies which react with the HA of virus subtypes to which the children have never been exposed. The antibodies reacting with the heterosubtypic HAs are presumably antibodies to the homologous HA which are cross-reactive among the HAs. They can be detected by ELISA but not by HAI. This may be due to the greater sensitivity of the ELISA. It is also possible that there are common HA epitopes which are masked on the antigen preparations used in HAI but which are exposed during HA purification or binding to plastic for the ELISA. When B cells are stimulated in vitro with an influenza A virus bearing one HA subtype, they can produce antibodies which react with the HA of another subtype as measured in an HA-specific ELISA (13). These heterosubtypic antibodies are presumably reacting with epitopes common to influenza A H1, H3, and H8 HA subtypes but which are not present on the influenza B HA. This seems to be a reasonable possibility since sequence analyses of the HA-1 subunit of various influenza A viruses have shown substantial regions of homology (1).

IgA and IgM anti-H1 HA-specific antibodies are also present in sera from adults who have never experienced infection with an H1N1 virus (3). However, heterosubtypic antibodies of the IgA and IgM classes were not detected in the sera of children convalescing from primary infection as described in this report (data not shown). The convalescent sera of only five children had IgM antibody, and there were relatively modest titers of IgA antibody to the homologous HA (geometric mean titer for IgA of 1:160 versus a geometric mean titer for IgG of 1:2,810). The titers of IgG H8 antibodies were 10- to 20-fold lower than those of homologous HA antibodies. If a similar quantitative relationship applied to IgA or IgM anti-H8 antibodies, they would not have been detected in the present study. Therefore, the IgA and IgM antibodies to heterosubtypic HA observed in adults may be the result of a cumulative increase in titer after several encounters with influenza A viruses.

The biological properties of these putatively subtype-cross-reactive HA antibodies are unknown, but they don't appear

to play a major role in protection against virulent influenza A virus infection. Individuals with these antibodies remain susceptible to natural or experimental infection with new influenza A virus subtypes (3, 9). It is possible, however, that the antibodies have a minor role in limiting the extent of infection. Epidemiological observations suggest that children undergoing infection with influenza A viruses have more severe illness than adults, even when preinfection HAI antibody titers for both groups are the same (5). Furthermore, children are more susceptible than adults to infection with an attenuated influenza A virus belonging to a subtype to which neither group had been previously exposed (2, 7). The human 50% infectious dose of an H1N1 attenuated vaccine virus was almost 100-fold less in children than in adults, and the level of virus replication was far more extensive in the children. It is possible that the heterosubtypic antibody present in the adults could have contributed to the restriction of virus replication, although other factors could also be responsible (2, 6–7).

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