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Neutralization Test in Influenza: Use in Individuals Without Hemagglutination Inhibition Antibody

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In influenza immunization trials, patients who were seronegative by the hemagglutination inhibition test could be divided into two groups according to pre-immunization levels of neutralizing (Neut) and neuraminidase inhibition (NI) antibodies. The group with low levels of Neut and NI antibodies usually developed adequate levels of hemagglutination inhibition antibody after one dose of vaccine, and the group with undetectable Neut and NI antibodies did not.

Immunity to influenza virus is usually determined by the presence of hemagglutination inhibition (HAI) antibody. The starting serum dilution is 1:8 or 1:10 to eliminate nonspecific inhibitors of hemagglutination. Seronegative individuals, defined as HAI titer <1:8, respond differently to inactivated influenza vaccines. The variable immune response may in part relate to the presence in some vaccinees of prior antigen priming undetectable at the 1:8 serum dilution in the HAI test. We examined neutralizing (Neut) and neuraminidase inhibition (NI) antibody titers in these seronegative individuals because the initial serum concentration in the Neut and NI tests is undiluted.

Serological tests were the standard microtiter HAI test (3) with an initial test serum dilution of 1:8 in our laboratory and 1:10 in the Respiratory Virology Branch, Center for Disease Control, Atlanta, Ga. Neut tests were done in duplicate in rhesus monkey kidney cell monolayer roller tubes with a challenge virus dose of 50 50% tissue culture infective doses (6). The NI test procedures have been described (1). Virus strains used for HAI, Neut, and NI tests have been described (2, 3); E. Kilbourne supplied influenza A recombinant strains X-42 (H1 equi N2) and X-54 (H1 equi N1). Antibody to the H antigen can cross-react with antibody to the N antigen from the same virus; recombinant strains were used because they contain an irrelevant hemagglutinin—an H antigen derived from a horse influenza virus. The neuraminidase antigens were derived from influenza A/Port Chalmers/73 (an H3N2 virus related to influenza A/Victoria/75) for the X-42 strain and from influenza A/Ft. Dix/76 (an HswN1 virus) for the X-54 strain. Vaccines used were split-product and whole-virus types previously described (2, 3).

Children with prevaccination HAI titers of <8 (or <10) were studied because children with titers of ≥8 usually respond to immunization with a rise in HAI antibody or maintainance of a high titer at ≥32. Seronegative individuals were divided into two groups: those who had no change in antibody and those who seroconverted to an HAI titer of ≥32 (or ≥40) after immunization. HAI titers of <8 (or <10) were by convention shown to be 4 (or 5), though the actual value could have been 0 to 4 (or 5). The initial test serum concentration for determining Neut and NI antibodies was undiluted; consequently, titers of under 8 (or 10) represented actual titers.

For the influenza B/Hong Kong/72 virus strain, the absence of Neut and NI antibodies before immunization was associated with a poor immune response after immunization (Fig. 1A). In comparison, children who developed significant HAI antibody titers (≥32) after immunization were observed to have a geometric mean Neut antibody titer of 6 and a geometric mean NI titer of 16 before immunization (Fig. 1B). The differences in prevaccination Neut and NI antibody titers in the two groups is significant ($P < 0.001$ using Student's t test and F test). A Neut or NI titer of 4 present before immunization was associated with an HAI titer of ≥32 after immunization in 25 of 29 individuals. The 11 poor responders had a mean age of 7 years, while the 18 good responders had a mean age of 12 years ($P < 0.001$).

For the influenza A/Victoria/75 virus strain, children who had no detectable serum HAI antibody after immunization were noted initially to have low levels of Neut and NI antibodies (geometric mean titers of 3 and 2, respectively, Fig. 2A). Conversely, children who developed significant HAI antibody titers of ≥40 after immunization were observed to have pre-immuni-

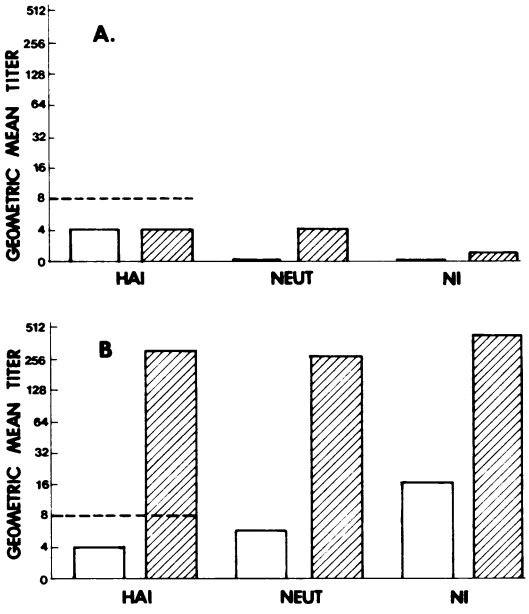

FIG. 1. *Geometric mean titer for HAI, Neut, and NI serum antibodies to influenza B/Hong Kong/72. All 29 individuals were seronegative before immunization by the HAI test; that is, no HAI antibody was detectable at the starting dilution of 1:8, and by convention this is represented by a 1:4 titer. The Neut and NI antibody titers shown are actual titers because the starting dilution is 0. Pre-immunization (□) and post-immunization (▨) titers are shown for the 11 individuals who remained seronegative by the HAI test after immunization (A) and for the 18 who developed an HAI titer of ≥32 after immunization (B).*

zation geometric mean titers of Neut and NI antibodies of 20 and 16, respectively (Fig. 2B). The difference in the pre-immunization geometric mean titers of Neut and NI antibodies in the two groups was significant ($P < 0.001$). A pre-immunization Neut or NI titer of 4 was associated with development of a post-immunization titer of ≥40 in all individuals. The mean age for the group of 13 poor responders was 8 years, while that for the group of 8 good responders was 9 years.

For the influenza A/New Jersey/76 virus strain, it was possible to determine in the same individual the immunogenicity of a vaccine in the unprimed and primed state. None of 15 children had HAI, Neut, or NI antibody detectable initially. After the first immunization, HAI antibody was still not detectable in any vaccine recipient (Fig. 3). However, Neut antibody was present in all but two individuals, with a geometric mean titer of 1:4 for the group. NI antibody was present only in two individuals and at very low levels.

After the second immunization, HAI antibody was readily detectable in all individuals with a geometric mean titer of 60 for the group and Neut antibody titers were even higher. NI antibody was still low because the NI antigen in the vaccine was weak (4).

Although more vaccinees received split-product vaccines than whole-virus vaccines, neither type of vaccine could consistently immunize with one dose individuals seronegative by the HAI, Neut, and NI tests.

The seronegative individual appears to be more clearly defined by examining two other types of antibody. Neut antibody usually parallels HAI antibody (5). Both types of antibody are stimulated by the viral hemagglutinin antigen. However, the Neut test appears to detect lower levels of viral antibody than does the HAI test. This difference may be related to the high serum concentrations and the additional viral antigens detected by the Neut test. The NI method tests for the host's response to a second

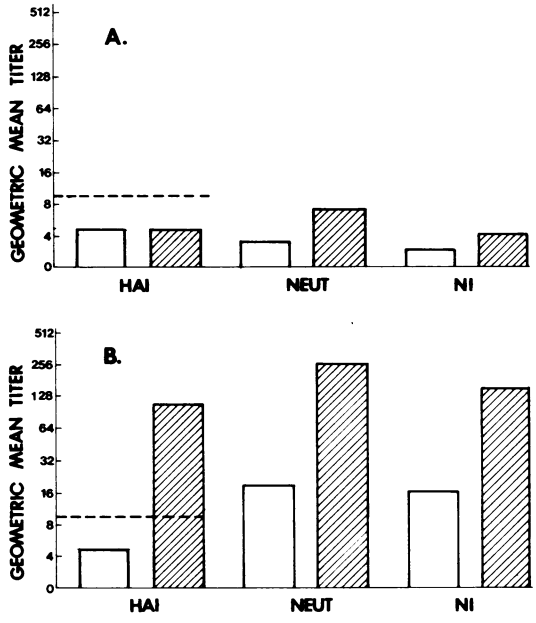

FIG. 2. *Geometric mean titer for HAI, Neut, and NI serum antibodies to influenza A/Victoria/75. All 21 individuals were seronegative before immunization by the HAI test; that is, no HAI antibody was detectable at the starting dilution of 1:10, and by convention this is represented by a 1:5 titer. The Neut and NI antibody titers shown are actual titers because the starting dilution is 0. Pre-immunization (□) and post-immunization (▨) titers are shown for the 13 individuals who remained seronegative by the HAI test after immunization (A) and for the 8 who developed an HAI titer of ≥40 after immunization (B).*

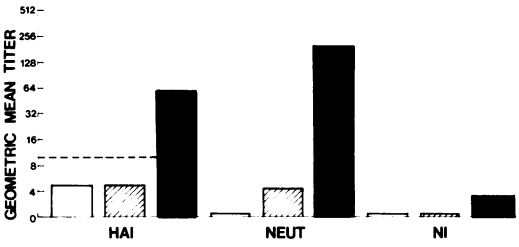

FIG. 3. *Geometric mean titer for HAI, Neut, and NI serum antibodies to influenza A/New Jersey/76. All 15 individuals were seronegative before immunizations by the HAI test; that is, no HAI antibody was detectable at the starting dilution of 1:10, and by convention this is represented by a 1:5 titer. The Neut and NI antibody titers shown are actual titers because the starting dilution is 0. The titers shown for HAI, Neut, and NI tests are preimmunization (□), 4 weeks after the first immunization (▨), and 2 weeks after the second immunization (■).*

viral antigen, neuraminidase. NI antibody levels do not necessarily parallel HAI antibody values (7).

The low levels of Neut antibody detected before immunization appear to represent virus-specific antibody because they correlated with a good antibody response post-vaccination. Enzyme-linked immunosorbant assays have increased the ability to detect very low levels of antibody. Simultaneous testing of Neut and enzyme-linked immunosorbant assay-detected antibodies will be useful to determine if low levels of antibody detectable by enzyme-linked immunosorbant assay can neutralize virus infectivity for influenza and other viruses.

In this study, individuals who were initially seronegative by the Neut and NI tests, as well as by the HAI test, did not develop significant levels of HAI antibody after immunization. These individuals most likely had never been exposed to the test influenza antigens. Individuals with initial Neut or NI titers of 4 or more developed significant HAI titers after immunization. Although they were "seronegative" by HAI testing before immunization, they were not immunological virgins as they had viral antibody detectable by other methods. Further testing of seronegative individuals should be done to confirm the findings described.

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